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Dietary Synbiotic Microcapsule Influence the Immune Responses, Growth Performance and Microbial Populations to White Spot Syndrome Virus in Pacific White Shrimp (*Litopenaeus vannamei*)

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ABSTRACT

White Spot Syndrome Virus (WSSV) is one of the most devastating shrimp pathogen which causes up to 70-90% mortality in commercial shrimp farming. This study was aimed to evaluate the effectiveness of synbiotic microcapsule (*Bacillus* NP5 Rf^R and mannan oligosaccharide combination) feed supplementation on the immune responses and growth performance of WSSV infected Pacific white shrimp. Shrimps (4.411±0.395 g) were cultured and fed by pellet containing synbiotic microcapsule at different dosages for 30 days, i.e., designated as 0.5% (M1), 1% (M2), 2% (M3) (w/w) and without synbiotic microcapsule addition (negative and positive controls). The synbiotic was encapsulated by spray drying method. Challenge test was performed at 31st of cultivation day by performing intramuscular injection of WSSV filtrate (10^4 copies mL^{-1}). Compared with control, the shrimp fed by M2 and M3 were found to have higher ($p<0.05$) value of Total Hemocyte Count (THC), pro Phenol Oxidase (proPO) and Respiratory Burst (RB) activity compared to positive control. The results showed that the Specific Growth Rate (SGR) of M1 and M2 were higher ($p<0.05$) than controls. Yet, the Feed Conversion Ratio (FCR) were lower ($p<0.05$) than controls. Above all, the survival rate of shrimps fed with microcapsule synbiotic were higher ($p<0.05$) than controls.

Key words: Synbiotic, microcapsule, immune response, *Litopenaeus vannamei,* WSSV

INTRODUCTION

Infectious disease is one of issues which directly affect the production of aquaculture activities. White Spot Disease (WSD) is one of infectious disease in shrimp caused by white spot syndrome virus. The WSSV infects various hosts and spread rapidly in aquaculture system (Paz, 2010). Moreover, WSSV caused a lethal infection reaching 70-90% of mortality level within 3-7 days of post-infection (Mai and Wang, 2010). The main target of WSSV infection is ectodermal and mesodermal tissues, especially epithelial cuticle and subcuticular connective tissue (Tang *et al*., 2013). The WSD infected shrimp is characterized by a rapid reduction in food consumption, become lethargic, have a loose cuticle with some showing characteristic white spots of 0.5-2.0 mm in diameter, which are most apparent inside the surface of the carapace. The white spots represent abnormal deposits of calcium salts by the WSSV infected cuticular epithelium. In many cases, moribund shrimps with WSD show a pink to reddish-brown coloration, due to the expansion of the cuticular chromatophores and few if any white spots (Lightner, 2011).

Probiotics, prebiotics, or even synbiotic application is proposed to be ideal and effective disease control strategies that encourage the sustainability in aquaculture. The probiotic in aquaculture defined as a live, dead or component of a microbial cells that administered through the feed supplementation or to the rearing media which could improve either disease resistance, health status, growth performance, feed utilization and stress response of the host. These are achieved at least in part through improving the microbial community balance in the host or in the ambient environment (Merrifield *et al*., 2010). Prebiotic defined as non-digestible food ingredients which beneficially affect the host by selectively stimulating the growth and/or activity of health promoting bacteria in the intestinal tract (Gibson, 2004). Moreover, synbiotic referred to combined application of probiotics and prebiotics based on the principle of providing a probiont with a competitive advantage (a fermentable energy source) over competing endogenous microbial populations (Merrifield *et al*., 2010).

One approach that widely used to improve the performance of synbiotic is by microencapsulation. Microencapsulation is a method that used to protect material through physicochemical processing which result in tiny particles layered by physical barrier against the harmful environmental conditions (Rathore *et al*., 2013). The goal of microencapsulation is to create a micro-environment in which the bacteria will survive during processing and storage and further released at appropriate sites in the digestive tract (Weinbreck *et al*., 2010). This technique benefits in stabilizing the core material, extending the shelf life and protecting components against environmental conditions (Anal and Singh, 2007; Ying *et al*., 2013). Spray drying of *Bifidobacteria* and oligofructose prebiotic enriched by inulin has maintained the cells viability (Fritzen-Freire *et al*., 2012). The dry product of microencapsulated synbiotic is important for simplify application in the field.

This study was aimed to evaluate the effectiveness of synbiotic microcapsules which consisting of combination of *Bacillus* NP5 Rf^R cells probiotic and the mannan oligosaccharide (MOS) prebiotic on the immune system and growth performances of WSSV-infected Pacific white shrimp.

MATERIALS AND METHODS

Bacterial strain and oligosaccharide: The bacterial strain used in this study, *Bacillus* NP5 Rf^R, was previously isolated from the digestive tracts of tilapia (Putra and Widanarni, 2015) that has been resistant to 50 ug mL^{-1} rifampicin. Prebiotic used in this study, mannan oligosaccharides (MOS), was derived from the cell wall of *Saccharomyces cerevisiae* (containing 30% protein, 1.4% fat and 13% crude fiber). The WSSV isolates used were obtained from the collection of Fish Health and Environment Laboratory of Brackish Water Aquaculture Center Situbondo, East Java.

Pacific white shrimp (*Litopenaeus vannamei*) used in this study (average weight of 4.411±0.395 g) were obtained from Gelung Shrimp Production Unit, Brackish Water Aquaculture Center Situbondo, East Java which provides the SPF (Specific-Pathogen Free)-licensed of WSSV and IMNV through previous PCR test.

Preparation of synbiotic and coating materials: The composition of synbiotic microcapsules in this study referred to the previous dose (Wang, 2007), i.e 1% w/w *Bacillus* NP5 Rf^R (10^9-10^{10} CFU g^{-1} wet weight) and 4 g kg^{-1} MOS of feed (Zhang *et al*., 2012) with some modifications. Probiotic biomass was produced by performing subculture technique on SWC (Sea Water Complete) broth media (5 g bacto peptone, 1 g yeast extract, 3 mL glycerol, 750 mL sea water and 250 mL distilled water) with 10% v/v of *Bacillus* NP5 Rf^R as inoculant (10^8 CFU mL^{-1}).

Incubation of bacteria was carried on thermoshaker at 200 rpm for 18 h at 29°C. The cells were harvested by centrifuging the culture at 6000 rpm for 15 min at 4°C and washed three times with sterile PBS (phosphate-buffered saline: 1.5 M NaCl, 15 mM KH_2PO_4, 100 mM Na_2HPO_4, 30 mM KCl and 1000 mL distilled water). Culture purity and identification were routinely checked during this study by spread plate method.

Coating materials used for microencapsulation synbiotic process in this study were Denatured Whey Protein (DWP) and maltodextrin. The DWP was obtained by enzymatic coagulation of pasteurized milk (De Castro-Cislaghi *et al*., 2012) through addition of rennet (0.05 g L^{-1}) (Enzimaks, IIEC Iran) and calcium chloride solution 40% (m/v) (0.4 mL L^{-1}). The solution was stirred for 10 min and was incubated at 37°C for 1 h. Later, the whey was separated from the curd and was heated at 90°C for 30 min to produce DWP. Afterwards, DWP was stored at 4°C for further use.

Before the microencapsulation process, prebiotic MOS was dissolved in sterile PBS as much as 0.4% (w/w) of wet weight of probiotics, then 10% (w/v) maltodextrin was added into it. This solution was heated at 80°C for 30 min (Fritzen-Freire *et al*., 2012) and used as the last solvent of probiotic biomass in the probiotic harvesting process before.

Synbiotic microencapsulation: The suspension of synbiotic consisted of 10% (w/v) maltodextrin, denaturated whey protein and core materials (probiotics and MOS) in 1:1 (v/v) ratio, respectively. Synbiotic suspension was prepared by mixing all of the ingredients and homogenizing them on a stirrer plate for 30 min before the spray drying process. The synbiotic microcapsules were produced by using laboratory scale spray dryer (Mini Bunchi 190 spray dryer, a constant air inlet temperature ranging from 100-110°C and outlet temperature ranging from of 55-58°C). The resulted powder (10^7 CFU g^{-1}) was collected from the base of the cyclone and stored in sealed sterile vials at -20°C.

Diets preparation: The feed used in this study was commercial shrimp feed (28% protein, 8% fat, 3% crude fiber, 8% ash content and 14% moisture content). Five types of diet were prepared by mixing synbiotic microcapsules with commercial feed manually using 2% (v/v) egg white as binder. The types of diet were M1 diet (0,5% (w/w) microcapsules), M2 diet (1% microcapsules), M3 diet (2% microcapsules), KN diet (control diet without challenge test) and KP diet (control diet with challenge test). Diets were prepared one day prior feeding time. Afterwards, the mixed diet were air-dried and stored at 4°C before use.

Preparation of WSSV filtrate: As much as 5 g WSSV infected tissue from Pacific white shrimp was homogenized in 20 mL TN buffer (200 mM Tris, 400 mM NaCl, pH 7.5), followed by centrifugation at 3000×g for 30 min at 4°C. The supernatant was collected and recentrifuged again at 3000×g for 30 min at 4°C. Furthermore, the final supernatant was filtered with 0.45 μm millipore and stored at -80°C until usage (Xie *et al*., 2005).

Culture conditions and experimental design: Prior to the start of the feeding trial, experimental shrimps were acclimatized for two weeks in the rearing tanks. The experimental design was completely randomized design with five treatments and three replications. For *in vivo* test, shrimps were stocked at tank (60×35×30 cm^3 within, volume of 0.04 m^3) with stocking density of 15 shrimps $tank^{-1}$. Feeding experiment was conducted for 30 days in a continually aerated system. The tank were covered with plastic cover to reduce the light intensity and to prevent

shrimp from escaping. Shrimps were fed four times a day with the feeding rate 6% body weight. Hence, feeding ratio was determined according to the daily feed consumption. After challenge test, shrimps were fed by commercial feed without synbiotic microcapsule supplementation. Water quality was maintained quite stable during experiment (temperature: 29±0°C, dissolved oxygen: 6.296±0.630 mg L^{-1}, TAN: 1.196±0.906 mg L^{-1}, salinity: 33±0 ppt, pH: 7.955±0.239; nitrite: 0.129±0.136 mg L^{-1} and nitrate: 7.866±2.107 mg L^{-1}). Moreover, residual feed and shrimp feces were removed by siphoning.

After feeding experiment for 30 days, shrimps were challenged with 100 μL/shrimp WSSV filtrate (10^4 copies mL^{-1}) on day 31 by performing intramuscular injection. Furthermore, during the feeding experiment (day 0 and 30) and after the challenge test (day 34), shrimps per tank were sampled to obtain intestine and hemolymph for determinations of bacterial counts and immune parameters.

Measurement of immune parameters: Immune system parameters observed consisted of Total Hemocytes Count (THC), prophenoloxidase activity (proPO) and Respiratory Burst activity (RB). Hemolymph was collected from the ventral sinus cavity by using a 26 gauge needle and 1 mL syringe. Syringe was previously mixed with precooled (4°C) anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA and pH 7) at 1: 2 (v/v). Later, intestinal shrimp in each tank were collected for bacterial quantification. Total hemocytes were calculated using hemocytometer at 400 times magnification on both grids sides.

The pro phenoloxidase activity was measured spectrophotometrically by recording the formation of dopachrome produced from L-dihydroxyphenylalanine (L-DOPA). A total of 1 mL mixture of hemolymph and anticoagulant were centrifuged at 700 g at 4°C for 20 min. The supernatant fluid was then discarded. Moreover, the pellet was rinsed, resuspended gently in 1 mL cacodylate citrate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate and pH 7.0) and further centrifuged. Later, the pellet was resuspended with 200 μL cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.01 M calcium chloride, 0.26 M magnesium chloride and pH 7.0). Moreover, 100 μL aliquot was incubated with 50 μL trypsin (1 mg mL^{-1}), which served as the activator for 10 min at 25-26°C. After 10 min, 50 μL of L-DOPA was added to the suspension, followed by 800 μL of cacodylate buffer addition 5 min later. The optical density at 492 nm wave length was measured using microplate reader. The optical density of the shrimp's phenoloxidase activity for all test conditions was expressed as dopachrome formation in 50 μL of hemolymph (Hsieh *et al*., 2008).

Hemocytes respiratory burst activity was calculated by using the principle of reduction of Nitro Blue Tetrazolium (NBT) which further formed formazon as a measurement of the amount of superoxide anion. About 50 μL of hemolymph was placed into the wells of the 'U' bottom microplate titre and was incubated at 37°C for 1 h to facilitate the cells adhesion. Afterwards, the supernatant was removed and the loaded wells were washed three times with 50 μL PBS. After the washing process, 50 μL of 0.2% NBT was added and the plate was incubated for further 1 h at 37°C. After 1 h incubation, the NBT solution was removed, the hemocyte cells were fixed with 50 μL of 100% methanol for 2-3 min and re-washed three times with 30% methanol. The plates were then air-dried. Around 60 μL of 2N potassium hydroxide and 70 μL of dimethyl sulphoxide (DMSO) were added into each well to dissolve the formazon blue precipitate formed. The OD of the turquoise blue colored solution was then read in a microplate reader at 630 nm (Singh *et al*., 2013).

Quantification of bacterial cells population: Shrimp intestines were collected from each tank (average weight of gut: 0.1 g) and homogenized in 0.9 mL of PBS. The quantification of bacterial cells population carried by serial dilution method (10-fold dilution), followed by plating on agar medium. Bacteriological media used to calculate the Total Bacterial Count (TBC) in this research was SWC (sea water complete) agar (5 g bacto peptone, 1 g yeast extract, 3 mL glycerol, 18 g bacto agar, 750 mL sea water and 250 mL distilled water). Moreover, SWC Rf^R (SWC enriched with 50 mg mL^{-1} rifampicin) was used to calculate Total Probiotic Bacteria (TPC) and selective media Thiosulphate Citrate Bile-salt Sucrose (TCBS) agar was used to calculate Presumptive *Vibrio* Count (PVC).

Growth performance: After 30 days of feeding experiment, sampling was performed to measure the effect of synbiotic microcapsule feeding to growth performance. The shrimp were counted and weighted. According to the result of sampling, SGR (Specific Growth Rate) and FCR (Feed Conversion Ratio) were calculated by the following equation (Bai *et al*., 2010; Ziaei-Nejad *et al*., 2006):

$$\text{SGR}\,(\%/\text{day}) = \frac{\text{LnWt} - \text{Ln Wo}}{\text{T}} \times 100$$

$$\text{FCR} = \frac{\text{Dry weight of ingested food}}{\text{Wet weight of produced shrimp}} \times 100$$

where, SGR is the specific growth rate (%), Wt is the weight of shrimp at certain time in the feeding experiment (g), Wo is the weight of shrimp on the initial feeding experiment (g), T is the number of rearing days (day).

Survival Rate (SR): Survival rate in this study was calculated at the end of the feeding experiment (30th day) and 72 h post infection with the following equation:

$$\text{Survival rate}\,(\%) = \frac{\text{Nt}}{\text{No}} \times 100$$

where, Nt is the number of live shrimp at the end of the observation and No is the number of shrimp in the initial observation.

PCR confirmatory test of WSSV infection: Confirmatory test of WSSV infection were conducted on shrimp which showed clinical sign after performing the challenge test. The WSSV confirmatory test was performed using PCR technique and based on methods as performed in previous studies (Nunan and Lightner, 2011).

Statistical analysis: Results are presented as Means±SD (standard deviation of means) and homogenic data was perform before analysis. The SPSS (Version, 16) programs were used for the statistical analyses. One-way analysis of variance (One-way ANOVA) was used to determine whether there were any significant differences between treatments. If so, all treatments were further tested by Duncan Multiple Range Test (DMRT). All tests were performed at the 95% confidence interval ($p \leq 0.05$).

RESULTS

Total Hemocytes Count (THC): Profile of hemocytes count is shown in Fig. 1. The total hemocytes before feeding experiment showed no difference ($p>0.05$) among treatments. However, the total hemocytes were increased after synbiotic feeding supplementation (30th day). Especially of treatment M2 and M3 which showed the highest THC ($p<0.05$) compared with other treatments. The value of THC in treatment M3 was the highest ($p<0.05$) compared with treatment M2. Seventy two hours post infection, the total hemocytes decreased except the negative control shrimp and showed no difference ($p>0.05$) among WSD-infected shrimps (M1, M2, M3 and positive control).

Respiratory Burst (RB) activity: Figure 2 shows the Respiratory Burst activity (RB) of Pacific white shrimp during the experiment. The RB activity before treatment showed that there was no significant difference ($p>0.05$) among treatments. After 30 days of feed supplementation, RB activity increased in all treatments. Treatment M2 and M3 showed higher RB activity ($p<0.05$) compared with negative and positive controls. Whereas, treatment M1 was found to have no difference ($p>0.05$) compared to both controls. After WSD infection, the RB activity in supplementation were lower than negative control. Furthermore, RB activities in negative control and M2 were higher ($p<0.05$) compared with treatment M1, M3 and positive control. However, the activity of RB in the negative control were higher ($p<0.05$) compared with treatment M2.

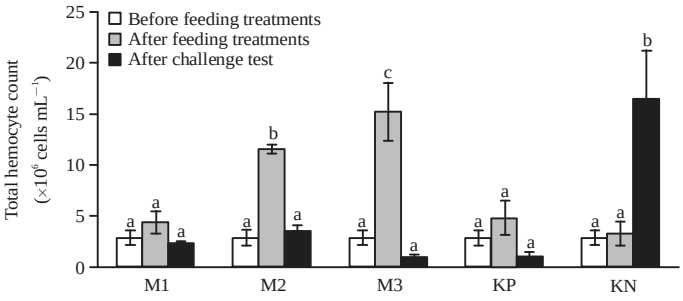

Fig. 1: Total hemocytes of Pacific white shrimp (*Litopenaeus vannamei*) of the experiment. Data is expressed as Mean±SD. Means in the same column sharing the same superscript are not significantly different determined by DMRT test ($p<0.05$)

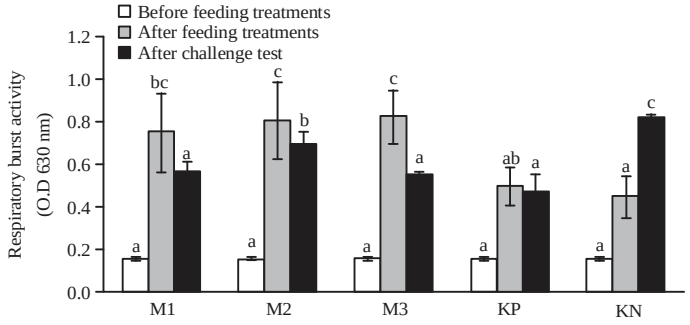

Fig. 2: Respiratory burst activity of Pacific white shrimp (*Litopenaeus vannamei*) of the experiment. Data is expressed as Mean±SD. Means in the same column sharing the same superscript are not significantly different determined by DMRT test ($p<0.05$)

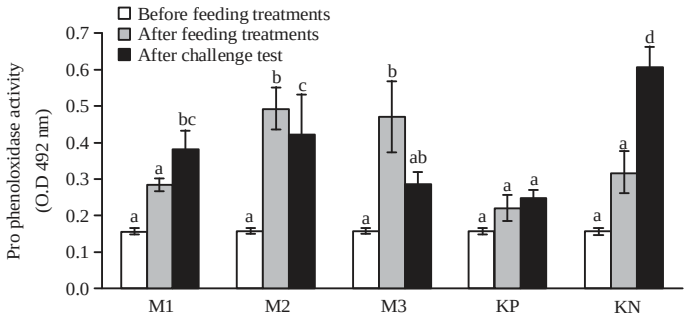

Fig. 3: Pro phenoloxidase (proPO) activity of Pacific white shrimp (*Litopenaeus vannamei*) of the experiment. Data is expressed as Mean±SD. Means in the same column sharing the same superscript are not significantly different determined by DMRT test ($p<0.05$)

Table 1: Total probiotic count, presumptive *Vibrio* count, total bacterial count in the intestinal track of Pacific white shrimp (*Litopenaeus vnnmei*)

Bacterial population (Log CFU g^{-1})	Treatments	Before synbiotic microcapsule feeding	After synbiotic microcapsule feeding	After challenge test
TPC	M1	ND**	3.550±0.447^{b}	2.720±0.448
	M2	ND**	4.279±0.228^{c}	3.034±0.028
	M3	ND**	2.834±0.575	2.955±0.439
	KP	ND**	ND**	ND**
	KN	ND**	ND**	ND**
PVC	M1	6.771±0.285	6.963±0.611	8.116±0.565^{b}
	M2	6.771±0.285	7.658±0.403	8.303±0.927^{bc}
	M3	6.771±0.285	7.217±0.719	6.156±0.055
	KP	6.771±0.285	7.002±0.548	8.632±0.497^{bc}
	KN	6.771±0.285	7.271±0.199	9.235±0.206^{c}
TBC	M1	7.872±0.333	7.445±0.600	8.741±1.202
	M2	7.872±0.333	8.464±0.077^{b}	8.879±0.880
	M3	7.872±0.333	8.399±0.122^{b}	8.687±1.320
	KP	7.872±0.333	8.891±0.259^{bc}	8.776±1.216
	KN	7.872±0.333	9.513±0.239^{c}	9.767±0.117

*Data is expressed as Mean±SD. Means in the same parameter and time of observation sharing the same superscript are not significantly different determined by DMRT test ($p<0.05$), **ND: Not determined, TPC: Total probiotic count, PVC: Presumptive *Vibrio* count, TBC: Total bacterial count

Pro phenoloxidase (proPO) activity: The pro phenoloxidase activity (proPO) is one of humoral immune response of crustace that can be quantified. The proPO activity in each treatments is presented in Fig. 3. The proPO activity prior to supplementation experiment showed no difference ($p>0.05$) among all treatments. However, after 30 days of feeding supplementation, proPO showed the increasing activity. At the end of supplementation, proPO activity in treatment M2 and M3 were higher ($p<0.05$) compared with treatment M1, positive and negative controls. After WSD infection, proPO activity in treatment M2 were higher ($p<0.05$) than treatment M3 and positive control, but showed no difference ($p>0.05$) with treatment M1.

Quantification of bacterial cells population: Table 1 shows the Total Probiotic Count (TPC) of *Bacillus* NP5 Rf^{R}, Presumptive *Vibrio* Count (PVC) and Total Bacterial Count (TBC) in the digestive tract of Pacific white shrimp. At the end of feeding experiment (day 30), TPC of treatment M2 were higher ($p<0.05$) compared with treatment M3 and M1. After challenge test, TPC showed no difference ($p>0.05$) among treatment M1, M2 and M3. PVC before (day 0) and after feeding experiment (day 30) showed no difference ($p>0.05$) among all treatment. Hence, after the challenge test (day 34), the minimum value of PVC ($p<0.05$) was detected in treatment M3. The PVC value

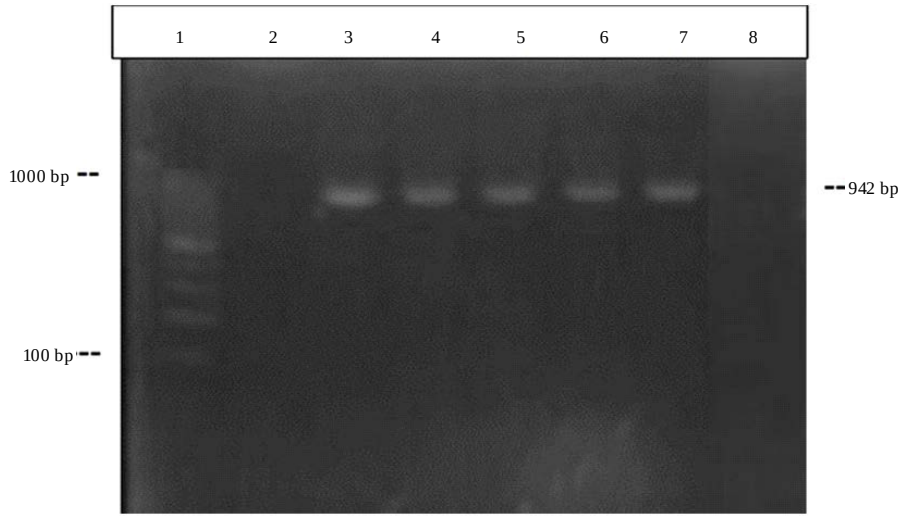

Fig. 4: Agarose gel electrophoretic patterns of PCR product amplification (942 bp) for the detection of WSSV-infected Pacific white shrimp. Lane 1: Marker, Lane 2: ddH_2O, Lane 3: Positive control, Lane 4: WSSV infected shrimp of sample M1, Lane 5: WSSV infected shrimp of sample M2, Lane 6: WSSV infected shrimp of sample M3, Lane 7: WSSV-infected shrimp of KP, Lane 8: Healthy shrimp of KN

Table 2: Specific growth rate and feed conversion ratio of Pacific white shrimp (*Litopenaeus vnnmei*) after feeding supplementation

	Treatments				
Growth performance	M1	M2	M3	KP	KN
SGR (% day^{-1})	2.191±0.287[b]	2.213±0.333[b]	2.074±0.250[b]	1.667±0.131	1.672±0.138
FCR	1.976±0.180	1.176±0.235	2.070±0.306[b]	2.420±0.120[b]	2.39±0.172[b]

*Means in the same row sharing the same superscript are not significantly different determined by DMRT test ($p<0.05$), SGR: Specific growth rate, FCR: Feed conversion ratio

in negative control were found to be highest one. Moreover, PVC value among treatment M2, negative and positive controls showed no difference ($p>0.05$). Total Bacterial Count (TBC) before feeding experiment showed no difference ($p>0.05$) among treatments. The highest TBC ($p<0,05$) after feeding experiment was detected in negative control while the lowest TBC ($p<0,05$) was found in treatment M1. Both of those treatments were different ($p<0.05$) from treatment M2 and M3. Above all, all treatments showed no difference ($p>0.05$) of TBC after WSD infection.

Growth performance: The maximum Specific Growth Rate (SGR) was detected in treatment M1 and M2. Both of these treatments were higher ($p<0.05$) than negative and positive control (Table 2), yet they showed no difference ($p>0.05$) compared with treatment M3. The minimum value of Feed Conversion Ratio (FCR) was found in treatment M2 and M1 (Table 2). The FCR of both of them were lower than negative and positive control ($p<0.05$), but showed no difference compared with treatment M3.

PCR confirmatory test of WSSV infection: Confirmatory of WSSV presence in the experimental shrimps was tested by performing PCR analysis (Fig. 4). In addition, samples were collected from all treatments (samples M1, M2, M3, positive and negative control). The results showed that the

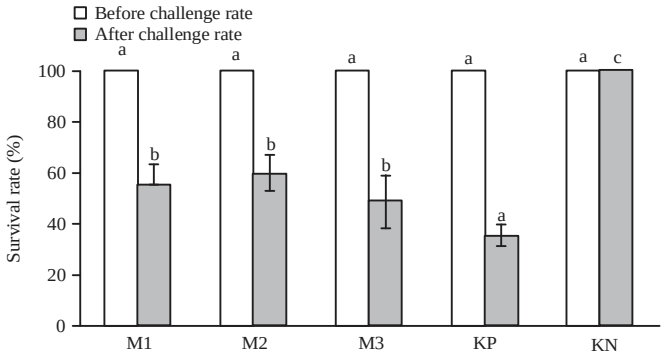

Fig. 5: Survival rate of Pacific white shrimp (*Litopenaeus vannamei*) before and after challenge test. Data is expressed as Mean±SD. Means in the same column sharing the same superscript are not significantly different determined by DMRT test ($p<0.05$)

four samples from M1, M2, M3 and positive control were positively infected WSSV as illustrated by the size of amplified DNA bands of 942 bp in the electrophoresis gel results, while negative control did not show amplified DNA band.

Survival Rate (SR): Survival Rate (SR) before the challenge test (Fig. 5) showed no difference among all treatments ($p>0.05$). After the challenge test, the shrimp fed by synbiotic microcapsule diet (treatment M1, M2 and M3) showed higher survival rate ($p<0.05$) than positive control. Whereas, no mortality was found in the negative control shrimps after the challenge test.

DISCUSSION

The results showed that dietary supplementation of synbiotic microcapsule (*Bacillus* NP5 Rf^R and MOS) was able to improve the immune responses of Pacific white shrimp against WSSV infection. The increasing value of immune responses in this study were observed through total hemocytes count, proPO activity and respiratory burst activity. In this study, the increasing of the total hemocytes (Fig. 1) after feeding supplementation occurred as a reaction of shrimp immune system to foreign particle i.e the increasing of synbiotic microcapsule doses. Hemocytes play a central role in crustaceans immune system. The changing of hemocyte count is one of indicators to detect stress and health status of shrimp. Foreign particles which enter into the shrimp body will be recognized by hemocytes cell and be responded through several mechanisms such as intracellular signaling cascade, phagocytosis, encapsulation and nodular aggregation (Rodriguez and le Moullac, 2000).

Decreasing of total hemocytes after WSSV infection caused by hemocytes migration from circulating system of the body to tissues where many cells were infected (Yeh *et al*., 2009). The decreasing of THC in immune system indicated that immune system worked on the infected areas through wound healing activities such as clumping of cells, initiation of the coagulation process through the release of plasma clotting factors, carrying and releasing factors in pro phenoloxidase system (proPO) (Smith *et al.,* 2003). In addition, cell apoptosis mechanism in hematopoietic tissues of shrimp caused the hemocytes declining after WSSV infection. This mechanism correlates with the increasing of serious infection and mortality of shrimp (Yeh *et al*., 2009).

Li *et al.* (2008) reported that supplementation of probiotic *Arthrobacter* XE-7 in white shrimp diet could increase the total hemocytes ($p<0.05$) at the end of the feeding period. Increasing total

hemocytes ($p<0.05$) was also found in *Cherax destructor* which were fed by synbiotic diet (Sang *et al*., 2011). Another study reported that the hemocytes count decreased ($p<0.05$) after challenge test with *Vibrio parahaemolyticus* (Li *et al*., 2008). Moreover, Yeh *et al.* (2009) found a similar case where WSSV infected shrimp hemocyte decreased after the challenge test. It was apparently caused by the accumulation of hemocytes at the infection site and the occurrence of cell apoptosis as a result of viral infection. You *et al*. (2010) also reported a similar case where the THC in *Marsupenaeus japonicus* shrimp that were cultured at 27 and 31°C decreased significantly after WSSV infection.

Respiratory burst are series of processes occurring in cells which conduct phagocytosis of foreign particles and produced Reactive Oxygen Intermediates (ROI) or oxygen radicals as the final product (Rodriguez and le Moullac, 2000). The increasing of RB activity (Fig. 2) after feeding experiment showed that dietary synbiotic microcapsule enhanced the immune system of Pacific white shrimp. In this study, RB activity was measured as the amount of superoxide anion (O_2^-) that was formed by hyaline cells. The first product resulted in RB process is superoxide anion (O_2^-). Furthermore, subsequent reactions will produce other ROI such as hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-) and singlet oxygen ($^1O_2$). The hydrogen peroxide can be transformed into hypochlorous acid ($HOCl^-$) through myeloperoxidase system (MPO-H_2O_2-Cl) to form an antibacterial system (Rodriquez and Moullac, 2000). The stress conditions caused by environmental factors, various types of toxic and biological stress (including pathogen infection) caused changes of ROI and led to oxidative stress in the cell (Mohankumar and Ramasamy, 2006; Castex *et al*., 2010).

Li *et al.* (2009) also showed similar results where RB activities of white shrimp supplemented with probiotic *Bacillus* OJ and isomaltooligosaccharide were increased ($p<0.05$) compared with control. Zhang *et al.* (2014) reported that *Trachinotus ovatus* fed with probiotic *Bacillus subtilis* (5.62×10^7 CFU g^{-1} feed) and 0.2% fructooligosaccharide (FOS) combinations showed higher RB activity ($p<0.05$) compared with control. While after WSD infection, RB activity decreased ($p<0.05$). Chang *et al.* (2003) also reported the same result where RB activity of white shrimp fed with β-1, 3-glucan decreased after WSSV infection.

The proPO system is acknowledged to be the most important immune system in crustaceans. According to Tassanakajon *et al.* (2013) the proPO-activating system is initiated by the recognition and binding of the Patogen Recognition Receptors (PRRs) to microbial cell wall components, such as peptidoglycan (PG), lipopolysaccharides (LPS) and β-glucans (BGs). The complex then triggers the activation of the serine protease (SP) cascade that converts proPO to active PO by a limited proteolysis. Active phenoloxidase (PO) oxidizes phenols into quinones that can nonspecifically cross-link neighboring molecules to form melanin. Melanin is a dark brown pigment that trap pathogens and prevent contact with the host (Amparyup *et al.,* 2013). The proPO activity after supplementation of synbiotic microcapsule (Fig. 3) showed the increasing tendency of proPO with the increasing of synbiotic microcapsule doses. Li *et al*. (2009) reported that the proPO activity of white shrimp increased as much as doses of probiotics were given. Nurhayati *et al.* (2015) also showed that the increasing of probiotic and prebiotic doses in white shrimp diet caused the increasing of proPO activity after synbiotic supplementation. The proPO activity after WSSV infection were positively correlated with decreasing of hemocytes count (Fig. 1). Immanuel *et al.* (2012) showed similar result where the proPO activity in *Penaeus monodon* fed fucoidan extracts of *Sargassum wightii* were decreased after challenge test with WSSV and also positively correlated with decreasing of the hemocytes.

The responses of several shrimp immune system parameters (THC, proPO and RB) in this study related to the activation of the innate immune response. The activation of the innate immune response were mediated by interaction of shrimp Pattern Recognition Receptors (PRRs) and Pathogen-Associated Molecular Patterns (PAMPs). This PAMPs could be polysaccharides and glycoproteins on the surface of microbes, such as lipopolysaccharide (LPS) from Gram negative bacteria, peptidoglycan (PGN) and lipoteichoic acid (LTA) from gram positive bacteria, glucans from yeast cells, as well as polynucleotides (Wang and Wang, 2013). The MOS availability in synbiotic microcapsuleswere also expected to have an essential role in the immune system through lectin pathway (Torrecillas *et al*., 2014). In *L. vannamei*, one of C-type lectin in Pacific white shrimp (LvLT) consists of two putative Carbohydrate-Recognition Domains (CRDs). The first CRD consists of an amino acid motif (QPD) for the binding of galactose and the other CRDs consist of amino acid motifs (EPN) for the binding of mannose. Lectins which bind carbohydrate could promote the agglutination of different cells, such as bacteria and other invading pathogens, it is reasonable to assume that these molecules may be regarded as having a potential role in invertebrate non-self-recognition reactions (Marques and Barracco, 2000). Some studies reported that supplementation of MOS in feed was able to improve the immune responses of *Cherax tenuimanus* against bacterial infections and environmental stress conditions and also *Sciaenops ocellatus* fed soybean-based diets (Sang *et al*., 2009; Buentello *et al*., 2010).

Probiotic cells (*Bacillus* NP5 Rf^R) were detected at the end of feeding supplementation and after WSSV infection (Table 1). This indicated that probiotics given as synbiotic microcapsules were able to reach and survive in the digestive tract of shrimp. The TBC were decreased after feeding supplementation also caused by the role of synbiotic. Li *et al.* (2009), reported that the total bacteria in the digestive tract of Pacific white shrimp decreased with increasing doses of probiotics and prebiotics. Nurhayati *et al.* (2015) reported the different results, where the increasing of synbiotic doses caused an increasing of total bacterial count in the digestive tract of *L. vannamei*. The PVC showed no difference value before and after feeding supplementation. However, Munaeni *et al.* (2014) reported the different result, the total *Vibrio* count of white shrimp fed by synbiotic microcapsule (combination of *Bacillus* NP5 Rf^R and oligosaccharide from sweet potato) were decrease than controls.

The probiotic and prebiotic have been widely reported to influence the growth of microbial in the digestive tract. The probiotic act through several mechanism include production of inhibitory compounds, competition for chemicals or available energy, competition for adhesion sites, inhibition of virulence gene expression or disruption of quorum sensing, improvement of water quality, enhancement of the immune response, source of macro or micronutrients and enzymatic contribution to digestion (Merrifield *et al*., 2010). Whereas prebiotics availability was to inhibit the adhesion and invasion of pathogens in the epithelium of the colon through the competition mechanism in obtaining glycoconjugates on the surface of epithelial cells, changing the pH of the colon, supporting the defense function, increasing the production of mucus, producing short chain fatty acids and stimulating cytokine production (Delgado *et al*., 2011).

In this study, dietary synbiotic microcapsules 0.5% (M1) and 1% (M2) gave the best results for specific growth rate and feed conversion ratio (Table 2). These were supported by the presence of *Bacillus* NP5 Rf^R cells population in the digestive tract of shrimp in treatment M1 and M2 which were higher than treatment M3 (Table 1). Previous study showed that exogenous enzymes produced by *Bacillus* NP5 probiotic have contributed in the digestion process, as one of growth supporting factor (Putra and Widanarni, 2015). The supplementation of probiotic *Bacillus* NP5 Rf^R

has been able to increase the activity of the amylase enzyme, the digestibility of carbohydrates and growth performance of tilapia. The MOS contained in microcapsules also influenced the growth performance indirectly through an improvement in the functional integrity of the enterocyte membrane in relation to an enhanced assimilation or digestion of specific nutrients or to possible changes on the levels of peptides that regulate either the peripheral satiation system (satiation and appetite signals) and also modification of the morphology of the digestive tract of shrimp (Torrecillas *et al*., 2014; Dimitroglou *et al*., 2013). The TEM analysis in Zhang *et al.* (2012) showed that MOS supplementation in diet could significantly increase the length of the intestinal microvilli of *L. vannamei*. Therefore, it could improve the absorption of nutrients and growth performance. Applications of synbiotic in some studies also known to increase growth performance including SGR and FCR in some aquatic organisms, such as *Acipenser baerii*, *Trachinotus ovatus* and *L. vannamei* (Geraylou *et al.,* 2013; Zhang *et al.,* 2014; Nurhayati *et al*., 2015).

Dietary synbiotic microcapsules improved the immune response and survival rate of white shrimp due to WSSV infection. The survival rates of shrimp fed by synbiotic microcapsule were higher ($p<0.05$) than positive control (Fig. 5). Endogenous *Vibrio* population reached 7 log CFU g^{-1} have probable contributed in the white shrimp mortality in this study. Phuoc *et al.* (2009) showed similar result where white shrimp which were single infected with *V. campbellii* (10^3-10^4 CFU mL^{-1}) did not cause mortality. However, after co-infection of WSSV and *V. campblellii,* mortality rate of shrimp increased compared to single infection with WSSV. Moreover, several studies regarding the use of probiotic *Bacillus* NP5, either as single probiotic or synbiotic application were also reported to have ability in enhancing the immune response of *V. harveyi* infected *L. vannamei* (Munaeni *et al*., 2014) and *Streptococcus* sp. infected tilapia (Tanbiyaskur *et al*., 2015).

CONCLUSION

Dietary synbiotic microcapsules at dose of 1% (*Bacillus* NP5 Rf^R and mannan oligosaccharides combination) gave the best effect on the immune responses and growth performance of WSSV infected Pacific white shrimp (*L. vannamei*).

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