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Original research article

Encapsulated Synbiotic Dietary Supplementation at Different Dosages to Prevent Vibriosis in White Shrimp, *Litopenaeus vannamei*

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ABSTRACT

The aim of this study was to evaluate the effect of encapsulated synbiotic (*Bacillus* sp. NP5 and oligosaccharide) dietary at different dosages on growth performance, survival rate, feed conversion ratio, and immune responses of *Litopenaeus vannamei* against *Vibrio* infection. The shrimps of the main treatments were fed by the diet that contained three different dosages of encapsulated synbiotic [0.5% (A), 1% (B), and 2% (C) (w/w)] with feeding rate of 5% of shrimp biomass (4 times a day). The shrimps of two control treatments (negative control and positive control) were fed only by commercial feed without supplementation of encapsulated synbiotic. The growth, feed conversion ratio, and survival rate were observed after 30 days of encapsulated synbiotic dietary. The shrimps were then challenged by injection of *Vibrio harveyi* (6 log colony forming units/mL) 0.1 mL/shrimp, excluded the negative control treatment. Afterward, the survival and immune responses were observed for 9 days after experimental infection. The shrimps treated with 2% encapsulated synbiotic (treatment C) in the diet showed the highest growth performance (2.98 ± 0.42%), feed conversion ratio (1.26 ± 0.19), and better immune responses i.e. total hemocyte counts, differential hemocyte count, phenoloxidase, and intestine bacteria observation compared to those of positive control treatment.

1. Introduction

The Pacific white shrimp (*Litopenaeus vannamei*) is an economically important commodity of Indonesian aquaculture. However, shrimp production decreased since 1990s and resulted in high economic losses due to viral and bacterial diseases (Austin and Zhang 2006; Flegel and Sritunyalucksana 2011; Li and Xiang 2012). One of the major bacterial disease problems is luminous vibriosis. Among genus *Vibrio, V. harveyi* is the major causative agent of luminous vibriosis (Robertson *et al.* 1998). Luminous vibriosis has been reported to be one of the bacterial diseases responsible for the larval mass mortality (Chrisolite *et al.* 2008). Vibrio infection caused even more shrimp mortality when the viral co-infection occurred (Hasan 2011).

Along with restriction of antibiotics application in shrimp culture, the luminous disease control has been done by feed supplementation of prebiotics (Li *et al.* 2007), probiotics (Guo *et al.* 2009; Rivera *et al.* 2014) and also synbiotics (Li *et al.* 2009; Arangure *et al.* 2013; Ramirez *et al.* 2013). Synbiotics which is a combination of prebiotics and probiotics could increase the health status by contributing the modulation of intestinal microflora in the host (Liong 2008; Chakraborti 2011). The synbiotic application as fresh preparation, however has been considered ineffective, because of the short period of cell viability (Weinbreck *et al.* 2010). The synbiotic supplementation as a dry form provides longer cell viability (Ross *et al.* 2005; Ubbink and Krueger 2006). Therefore, synbiotic microencapsulation process is required. This technique is attemptable, more efficient for practical uses, and protects longer the cells viability (Anal and Singh 2007). Until the current studies the research of supplementation of encapsulated synbiotic to *L. vannamei* is still limited, where the optimum dosage could be an important factor to get optimal host performance.

2. Materials and Methods

This study was conducted within two steps. First, the microencapsulation process of synbiotic and second, the application of encapsulated synbiotic for *in vivo* test. Synbiotic was prepared from oligosaccharide extract of sweet potato (*Ipomoea batatas* L.) as the prebiotic, and *Bacillus* sp. NP5 as probiotic bacteria which were

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marked by rifampicin resistancy (50 μg/mL). Sweet potato as prebiotic source used in this study was obtained from sweet potato research center (Balitkabi), East Java. The preparation of prebiotic according to the method of Marlis (2008) was conducted at Nutrition Laboratory, and the probiotic was prepared at Fish Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agriculture University. Synbiotic microencapsulation process was done at SEAFAST Center Laboratory, Bogor Agricultural University. The selected materials used for microencapsulation process were whey protein and maltodextrin.

The shrimps (*L. vannamei*) with initial weight of 2.43 ± 0.26 g were obtained from Situbondo Brackishwater Aquaculture Development Center (BBAP), East Java, Indonesia. The shrimps were cultured in plastic aquaria (each with a dimension of $60 \times 40 \times 35$ cm^3). This experiment was conducted in a completely randomized design with five treatment diets including three dosages of encapsulated synbiotic diet [0.5% (A), 1% (B), and 2% (w/w) (B), as well as positive control (PC), and negative control (NC)]. Each treatment was conducted in four replications. The initial densities were 10 shrimps of each tank. Each tank containing 35 L of sea water and kept under controlled conditions (temperature was ranging from 28 to 29 °C, salinity was from 33 to 34 ppt, total ammonia nitrogen was ranging from 0.02 to 0.66 mg/L, dissolved oxygen was ranging from 5 to 6.5 mg/L, and pH was from 7.6 to 7.9). Faeces and uneaten food were sucked out at the same time of water replacement, which was done every day (after the first feeding) up to 10 L.

The experimental diets were prepared by adding the encapsulated synbiotic (0.5%, 1%, and 2% g/kg) to the diet. The control treatment diets were added only with egg white and without supplementation of encapsulated synbiotic. Feed used in this study was commercial shrimp feed pellet (containing 36% protein, 5% fat, 4% fiber, 12% moisture and 15% ash). Feed mixing process was sprayed manually for each treatment. Encapsulated synbiotic weighed according to the treatment and then egg white added as the binder [2% (v/w)] from the total feeding rate (Wang 2007). Feed that has been weighed according to the feeding rate was then put in the mixture homogenously. Feeding was done four times a day (at 07.00; 11.00; 15.00 and 19.00) for 30 days.

After 30 days of encapsulated synbiotic dietary, specific growth rate (SGR) and feed conversion ratio (FCR) were observed. On 31st day, shrimps in the treatments A, B, C, and PC were challenged by injecting 0.1 mL/shrimp with *V. harveyi* intramuscularly in the cell density of 6 log colony forming unit (CFU)/mL, whereas treatment NC was only injected by phosphate buffer saline 0.1 mL/shrimp. *V. harveyi* used in this study was genetically marked by rifampicin resistancy (50 μg/mL). Observations of immune response parameters included the total hemocyte counts (THCs), the differential hemocyte count (DHC) according to the method of Hai and Fotedar (2009) and the phenoloxidase (PO) according to the method of Liu and Chen (2004). Immune responses were observed on 30th, 32nd and 40th day after the initial treatment, while intestine bacteria (total plated) was observed on 0, 30th, 32nd, 35th and 40th day.

Shrimp intestine was isolated and weighed (g) and then put in a microtube that contained 1 mL of phosphate buffer saline (NaCl 0.8%, K_2HPO_4 0.15%, Na_2HPO_4 0.02% and KCl 0.02%). After homogenization, mixture was serially diluted and plated by performing total plate count (Li *et al.* 2009). The media used for total plate count were sea water complete (SWC) (bacto peptone 0.5%, yeast extract 0.1%, glycerol 0.3%, bacto agar 2%, sea water 75%, and distilled water 25%) without rifampicin for total viable bacterial count, SWC with rifampicin (50 mg/mL) for counting the Bacillus NP5 which resistant to Rifampicin (RfR) and thiosulphate citrate bile-salt sucrose (TCBS Criterion, USA) for counting the V. harveyi Rf^R.

The experiment was conducted in a completely randomized design. Significant differences of regressions of survival rate (SR), SGR and FCR were tested by analysis of variance. The results which showed differences were tested by least significance different test. The immune responses are showed in graphs and analyzed descriptively.

3. Results

SR of shrimp was observed for 30 days before and 9 days after *V. harveyi* infection (Figure 1). The encapsulated synbiotic dietary before challenge test did not show a significant different effect on SR. However, after challenge test it showed a significant difference ($p < 0.05$) among shrimps fed with encapsulated synbiotic dietary and infected with *V. harveyi* i.e. treatment A (93.33 ± 5.77%); B (93.33 ± 5.77%); and C (93.33 ± 11.55%); and shrimps without encapsulated synbiotic dietary and infected with *V. harveyi* (PC), that is 63.33 ± 5.77%. NC treatment showed the highest SR (100 ± 0%).

No mortality occurred during encapsulated synbiotic dietary (30 days before challenge test) for all treatments (Figure 2). Mortality started to occur from 32nd day (1st day after *V. harveyi* infection) at PC treatment and continued until 36th day. The mortality of shrimps fed with encapsulated synbiotic dietary (treatment A, B, and C) occurred at 33rd day and until 35th day.

SGR and FCR were observed after encapsulated synbiotic dietary (30 days before infection). The result of feeding, growth performance (SGR), is presented in Figure 3. The result clearly showed the beneficial effects of encapsulated synbiotic dietary on SGR of *L. vannamei*. The shrimps that supplemented with encapsulated synbiotic have significant increase of SGR in comparison to the control treatments (both negative and PC) ($p < 0.05$). The experimental treatments of this study were significantly different for all treatments. The greatest effect was obtained in treatment C [2% (w/w) encapsulated synbiotic dietary] which had a value of 2.98 ± 0.42%, and then treatment B (2.69 ± 0.3%), treatment A (2.23 ± 0.16%), NC treatment (2.12 ± 0.31%), and PC treatment (2.09 ± 0.23%). Both NC and PC control treatments before challenged test were fed by the same treatments, so there was no significant difference between them.

The FCRs of shrimps fed by encapsulated synbiotic were lower compared with the control treatment ($p < 0.05$) (Figure 4). The lowest FCR value was showed by treatment C (1.26 ± 0.19), followed by treatment B (1.56 ± 0.25), then treatment A (1.89 ± 0.08), PC (1.97 ± 0.27), and NC (1.99 ± 0.25).

Immune response of *L. vannamei* was observed at the pre-challenge test of *V. harveyi* (30th day), 1 day after challenge test (32nd day), and 9 days after challenge test (40th day). Observations

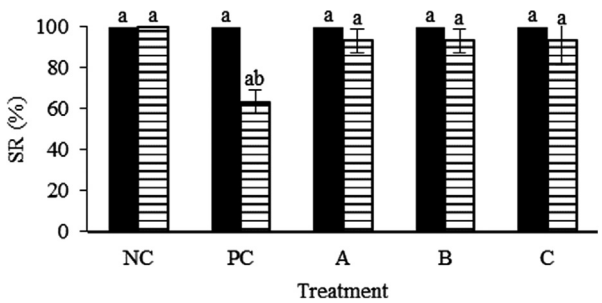

Figure 1. SR of *L. vannamei*. Different letters over each treatment bar (mean ± standard error) indicate significant difference ($p < 0.05$). A: 0.5% encapsulated synbiotic dietary; B: 1% encapsulated synbiotic dietary; and C: 2% encapsulated synbiotic dietary. (■) Before *V. harveyi* infection, (▤) after *V. harveyi* infection. NC = negative control; PC = positive control; SR = survival rate.

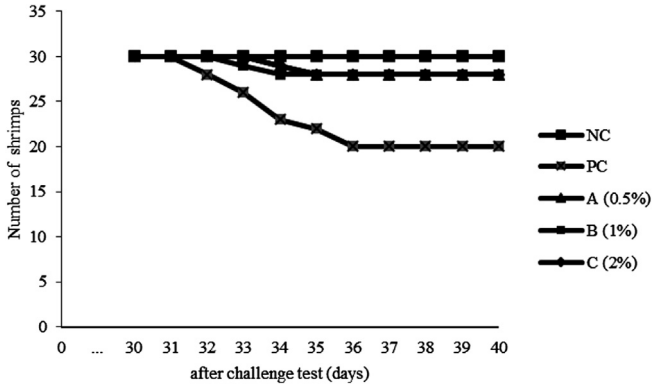

Figure 2. Mortality rate after the challenge test of *L. vannamei* at differential dosages of encapsulated synbiotic dietary. A: 0.5% encapsulated synbiotic dietary; B: 1% encapsulated synbiotic dietary; and C: 2% encapsulated synbiotic dietary. NC = negative control; PC = positive control.

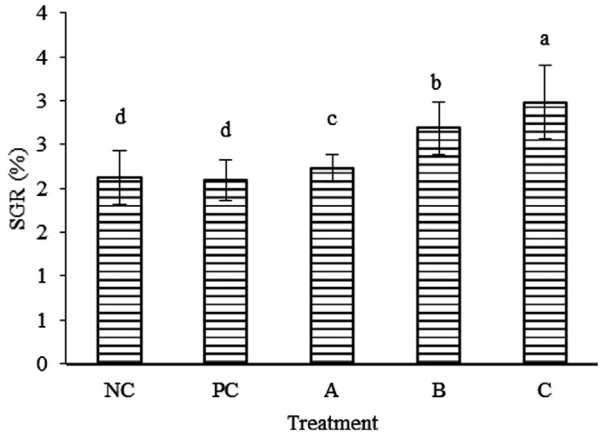

Figure 3. SGR of *L. vannamei*. Different letters over each treatment bar (mean ± standard error) indicate significant difference ($p < 0.05$). A: 0.5% encapsulated synbiotic dietary; B: 1% encapsulated synbiotic dietary; and C: 2% encapsulated synbiotic dietary. NC = negative control; PC = positive control; SGR = specific growth rate.

of immune responses include the THC, PO activity, DHC, and intestinal bacterial population. Total hemocyte after 30 days of encapsulated synbiotic dietary on treatment A, B and C has increased higher than the control treatments (Figure 5). The

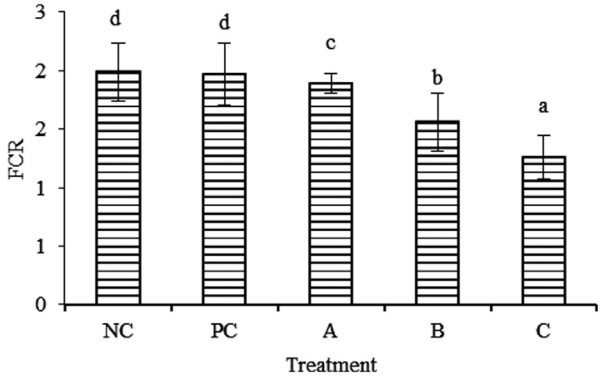

Figure 4. FCR of *L. vannamei*. Different letters over each treatment bar (mean ± standard error) indicate significant difference ($p < 0.05$). A: 0.5% encapsulated synbiotic dietary; B: 1% encapsulated synbiotic dietary; and C: 2% encapsulated synbiotic dietary. FCR = feed conversion ratio; NC = negative control; PC = positive control.

increase also occurred after the challenge test (32nd day) in all treatments. However, at the 40th day, total hemocytes decreased in all treatments.

PO values of encapsulated synbiotic dietary (30th day) showed improvement, especially in treatment C (Figure 6). These results indicated that encapsulated synbiotic dietary was able to stimulate the shrimp immune system by increasing the activity of PO. After challenge test the PO value also increased in all treatments, including control treatments, except for treatment C. Before *V. harveyi* infection, the treatment of encapsulated synbiotic dietary resulted in higher granular cells compared with control treatments (Figure 7). The same results were showed at the time after challenge test and at the end of observation (40th day).

The intestinal bacterial population were observed at 0 (before treatment), 30th, 32th, 35th, and 40th day (the last day of treatment). The observation included bacterial abundance/TVBC, *V. harveyi* Rf^{R} count, and *Bacillus* NP5 Rf^{R} count. The number of bacteria in the intestine was ranging from 7 up to 9 log CFU/g (Figure 8). Intestinal bacterial populations increased with the encapsulated synbiotic dietary treatments (30th day), but did not occur in control treatments. The intestinal bacterial population also increased after challenge test. However declined intestinal bacterial population was noted at 35th day and 40th day in treatments A, B, and C, but not in the PC. The lowering of the intestinal bacterial population was most probably caused by the decreasing population of *Bacillus* NP5 Rf^{R} and *V. harveyi* Rf^{R} in the intestine (Figure 9). *Bacillus* NP5 Rf^{R} populations in the intestine were relatively low on 35th and 40th day, but the B and C treatments of *Bacillus* NP5 Rf^{R} population were still higher than A treatment. On the other hand, the population of *V. harveyi* Rf^{R} at all shrimps treated with encapsulated synbiotic diet was not found in intestines (Figure 9).

4. Discussion

SR of treatment A, B, and C was higher than PC which indicated that encapsulated synbiotic dietary gave positive effect to increase resistance against *V. harveyi* infection. The applications of synbiotic with combination of 0.2% isomaltooligosaccharide prebiotic and probiotic *Bacillus* OJ (PB) 8 log CFU/g of feed was significantly higher ($p < 0.05$) and able to produce a positive synergistic effect on the immune system of shrimp against white spot syndrome virus infection (Li *et al.* 2009).

The results of this study also showed that the encapsulated synbiotic dietary on feed provide a beneficial effect on growth performance and FCR. Increasing growth rate was found along increasing dosage. Latest research done by Widanarni *et al.* (2014)

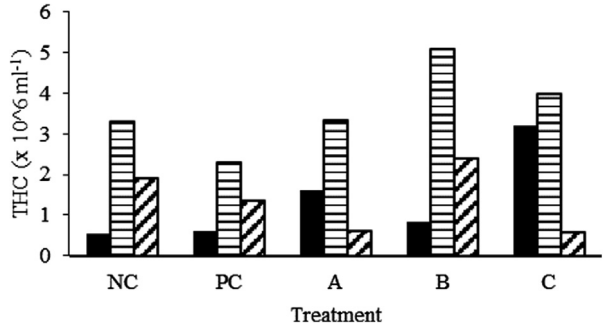

Figure 5. THC of *L. vannamei*. A: 0.5% encapsulated synbiotic dietary; B: 1% encapsulated synbiotic dietary; and C: 2% encapsulated synbiotic dietary. (■) Before *V. harveyi* infection (the 30th day), (▤) 1 day after *V. harveyi* infection (the 32nd day); (◪) 9 days after *V. harveyi* infection (the 40th day). NC = negative control; PC = positive control; THC = total hemocyte count.

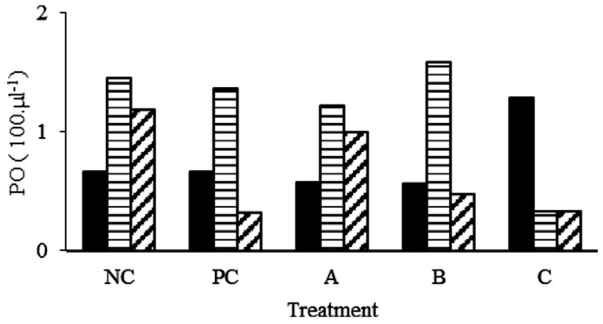

Figure 6. PO of *L. vannamei*. A: 0.5% encapsulated synbiotic dietary; B: 1% encapsulated synbiotic dietary; and C: 2% encapsulated synbiotic dietary. (■) Before *V. harveyi* infection (the 30th day), (▤) 1 day after *V. harveyi* infection (the 32nd day); (◪) 9 days after *V. harveyi* infection (the 40th day). NC = negative control; PC = positive control; PO = phenoloxidase.

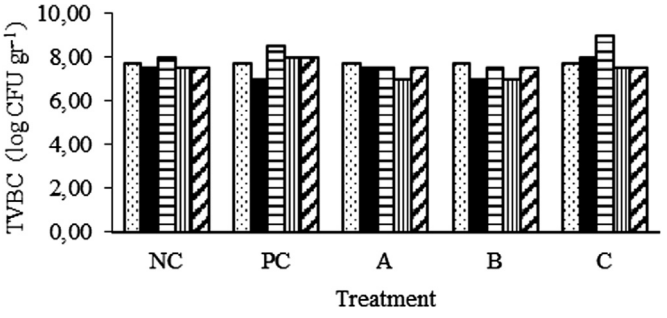

Figure 8. TVBC in the intestine of *L. vannamei*. A: 0.5% encapsulated synbiotic dietary; B: 1% encapsulated synbiotic dietary; and C: 2% encapsulated synbiotic dietary. (▣) Before treatment of encapsulated synbiotic dietary (the 0 day); (■) before *V. harveyi* infection (the 30th day); (▤) 1 day after *V. harveyi* infection (the 32nd day); (▥) 4 days after *V. harveyi* infection (the 35th day); (◪) 9 days after *V. harveyi* infection (the 40th day). NC = negative control; PC = positive control; TVBC = total viable bacterial count.

showed that growth rate increased with supplementation of probiotic *Bacillus* NP5 at a dose of 8 log CFU/mL. The increase of growth rate was assumed because of enzymatic activity in shrimp intestine. Probiotic *Bacillus* NP5 that were used in this study were isolated from tilapia intestine which is capable of secreting amylase enzyme (Putra *et al.* 2015) and has been adapted to SWC medium to survive when exposed to sea water and in shrimp intestine. The amylase enzyme acts as an exogenous enzyme (Taoka *et al.* 2007; Wang 2007). This enzyme was suggested to have capability to stimulate endogenous enzyme that is produced by shrimps (Saeed *et al.* 2006), so feed that is absorbed in shrimp intestine can be degraded effectively, then the optimum nutritional absorption can be reached. Besides that, prebiotic also provided as the additional nutrients for probiotic bacteria (Evivie 2013).

The lower FCR value in this study indicated that the shrimps fed by encapsulated synbiotic dietary showed the effectivity in nutrient digestibility. Similar result reported by Nurhayati *et al.* (2015) that supplementation of synbiotic SKT-b gave a significant effect on the growth and feed conversion of shrimp (*L. vannamei*).

In observation of immune responses, THC value in this study showed an increase after infection of *V. harveyi* for all treatment and then declined. This means a rapid reaction of shrimp immunity to infection. The treatment C showed no significant different before and after infection of *V. harveyi*. This suggested that supplementation of encapsulated synbiotic with optimum dosage have the capability to stimulate the production of hemocyte cell, therefore, the infection did not affected the THC value. A study presented by Chiu *et al.* (2007) showed that the probiotics was capable of increasing the THC value as well as enhancing the immune response during the period of stress because of pathogen infection. Hemocyte cell count decline is an effect of the body's defense mechanisms such as the infiltration of the networks of infected hemocytes, and hemocyte cell death due to apoptosis mechanism (Costa *et al.* 2009). Pro-PO activity system and other humoral body defense mechanisms also affect the number of hemocyte cells (Huang *et al.* 2013; Tassanakajon *et al.* 2013).

The increase and decrease of THC were due to the increase and decrease components of its hemocyte cells. Hemocyte consists of three types of granules in the cytoplasm, i.e. the hyaline, granular and semigranular hemocytes. The percentage of granular cells and semigranular in this study was made into one group, namely the percentage of granular cells. The hyaline and granular cells contributed to destroy the antigen at shrimp body through phagocytosis, encapsulation, nodule formation and produced humoral components. Humoral components are stored in granule hemocyte which include anticoagulant protein, aglutinin, PO enzymes, antimicrobial peptides, and protease inhibitors (Jiravanichpaisal *et al.* 2006).

PO is an enzyme responsible for melanization process in crustaceans as response to antigen and for pigmentation (Zufelato *et al.* 2004). PO system can be activated by several microbe polysaccharides and specific pattern recognition proteins, such as LPS (lippo polysaccharides) and β-1, 3-glucan-binding protein and peptidoglycan-binding proteins (Wang 2007). The treatment C

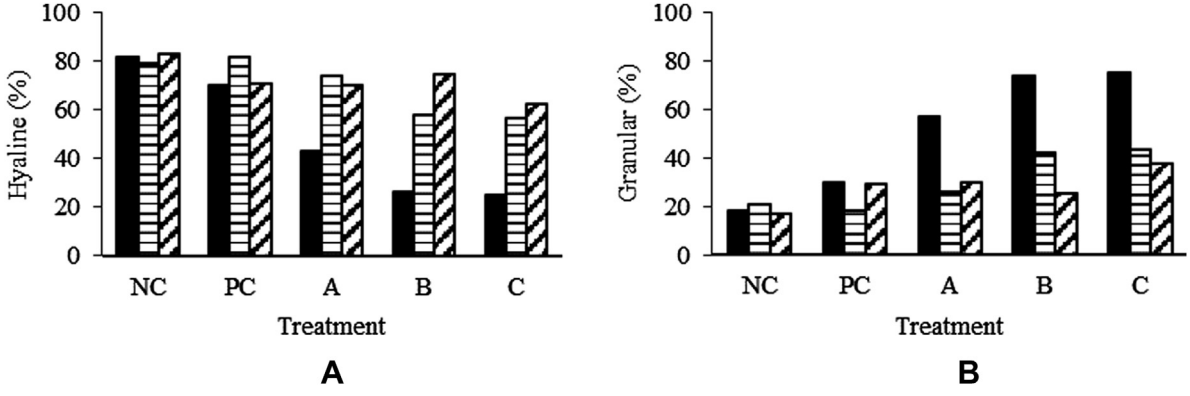

Figure 7. Differential hemocyte count: (A) hyaline count; and (B) granular count. A: 0.5% encapsulated synbiotic dietary; B: 1% encapsulated synbiotic dietary; and C: 2% encapsulated synbiotic dietary. (■) Before *V. harveyi* infection (the 30th day), (▤) 1 day after *V. harveyi* infection (the 32nd day); (◪) 9 days after *V. harveyi* infection (the 40th day). NC = negative control; PC = positive control.

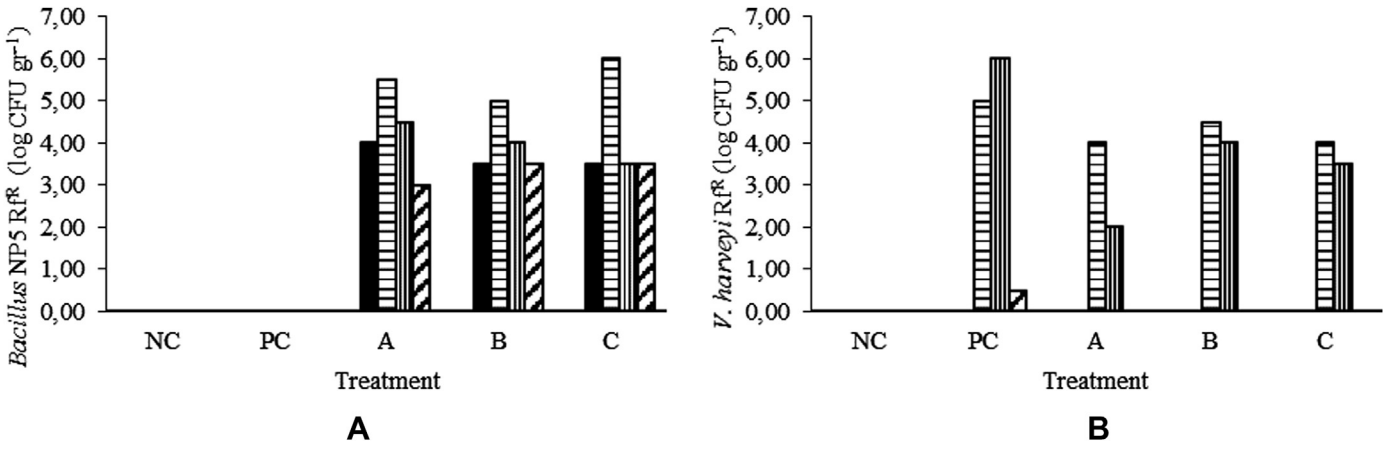

Figure 9. (A) *Bacillus* NP5 Rf^R count; and (B) *V. harveyi* Rf^R count in the intestine of *L. vannamei*. A: 0.5% encapsulated synbiotic dietary; B: 1% encapsulated synbiotic dietary; and C: 2% encapsulated synbiotic dietary (the 0 day); (■) before *V. harveyi* infection (the 30th day), (▤) 1 day after *V. harveyi* infection (the 32nd day); (▥) 4 days after *V. harveyi* infection (the 35th day); (◪) 9 days after *V. harveyi* infection (the 40th day). NC = negative control; PC = positive control.

showed higher increase of PO value before infection and then declined after infection. Increasing of PO activity has caused the shrimp's ability to recognize antigen that enters the body's system (Garcia-Carreno *et al.* 2008). The increase of PO value before infection was the effect of dietary supplementation by encapsulated synbiotic. Probably, the supplementation of probiotic *Bacillus* NP5 has increased the β-1, 3-glucan-binding protein content in the gut shrimp, as it has been reported by Hao *et al.* (2014) which resulted in improvement of PO system. Other study reported that the decline of PO is a sign of recovery period from *V. harveyi* infection (Huang *et al.* 2013).

Encapsulated synbiotic dietary increased the population of bacteria in shrimp intestine up to 9 log CFU/g. Similar result was reported by Li *et al.* (2007) that the addition of short-chain fructo-oligosaccharides was able to increase the growth of bacteria in the shrimp digestive tract. The growth of bacteria in shrimp intestine dominated by probiotic *Bacillus* NP5 Rf^R indicated by its population which reached 5 log CFU/g. This suggested that *Bacillus* NP5 Rf^R, supplied with prebiotics which has been encapsulated, was able to stick well and utilizes prebiotics on shrimp intestine. The addition of prebiotic oligosaccharides can improve the shrimp health because the existence of beneficial intestinal bacteria suppresses potentially pathogenic bacteria (Ringo *et al.* 2010). This statement reinforces findings that encapsulated synbiotic dietary was also able to suppress the growth of *V. harveyi* in shrimp intestine.

In summary, the encapsulated synbiotic dietary for 30 days with different dosages gives significant effects on the growth performance and FCR. The best dosage was shown by application of 2% encapsulated synbiotic dietary (treatment C). The supplemented diet by 2% encapsulated synbiotic showed significantly higher growth performance (2.98 ± 0.42%), FCR (1.26 ± 0.19), and immune responses to *V. harveyi* infection than control treatments.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgments

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