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# The Use of Synbiotics to Prevent IMNV and *Vibrio harveyi* Co-Infection in *Litopenaeus vannamei*

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**This study evaluated the effects on viral immune responses and bacterial co-infection, of different feeding frequencies of a synbiotic supplemented diet given to Pacific white shrimp (*L. vannamei*). A synbiotic-supplemented diet was formulated from probiotic *Vibrio alginolitycus* SKT-b$^R$ and prebiotics from sweet potato (*Ipomoea batatas* L.) oligosaccharide. Pacific white shrimp were fed with synbiotic diet at different frequencies, i.e. daily (P1), twice a week (P2), and once a week (P3) for a 30 day pre-challenge test. After the 30 day feeding period, the shrimps were challenged by intramuscular injection of Infectious Myonecrosis Virus (IMNV) and *Vibrio harveyi*. The results showed that shrimp treated with a synbiotic-supplemented diet showed significantly higher growth performance than control groups (P<0.05). Shrimp treated under regime P1 showed the highest values for phenoloxidase (PO) and respiratory burst (RB) parameters compared to shrimp given with other treatments. Following the challenge test, higher survival rate were seen in the P1 treatment group, in comparison to the positive control, and the P1 treatment group showed the highest values in total haemocyte count (THC), PO, and RB.**

Key words: synbiotic, IMNV, *Vibrio harveyi*, co-infection, *Litopenaeus vannamei*

## INTRODUCTION

The Pacific white shrimp (*L. vannamei*) is the most cultivated shrimp species in the world (Teixeira-Lopes *et al*. 2011). Shrimp production in Indonesia has significantly increased in the past three years and the Government of Indonesia is promoting even greater rates of shrimp production in 2014. However, the current system of shrimp cultivation in Indonesia face several challenges, such as high rates of infectious disease.

The diseases that most often impact intensive shrimp cultivation are bacterial, viral, and co-infectious diseases (Teixeira-Lopes *et al*. 2011). According to Poulos *et al*. (2006), one of the most lethal and problematic viral diseases found in white shrimp culture is the Infectious Myonecrosis Virus (IMNV). IMNV can cause massive mortality of up to 70% in shrimp populations (Hasan 2011) and IMNV spread to Indonesia in 2006 (Senapin *et al*. 2007). Shrimp production is also hampered by *Vibrio* spp., a bacterial infectious disease often acting as a cause of secondary infection (Liu & Chen 2004). Teixeira-Lopes *et al*. (2011) reported that, in a farm of northeast brazil, white shrimp may naturally be infected by two different kinds of viruses, the *Infectious Hypodermal and Hematopoietic Virus* (IHHNV) and IMNV. Phuoc *et al*. (2009) reported in shrimp pond, shrimp body that infected already by *White Spot Syndrome Virus* (WSSV) could be easily infected by *Vibrio* spp. bacteria.

Infection by several pathogens at the same time (co-infections) can accelerate and increase mortality (Hasan 2011). Therefore, a specific method is needed to prevent these co-infections. According to Li *et al.* (2009), synbiotics represent an alternative method that can be used to decrease disease incidence, since they have been proven to increase the shrimp immune response and resistance to disease. Synbiotics are usually comprised of a balanced combination of probiotics and prebiotics for supporting the survival and growth of beneficial bacteria in the digestive tract.

Probiotics are usually live microorganisms which, when administered in adequate amounts, confer health benefits to their host (Nayak 2010). A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Ringo *et al*. 2010). Some studies have shown the benefits of using probiotics (Panigrahi *et al*. 2005; Watson *et al*. 2008; Nayak 2010) and prebiotics (Soleimani *et al*. 2012) separately for addressing disease in shrimp aquaculture. However, Merrifield

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*et al.* (2010) has reported that the application of synbiotics is better than the separate application of probiotics and prebiotics for reducing disease rates in shrimp aquaculture. Results of some studies indicate that administration of synbiotic through feed can effectively increase growth performance (Geraylou *et al*. 2013), increase immunity in fish and shrimp, and increase host resistance to pathogenic infection (Li *et al*. 2009; Ai *et al*. 2011; Lin *et al.* 2012).

IMNV and *V. harveyi* co-infection can cause a higher mortality rate in shrimp cultivation than other types of infection or than infection by either pathogen alone. Therefore, an application of synbiotics is suggested as an alternative method to conventional antibiotic treatment, for preventing the co-infection. The conventional antibiotic treatment considered to be bad due to antibiotic resistance, pollution, health or cost concern. Duration of supplementation is an important factor in increasing effectiveness of supplement application in cultured organisms at the commercial level (Merrifield *et al*. 2010). In addition, work by Nayak (2010) indicated that the frequency of synbiotic administration is an important factor which can affect the establishment and subsequent induction of immune responses in the host. This study aimed to evaluate the effect of different frequencies of synbiotic dietary supplementation given to Pacific white shrimp (*L. vannamei*) to boost viral immune response and prevent bacterial co-infection.

## MATERIALS AND METHODS

**Experimental Design.** This study was conducted for six weeks in the Fish Health Laboratory, the Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University. The experiments used pasific white shrimp *L. vannamei* in post-larval stadia (PL) 15 which were obtained from a commercial hatchery in Carita, Banten. Prior to the study, the post-larvae were acclimatized to laboratory conditions for 14 days. Then the shrimp were weighed (0.3 g $\pm$ 0.03 mean initial weight), and randomly distributed to five experimental groups, each of which had three replications. Each replication contained 15 shrimp, reared with the experimental diet for 30 days. The experimental diets were provided at amounts equal to 10-12% body weight. Shrimp were fed to apparent satiation four times daily. Water quality during the experiment was maintained by siphoning out shrimp faeces and exchanging culture media at a rate of 10% daily. Water quality during the experiment was kept at the following parameters: temperature 28-29 $^{o}$C, salinity 29-32 ppt, dissolved oxygen 4.5-6.5 mg/L, pH 7.4-7.5, and ammonia-nitrogen 0.005-0.016 mg/L.

**Virus and Bacteria Stock.** The virus used in this study was *Infectious Myonecrosis Virus* (IMNV). The virus stock was obtained from IMNV-infected White shrimp from Situbondo Brackish Water Aquaculture Development Center (BBAP) Indonesia. The virus was extracted and stored -80 $^{o}$C of temperature according to Rodriguez *et al*. (2007). The bacteria stock was *Vibrio harveyi* (MR 5339) which has been tagged with rifampicin-resistant gene sequences. The *V. harveyi* isolates were obtained from the Fish Health Laboratory, the Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University.

**Probiotic and Prebiotic Preparation.** The probiotic bacteria used in our experimental supplement was *Vibrio alginolitycus* SKT-b; its isolates had been tagged with rifampicin-resistant gene sequences (SKT-b$^{R}$) according to Widanarni *et al*. (2003), and re-characterized. Culturing of SKT-b$^{R}$ probiotic bacteria was performed in Sea Water Complete (SWC-broth) (5 g bactopeptone, 1 g yeast extract, 3 mL glycerol, 750 mL sea water, and 250 mL aquades) that incubated in a waterbath shaker at a temperature of 29-30 $^{o}$C, 160 rpm for18 h. We obtained cell bacterial pellets which were then washed twice in Phosphate Buffer Saline (PBS) solution.

The preparation of the prebiotic was conducted in several stages according to the method described by Marlis (2008). Production of probiotic was started with production of sweet potato starch, extraction of oligosaccharide using ethanol 70%, and measurement of total dissolved solids. Then the type and content of oligosaccharides were analyzed using High Performance Liquid Chromatography (HPLC) with the following results: 1.115% inulin, fructooligosaccharides (FOS) 1.015%, and galactooligosaccharides (GOS) 1.488%.

**Experimental Diet Preparation.** The experimental diet was prepared by adding 1 dose (1% probiotic and 2% prebiotic of feed weight) of synbiotic into commercial pellets contained 40% crude protein (Nurhayati *et al*. 2014). Combining of feed and synbiotic was carried out by adding 2% egg white as binder; the feed for control groups contained only the 2% egg white without synbiotics. The shrimp larvae were fed this diet for 30 days using a Completely Randomized Design consisting of five treatments in triplicate: control (-) (untreated diet without challenge test); control (+) (untreated diet with challenge test),

P1 (daily synbiotic supplementation with challenge test), P2 (twice a week synbiotic supplementation with challenge test), and P3 (once a week synbiotic supplementation with challenge test).

**Challenge Test.** Ten shrimp from each replication were challenged after receiving the experimental diet for 30 days. The challenge test aimed to study the performance of synbiotics treatment in increasing IMNV and *V. harveyi* resistance in white shrimp. The shrimp were first infected with IMNV at a dose of 100 µL, and then infected three days later with $10^3$ CFU/mL *V. harveyi*. Infection was conducted by intramuscular injections using 1 mL syringe in the ventral side between the second and third segments (Burge *et al*. 2007). The negative (-) control group was injected with PBS instead. The shrimp taking part in the challenge test were observed for seven days after the first infection with *V. harveyi*.

**Experimental Parameters.** Experimental parameters measured consisted of growth performance, number of bacteria in the intestines, immune response, and shrimp resistance to co-infection. The growth parameters observed included daily growth rate (DGR) and feed conversion ratio (FCR) (Soleimani *et al*. 2012). The number of bacteria in the intestines were measured via total viable bacterial counts (TBC), presumptive vibrio counts (VBC) and the presumptive SKT-$b^R$ counts (Li *et al*. 2009). The growth performance and number of bacteria in the intestines were measured directly after 30 days of synbiotic treatment. Survival is expressed as a percentage of the number of shrimp at the end of the experiment compared to the number of shrimp at the beginning of the experiment.

The immune response parameters measured included total haemocytes count (Hai & Fotedar 2009), phenoloxidase activity (Hsieh *et al*. 2008), and respiratory burst activity (Akrami *et al*. 2013). Shrimp resistance was observed by calculating the number of shrimp surviving co-infection of IMNV and *V. harveyi,* seven days following the challenge test. Observation of the shrimp's condition for seven days revealed symptoms of IMNV infection among all the shrimp injected with the pathogen. This finding was confirmed by the PCR results, which confirmed that the virus injected was indeed the IMN. Immune response and shrimp survival were measured twice: directly after the 30 day feeding period/pre-challenge, and seven days after the challenge test injections.

**Data Analysis.** The data obtained were statistically analyzed using the SPSS 16 statistical software and were further tested using Duncan test at $P<0.05$.

## RESULTS

**Growth Performance.** There was a significant difference in DGR between synbiotics treatments and controls ($P<0.05$). However, there was no such difference among synbiotics treatments ($P>0.05$). The highest DGR recorded was 8.12%, in the P1 group, with the next highest measures seen in the P2, P3, and control groups(7.78%, 7.66%, control positive 7.21%, and control negative 7.18%; respectively) (Figure 1A).

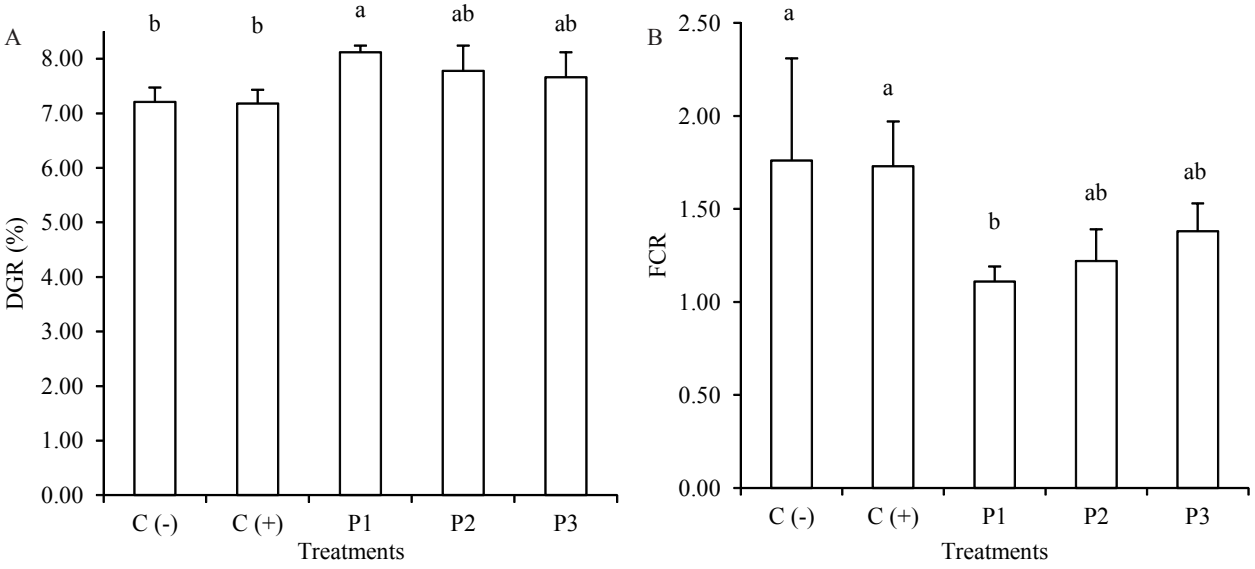


Figure 1. Daily growth rate (DGR) (A) and feed conversion ratio (FCR) (B) of Litopenaeusvannamei. Different letters over each treatment bar (mean ± SE) indicated significant difference (Duncan; $P<0.05$). C(-): control negative; C (+): control positive; P1: daily supplementation of synbiotic in diet; P2: twice a week supplementation of synbiotic in diet; P3: once a week supplementation of synbiotic in diet.

FCR value for P1 treatment was significantly lower (P<0.05) than those of control (both positive and negative), but were not significantly different compared to those of P2 and P3. The lowest FCR (1.11) was found in P1 treatment (Figure 1B).

**The Number of Bacteria in the Shrimp's Intestines.** Dietary supplementation with synbiotics for 30 days may increase the number of bacteria in treated shrimp intestines. In our study we found enhanced numbers of bacteria in total viable bacterial counts (TBC), presumptive vibrio counts (TVC), and presumtive SKT-$b^R$ counts. The highest measures for those parameters were identified in the intestines of the shrimp in the P1 group (Table 1).

**Immune Response.** Total Haemocyte Counts (THC) detected during the pre-challenge test were significantly different (P<0.05) between all groups receiving synbiotics treatment, and both controls. The THC value for the negative control group was 3.93 x $10^6$ cells/mL and for the positive control was 3.73 x $10^6$ cells/mL, while values for the shrimp in treatment groups P1, P2, and P3 were 6.40 x $10^6$ cells/mL, 6.80 x $10^6$ cells/mL and 6.92 x $10^6$ cells/mL, respectively. The shrimp THC values during the post challenge test for P1 was significantly higher (P<0.05) than those of positive control, P2 and P3; but was not significantly different to negative control.

Phenoloxidase activity (PO) values measured during the pre-challenge test in P1 and P2 treatments were significantly higher (P<0.05) than those of P3 and both controls (Figure 2B). After the challenge test, PO values in treatments P1 was higher than those of P3 and both control.

Respiratory burst activity (RB) values measured during the pre-challenge test did not show a significant difference among treatments. By contrast, RB values during the post challenge test were significantly different (P<0.05) between the groups receiving synbiotics treatment, and both controls. The RB values of each of the treatment groups P1, P2, and P3 were also significantly different from each other (P<0.05) (Figure 2C). The highest RB value was recorded in the P1 treatment group.

**Survival.** The survival of shrimp at the pre-challenge test was 100% for all treatment groups. However, the survival of shrimp at the post challenge test, all treatments including negative control were significantly higher (P<0.05) than those of positive control treatments. The survival rates obtained for negative control, P1, P2, and P3 groups were 100, 93.33, 80.00, and 73.33%, respectively (Figure 3 & 4); whereas the positive control was 46.67%.

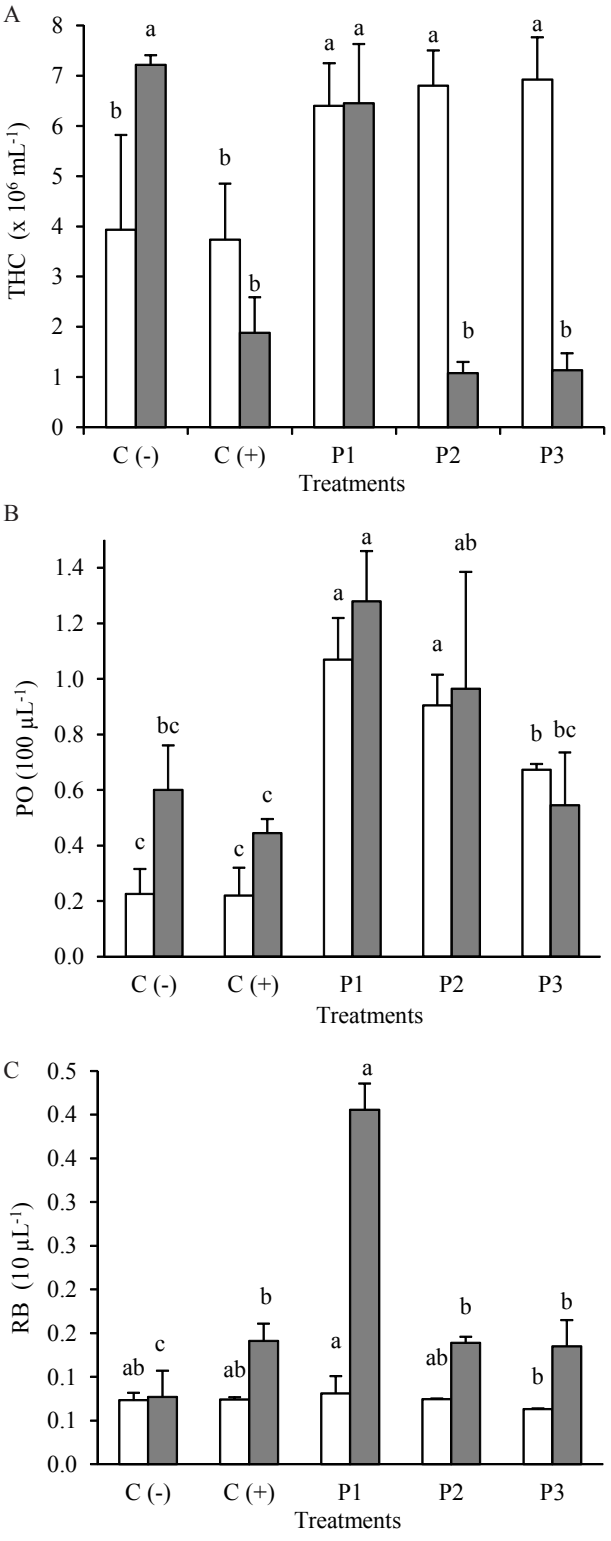


Figure 2. Total haemocytes (THC) (A); Phenoloxidase activity (PO) (B); and Respiratory burst activity (RB) (C) of *Litopenaeus vannamei*. Different letters over each treatment bar in same colors (mean ± SE) indicated significant difference (Duncan; P<0.05). C(-): control negative; C (+): control positive; P1: daily supplementation of synbiotic in diet; P2: twice a week supplementation of synbiotic in diet; P3: once a week supplementation of synbiotic in diet. □ Pre-challenge test, ■ Post challenge test.

Table 1. The number of bacteria in the intestines of the test shrimp (*L. vannamei)*

| Treatment | Total bacteria (CFU/g) | Total vibrio bacteria (CFU/g) | Total SKT-bR probiotic bacteria (CFU/g) |
|---|---|---|---|
| (-) Control | $6.00 \times 10^5$ | $2.00 \times 10^3$ | - |
| (+) Control | $5.00 \times 10^5$ | $3.00 \times 10^3$ | - |
| P1 | $2.21 \times 10^7$ | $2.31 \times 10^5$ | $1.83 \times 10^4$ |
| P2 | $1.34 \times 10^7$ | $1.73 \times 10^5$ | $1.23 \times 10^2$ |
| P3 | $3.22 \times 10^6$ | $1.03 \times 10^4$ | $9.51 \times 10^1$ |

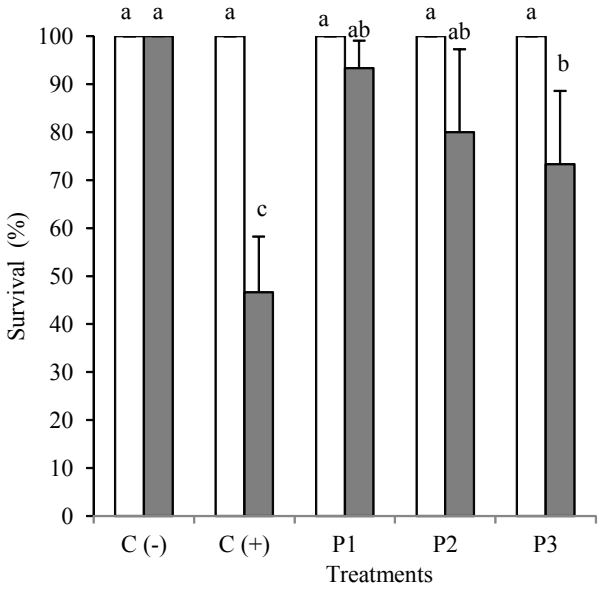


Figure 3. Survival of *L. vannamei*. Different letters over each treatment bar in same colors (mean ± SE) indicated significant difference (Duncan; $P<0.05$). C(-): control negative; C (+): control positive; P1: daily supplementation of synbiotic in diet; P2: twice a week supplementation of synbiotic in diet; P3: once a week supplementation of synbiotic in diet. □ Pre-challenge test, ■ Post challenge test.

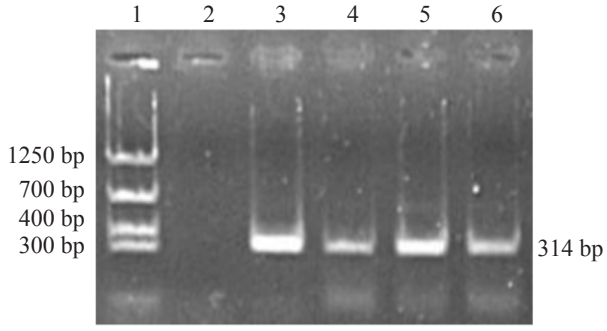


Figure 4. The PCR test results for the test shrimp (*L. vannamei*) post challenge test by IMNV and *V. harveyi*. Agarose gel of amplicons with 3 shrimp samples showing gross signs of white muscle tissue. Lane 1: marker; Lane 2: negative control; Lane 3: positive control; Lane 4: sample A IMNV positive; Lane 5: sample B IMNV positive; and Lane 6: sample C IMNV positive. The band at 314 bp for sample indicates a light IMNV infection according to the kit instructions.

## DISCUSSION

The shrimp which were fed an experimental diet containing synbiotics supplements showed better growth performance compared to those in the control groups. The highest growth was measured in the P1 treatment group. Other research has shown that the dietary administration of synbiotics can influence the growth performance of shrimp. Lesmanawati (2013) suggested that the administration of SKT-b bacteria via dietary supplements in white shrimp may beneficially impact the shrimps' digestive enzyme activity. Ai *et al*. (2011) reported that gastrointestinal bacteria take part in the decomposition of nutrients, providing the host organism with physiologically active materials such as enzymes, amino acids, and vitamins that enhance food utilization and digestion. Improvement in growth and FCR as a result of dietary supplementation with synbiotics has been previously established, and has been credited to physiological and biological changes in the gastrointestinal environment and morphological changes to the epithelium (Daniels *et al*. 2010). Other studies indicate that the application of synbiotics allow for more efficient conversion of ingested food into structural protein, with subsequent improved growth (Hai & Fotedar 2009).

In this study, the administration of synbiotics via diet for 30 days led to an increase in the number of bacteria, *Vibrio,* and probiotic SKT-b in shrimp intestines. We found the highest number of bacteria in the P1 treatment group. Other research supports this finding that synbiotic supplements increase the function and number of beneficial bacteria in the intestines (Hai & Fotedar 2009). According to Daniels *et al*. (2010), the administration of mannan oligosaccharides (MOS) in lobsters resulted in improved bacterial stability in the gastrointestinal tract, compared to diet without MOS. Li *et al*. (2009) reported that increased numbers of *Bacillus* and isomaltooligossacharides (IMO) in white shrimp led to a reduction in the viable count of total bacteria and *Vibrio* in shrimp intestines, yet to an increase in shrimp immuno-modulatory function.

Some studies have reported that the dietary administration of synbiotics in shrimp may decrease high mortality rates, and improve host immunity (Li *et al*. 2009; Zhang *et al*. 2012). In the present study, shrimp fed a synbiotics-supplemented diet through daily administration showed the highest survival following the post challenge test. Our results showed that the probiotics SKT-$b^R$ and the oligosaccharides from sweet potatoes could increase the shrimp resistance against co-infection by multiple pathogens. Septiani (2011) similarly found that the administration of the probiotics SKT-b and prebiotics oligosaccharide from the sweet potatoes (*I. batatas* L.) increased the immune response of white shrimp against IMNV infection. Synbiotics mechanism in stimulating the immune response in fish and shrimp have not been widely studied. In mammals, the mechanism synbiotics begins with the metabolism of prebiotics by probiotics produce short-chain fatty acids/short chain fatty acids (SCFA). SCFA lowers intestinal pH in the colon, resulting in conditions that do not suit the needs of pathogenic bacteria (Delgado 2011). Nayak (2010) explained that the gut is an organ of a growing probiotics, the probiotics which are associated with lymphoid tissue that could activate the immune system (Gut Associated Lymphoid Tissue [GALT]).

Our results suggest that administration of the dietary symbiotics supplements at less frequent intervals may increase the THC values of treated shrimp compared to the control ones. Haemocytes play an important role in the crustacean immune system because haemocytes are able to eliminate foreign particles in the body (Hauton 2012). High numbers of haemocytes in the haemolymph may minimize the IMNV infection and increase the survival of IMNV-infected shrimp (Lesmanawati 2013). For the post challenge test, the THC values obtained decreased from pre-challenge levels in the P2 and P3 treatment groups, and the positive control. The decrease in haemocytes numbers is an effect of crustacean defense mechanism in response to viral infections (Hauton 2012). Unexpectedly, P1 treatment did not result in a decrease in haemocytes in the post challenge test. Apparently daily synbiotics application might actually increase host ability to produce new haemocytes cells compared to P2 and P3 treatments. Sang *et al*. (2009) stated that shrimp haemocytes have the ability to proliferate, the proliferation rate can be improved by giving immunostimulant and lipopolysaccharide as well as prebiotics. Response to infection causes loss of free circulating haemocytes cells in large number, after that followed by recovery through rapid synthesis and release of new haemocytes by hematopoietic tissue (HPT) (Lin & Soderhall 2011). Formation of new sustainable haemocytes very important for the survival of the host.

Phenoloxidase activity plays a crucial function in invertebrate immune defense, by activating the melanin synthesis pathway and producing cytotoxins to kill invading microorganisms. PO and immuno-competence have been shown to be positively correlated in numerous invertebrates where organisms with higher PO are less susceptible to infection (Zhang *et al*. 2012). In the present study, the P1 and P2 treatment groups showed the highest PO measures during observation in the pre-challenge test. This result indicates that the administration of synbiotics either daily or every three days, may improve shrimp resistance against infection. Li *et al.* (2009) reported that PO increased with increased doses of probiotic bacteria and prebiotics. Phagocytosis is the most common cellular defense system used by fish and shrimp. During phagocytosis, particles or microorganisms are engulfed by host cells, which then form digestive vacuola called phagosomes. Phagocyte cells have various mechanisms for eliminating pathogens. The mechanism of Respiratory Burst (RB) involves the release of degradative enzymes by the phagocytes cell to the phagosome to eliminate particles (oxygen dependent killing mechanism). There were no significant differences ($P>0.05$) in RB value detected between treatment groups at the pre-challenge period. After the challenge test, the highest RB value was detected in the P1 treatment group. This suggests that increased frequency of synbiotics application leads to increased respiratory burst activity, and apparently can play a role in ensuring immune response stability that affects shrimp resistance to diseases attack. Chiu *et al*. (2010) reported that RB values in *Epinephelus coioides* fed with a diet containing probiotics *Saccharomyces cerevisiae* were higher than those measured in the control group. Similarly, Li *et al.* (2009) reported that the RB values in white shrimp treated with the *Bacillus* OJ probiotics and the isomaltooligosaccharide prebiotics were higher than those measured in the control group. Li *et al.* (2009) also found that RB value increased after the shrimp was co-infected. RB values increased also in Senegalese sole (*Solea senegalensis*, Kaup) after infection with *Photobacterium damselae* subsp. *piscicida* (Diaz-Rosales *et al*. 2009). The increase in RB is considered to be beneficial with respect to enhancing immunity against viral infection (PyngYeh

*et al*. 2009). The conclusion from this study is that the daily administration of synbiotic dietary supplements demonstrated the best results for growth performance, with a daily growth rate of 8.12 ± 0.12% and a feed conversion ratio of 1.11 ± 0.08. It also showed the best results for immune response and resistance to co-infection of IMNV and *V. harveyi.*

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