Effects of Microencapsulated Synbiotic Administration at Different Dosages Against Heavy Co-infection of White Spot Disease (WSD) and *Vibrio harveyi* in Pacific White Shrimp (*Litopenaeus vannamei)*

Yunarty[1], Munti Yuhana[2]* and Widanarni[2]

[1]*Study Program of Aquaculture Science, Graduate School, Bogor Agricultural University, Jl. Agatis, Darmaga Campus, Bogor, 16680 Indonesia*
[2]*Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University, Jl. Agatis, Darmaga Campus, Bogor, 16680 Indonesia*
E-mail : myhn@gmx.ch

Abstract

White spot disease (WSD) is one of infectious disease in shrimp caused by white spot syndrome virus (WSSV). This study aimed to determine the dosage immunological effects and growth performances of microencapsulated synbiotic (Bacillus NP5 and mannan oligosaccharide) at different dosages on Pacific white shrimp. The microencapsulated synbiotic was administered as feed supplementation against the co-infection of WSSV and Vibrio harveyi. Synbiotic was encapsulated by spray drying method, further feed supplemented to Pacific white shrimp for 30 days at a dosages of 0.5% (A), 1% (B), 2% (C) and control treatments, i.e. without any microencapsulated synbiotic administration as positive control (D) and negative control (E). The challenge test was performed on day 30 after feeding supplementation, then the experimental shrimps were injected by WSSV intramuscularly at the infective dosage of 10^4 copies.-ml^{-1}. Afterwards, 24 hours after WSSV injection the shrimps were immersed in water contained cells suspension of V. harveyi at the cells population dosage of 10^6 CFU-.ml^{-1}. All synbiotic treatments showed better results with the values of Total Haemocyte Count (THC), Phenoloxidase (PO) and Respiratory Burst (RB), were higher ($P<0.05$) compared to positive control. The specific growth rates (SGR) of A, B and C showed higher than both controls of D and E. The feed conversion ratio (FCR) value of synbiotic treatments were lower ($P<0.05$) than both controls. However, the administration of microencapsulated synbiotic have not been able to prevent heavy impact of WSSV and V. harveyi co-infection due to lower SR and mortality pattern which continued to increase.

***Keywords**: Synbiotic, Litopenaeus vannamei, WSSV, Vibrio harveyi, co-infection*

Introduction

Infectious diseases caused by bacteria and/or viruses is one of factors caused the decrease production of Pacific white shrimp (*Litopenaeus vannamei*) (Phuoc *et al.*, 2009). One of causative agent of viral disease that currently widely attacks the Pacific white shrimp is white spot syndrome virus (WSSV). WSSV outbreak caused the shrimp mortality up to 90-100% within 3-10 days after exhibiting clinical signs (Sanchez-Martinez *et al.*, 2007). Beside the viral infections, the bacterial infections were also commonly found, such as *Vibrio* sp. *which* often found as the causative agent of secondary infection in shrimp (Liu and Chen, 2004).

Several studies reported that some pathogens are not always become a single causative agent in the shrimp diseases.There are also joint infection between pathogens in the same host which is called co-infection (Teixera-Lopes *et.al* 2011). The co-infection diseases between virus and bacteria have been reported by Phuoc *et al.* (2009); either the co-infection by WSSV with *V. harveyi* or by WSSV with *V. campbellii*. Therefore, the alternative and effective control measures are needed to prevent co-infection disease by enhancing the immune system of shrimp. One of alternative methods is by using the synbiotic as feed supplementation. Synbiotic (a combination of probiotic and prebiotic) applicationhas been proven to be able in improving immune response, resistance, and growth performance of shrimp (Li *et al.*, 2009) and fish (Geraylou *et al.*, 2013).

The synbiotic application in the form as the fresh culture cells suspension has provided in limited cells lifetime. The technique for improving the cells life time need to be applied is the microencapsulation method (Anal and Singh, 2007). Microencapsulation technology is used for coating or lining a core material with polymeric substances

*) Corresponding author

ijms.undip.ac.id
DOI: 10.14710/ik.ijms.21.4.169-176
Received : 28-10-2016
Accepted : 11-11-2016

aslayer or coating material, so that it becomes small particles within microscopic sizes. The layer of the microcapsule granules will protect the probiotic bacterial cells from the damaging effects of extreme environmental condition (Widodo *et al.*, 2003; Weinbreck *et al.*, 2010). The common method applied for microbial cells microencapsulation is by spray drying method. This method has been reported to be able to maintain the cells viability of microencapsulated *Bifidobacteria* cells and oligofructose enriched with inulin (Freire *et al.*, 2012).

This study was carried out to evaluate the effects of the combination of probiotic *Bacillus* NP5 and commercial prebiotic of mannan oligosaccharide (MOS). Probiotic *Bacillus* NP5 was isolated from the digestive tract of tilapia (*Oreochromis niloticus*) (Putra and Widanarni, 2015). Prebiotic MOS was derived from *Saccharomyces cerevisiae* cell wall produced commercially was an additive prebiotic for animal feed. The addition of prebiotic mannan oligosaccharide can improve survival rate, health status and immunity of lobster (*Cherax tenuimanus*) (Sang *et al.,* 2011).

In the previous studies (Zubaidah *et al.,* 2015; Munaeni *et al.*, 2014) have reported that the combination of probiotic cells of *Bacillus* NP5 Rf^R and prebiotic from sweet potato extract produced in the form of microencapsulated synbiotic, could improve the growth performance of Pacific white shrimp challenged by *V. harveyi*. On the other hand, the efficacy of microencapsulated synbiotic, combination of *Bacillus* NP5 and MOS on Pacific white shrimp co-infected by WSSV and *V. harveyi*, has not been known. Based on this approach, this study was conducted to determine the best dosage of microencapsulated synbiotic administration through feed on growth performance and immune responses of Pacific white shrimp co-infected by WSSV and *V. harveyi*.

Materials and Methods

Experimental shrimps used were Pacific white shrimp post-larvae with average weight of 5.44 ± 0.44 g individu^{-1}, obtained from Shrimp Production Unit Gelung, Brackish Water Aquaculture Center Situbondo, East Java. The shrimp were regurlarly PCR checked as specific-pathogen free (SPF) for WSSV and IMNV. Probiotic used was probiotic *Bacillus* NP5, which has been marked as antibiotic rifampicin resistant at a dosage of 50 µg ml^{-1}. Prebiotic used was commercial prebiotic MOS. Isolate of WSSV and *V. harveyi* were obtained from collection of Brackish Water Aquaculture Center Situbondo, East Java.

Rearing Condition and Experimental Design

Pacific white shrimp were reared in tanks with a size of 60 cm x 35 cm x 30 cm with a density of 15 individu per tank. Pacific white shrimp were acclimatized for two weeks before feeding trial. Feed used was commercial feed with a protein content of 28%. The feeding were conducted four times a day, with feeding rate (FR) was 6%. The feed was mixed with microencapsulated synbiotic powder. The dosages were taken according to treatment and was coated by egg albumine as the binder. The test feed was air-dried for 5 to 10 minutes or can be stored in the refrigerator prior to be used. The feeding trial was conducted for 30 days.

The challenge test with WSSV filtrate was performed on day 31, by injecting the virus filtrate, as much as 100 µL individu^{-1} (10^4 copies ml^{-1}; it was measured using real time PCR) and negative control were injected with PBS (phosphate buffer saline) in the same concentration. Artificial co-infection of *V. harveyi* was carried out 24 hours after virus injection by immersing the shrimp in 10^6 CFU ml^{-1} of the *V. harveyi* cells suspension (Phuoc *et al.*, 2009), except for the shrimps of negative control, which were immersed in PBS. At feeding trial period (day 0 until day 30) and after the challenge test, there were intestinal bacterial samplings to determine total bacterial count and total haemolymph count observation for immune parameters observation.

Experimental design used was a completely randomized design (CRD), with five treatments and three replications; the administraton of microencapsulated synbiotic at a dosage of 0.5% (A), 1% (B), 2% (C) and controls (without the administration of microencapsulated synbiotic) including positive control (D) and negative control (E). Water quality was maintained in optimum condition (temperature at 29 ± 0 °C; dissolved oxygen at 6.296 ± 0.630 mg L^{-1}; TAN at 1.196 ± 0.906 mg L^{-1}; salinity at 33 ± 0 ppt; pH at 7.955 ± 0.239; nitrite at 0.129 ± 0.136 mg L^{-1}; nitrate at 7.866 ± 2.107 mg L^{-1}). Siphoning was done every day to remove feces and unconsumed feed.

Synbiotic Microencapsulation

The composition used in the production of microencapsulated synbiotic consisted of probiotic *Bacillus* NP5 RfR with cells density of 10^9-10^{10} CFU g^{-1} of cells wet weight (Wang, 2007) and prebiotic MOS 0.4% (w/w) (Zhang *et al.*, 2012). Probiotic biomass production was done by subculture technique in SWC-broth medium using 10% inoculant with a density of 10^8 CFU ml^{-1} (v/v) (Munaeni *et al.*, 2014; Zubaidah *et al.,* 2015).

The coating materials used on synbiotic microencapsulation were denatured whey protein (DWP) and maltodextrin. DWP process was obtained through enzymatic reaction according to procedure that have been implemented by De Castro-Cislaghi *et al.* (2012). Before microencapsulation process, 0.4% prebiotic MOS (w/w)wet weight was dissolved in sterile PBS, then added by 10% maltodextrin (w/v). MOS and maltodextrin was heated at a temperature of 80 °C for 30 minutes (Freire *et al.*, 2012). Formulation of synbiotic (probiotic and MOS), whey protein and maltodextrin was 1:1 (v/v):10% (w/v) (Munaeni *et al.*, 2014; Zubaidah *et al.,* 2015). The materials were further homogenized using stirrer plate for 30 minutes. Furthermore, the spray drying process was performed using a spray dryer (Mini bunchi 190) with temperature ranging from 100 up to 110 °C at the inlet and 55-58 °C at the outlet.

Parameters Observation

Parameters observed were immune response parameters including total haemocyte count, phenoloxidase activity (Liu and Chen, 2004) and respiratory burst activity (Singh *et al.*, 2013), total bacterial count in the intestine, survival rate (SR) (Sang *et al.,* 2011), growth performance parameters including specific growth rate (SGR) and feed conversion ratio (FCR) (Akrami *et al.*, 2012).

Statistical Analysis

Data were tested by analysis of variance (ANOVA), if there were significantly differences then continued with Duncan test using SPSS version 16.

Result and Discussion

Total hemocyte count (THC) at the end of treatment or 30 days after the feeding trial showed that all synbiotic treatments (A, B, C) were significantly different (P<0.05) from positive control (D) and negative control (E). The highest THC was found in B (1%). Increasing of THC at the end of treatment was caused by administration of microencapsulated synbiotic. The addition of synbiotic to the feed will increase total hemocyte and survival of Pacific white shrimp (Partida-Arangure *et al.*, 2012). Hemocyte will recognize the foreign particles which get into the shrimp body. Hemocyte cells will respond through several mechanisms such as intracellular signaling cascades, phagocytosis, encapsulation and nodular aggregation (Rodriguez and Le Muollac, 2000). After the challenge test, THC decreased except in negative control treatment (Figure 1). This was due to the functioning of shrimp immune system. The reduction of hemocytes is the effect of immune system work

Table 1. Total viable bacterial count (TBC), presumptive *Vibrio* count (PVC), total *Bacillus* NP5 Rf^R and total *V.harveyi* Rf^R

Bacterial population	Treatments	Before feeding trial (Log CFU g^{-1})	After feeding trial (Log CFU g^{-1})	After the challenge test (Log CFU g^{-1})
TBC	A	5.33 ± 0.11^a	4.77 ± 0.10^a	5.31 ± 0.02^a
	B	5.33 ± 0.11^a	4.49 ± 0.22^a	5.26 ± 0.04^a
	C	5.33 ± 0.11^a	4.78 ± 0.27^a	5.19 ± 0.05^a
	D	5.33 ± 0.11^a	5.38 ± 0.21^b	5.49 ± 0.30^{ab}
	E	5.33 ± 0.11^a	5.56 ± 0.33^b	5.63 ± 0.30^b
PVC	A	5.48 ± 0.18^a	4.87 ± 0.21^a	5.74 ± 0.13^a
	B	5.48 ± 0.18^a	4.91 ± 0.07^a	5.96 ± 0.15^a
	C	5.48 ± 0.18^a	5.37 ± 0.03^b	5.68 ± 0.12^a
	D	5.48 ± 0.18^a	5.38 ± 0.12^b	5.74 ± 0.13^a
	E	5.48 ± 0.18^a	5.47 ± 0.10^b	5.86 ± 0.30^a
Total *Bacillus* NP5 Rf^R	A	*nd*	4.17 ± 0.31^a	3.83 ± 0.29^a
	B	*nd*	5.00 ± 0.04^b	4.39 ± 0.19^b
	C	*nd*	4.08 ± 0.28^a	3.88 ± 0.15^a
	D	*nd*	*nd*	*nd*
	E	*nd*	*nd*	*nd*
Total *V.harveyi* Rf^R	A	*nd*	*nd*	3.95 ± 0.37^a
	B	*nd*	*nd*	4.01 ± 0.32^a
	C	*nd*	*nd*	3.97 ± 0.13^a
	D	*nd*	*nd*	4.55 ± 0.16^b
	E	*nd*	*nd*	*Nd*

Note: different superscript letters in the same column and row indicate significant different results huruf (P<0.05), *nd; not determined.*

due to hemocyte infiltration in infected tissues (Costa *et al.*, 2009). Phenoloxidase (PO) activity (Figure 2) of all synbiotic treatments were significantly different (P<0.05) from controls. The highest PO activity was found in B, followed by C and A. At the end of treatment or after 30 days of feeding trial, there was increasing of PO activity that was suspected due to the addition of microencapsulated synbiotic, in which synbiotic is a combination of probiotic and prebiotic which showed improvement of PO activity (Chiu *et al.*, 2007). Reduction of PO activity occurred after the challenge test, except in negative control. PO activity and THC were correlated to each other. The more hemocyte count, the more ProPO production, and vice versa (Smith *et al.*, 2003).

RB activity (Figure 3) of all synbiotic treatments were significantly different (P<0.05) from controls, with the highest RB activity was found in B, followed by C and A treatments. Increasing of RB activity at the end of treatment indicated that administration of synbiotic at different dosages to Pacific white shrimp could boost the shrimp immune system. Li *et al.* (2009) reported that feed supplementation with *Bacillus* OJ gave significant influence on the immune response and disease resistance. Reduction of RB activity after the challenge test, allegedly associated with the declining of THC after challenge test. RB activity occurs in hemocyte cells which perform elimination activity of foreign particles within phagocytosis process (Rodriguez and Le Muollac, 2000). The total

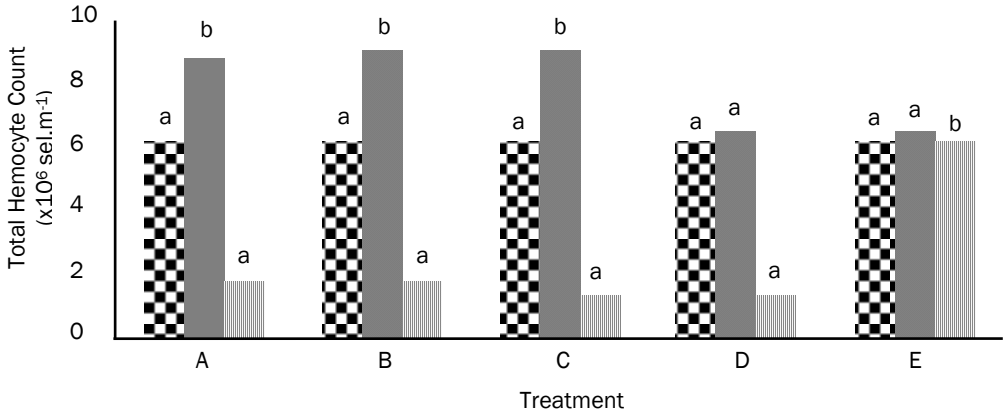

Figure 1. Total haemocyte count (THC) of Pacific white shrimp. Administration of microencapsulated synbiotic 0.5% (A), 1% (B), 2% (C), without administration of microencapsulated synbiotic as positive control (D) and negative control (E). Different superscript letters on the same observation period indicate significant different results (P<0.05).
Note : = before feeding trial, = After feeding trial, = After the challenge test

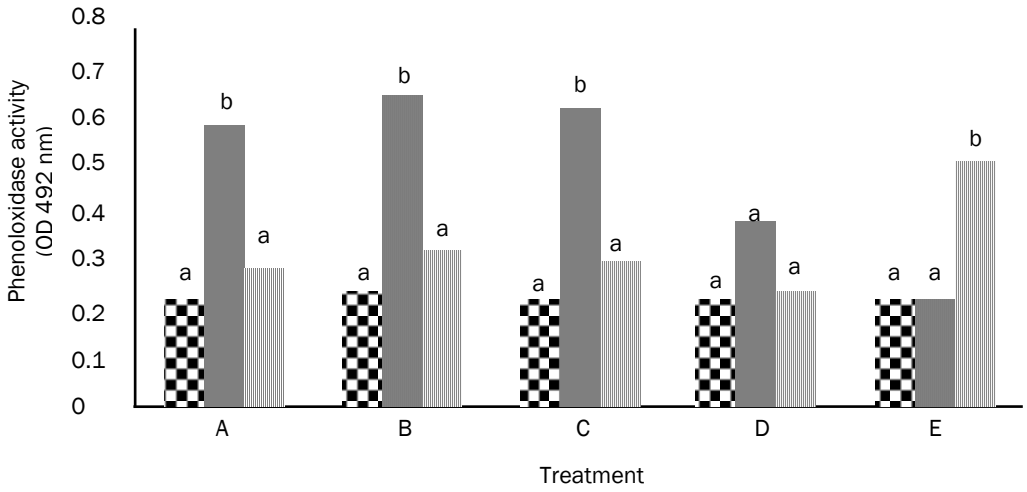

Figure 2. Phenoloxidase (PO) activity of Pacific white shrimp. Administration of microencapsulated synbiotic 0.5% (A), 1% (B), 2% (C), without administration of microencapsulated synbiotic as positive control (D) and negative control (E). Different superscript letters on the same observation period indicate significant different results (P<0.05).
Note : = before feeding trial, = After feeding trial, = After the challenge test

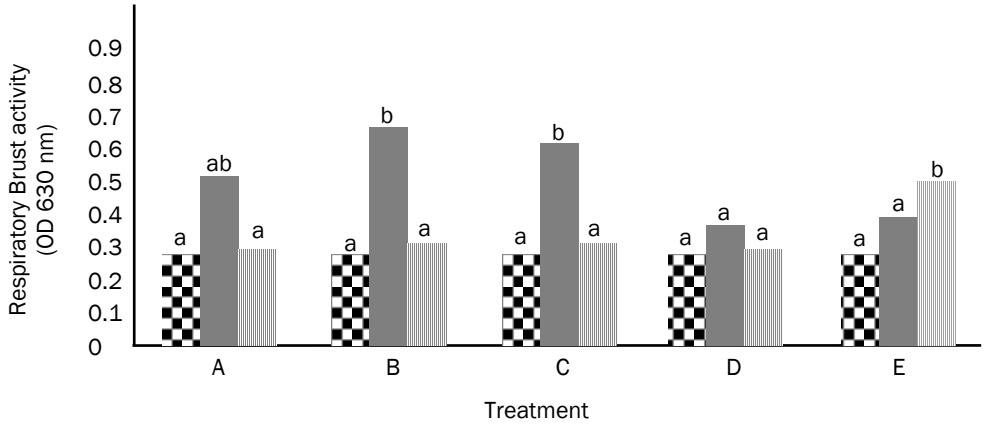

Figure 3. Respiratory burst (RB) activity of Pacific white shrimp. Administration of microencapsulated synbiotic 0.5% (A), 1% (B), 2% (C), without administration of microencapsulated synbiotic as positive control (D) and negative control (E). Different superscript letters on the same observation period indicate significant different results (P<0.05).
Note : = before feeding trial, = After feeding trial, = After the challenge test

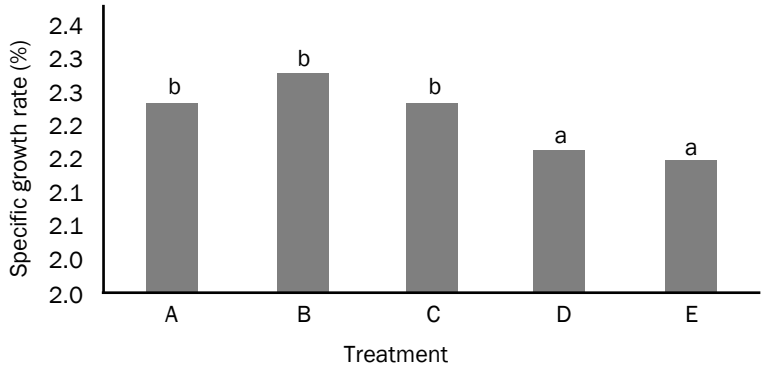

Figure 4. Specific growth rate (SGR) of Pacific white shrimp. Administration of microencapsulated synbiotic 0.5% (A), 1% (B), 2% (C), without administration of microencapsulated synbiotic as positive control (D) and negative control (E). Different superscript letters on each bar indicate significant different results (p<0.05).

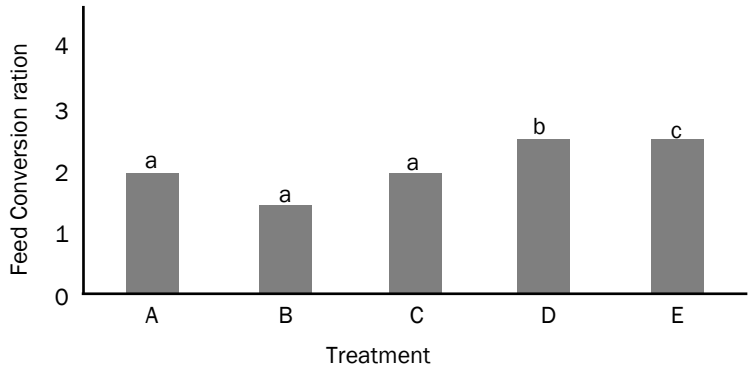

Figure 5. Feed conversion ratio (FCR) of Pacific white shrimp. Administration of microencapsulated synbiotic 0.5% (A), 1% (B), 2% (C), without administration of microencapsulated synbiotic as positive control (D) and negative control (E). Different superscript letters on each bar indicate significant different results (p<0.05).

bacterial cells count (TBC) at the end of microencapsulated synbiotic treatment showed a decline of TBC in all microencapsulated synbiotic treatments that were significantly different (P<0.05) compared to D (positive control) and E (negative control). The lowest TBC was found in B and the highest was found in E; it is likely due to the role of the present of prebiotic materials in the shrimp's digestive tract. According to Ringo *et al.* (2010) the prebiotic oligosaccharides showed its ability to

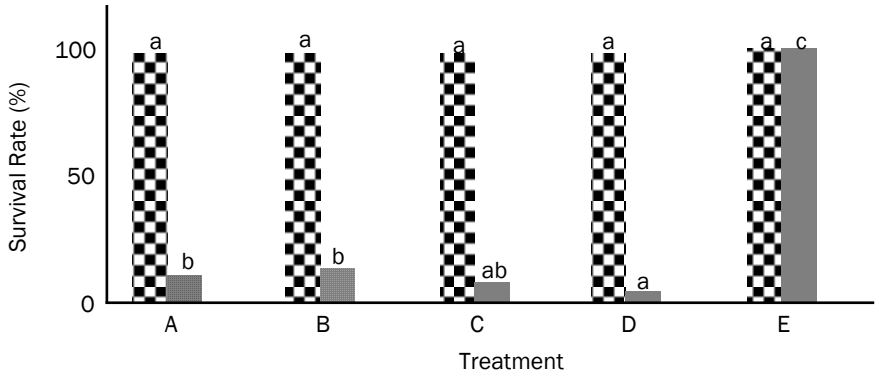

Figure 6. Survival rate of Pacific white shrimp. Administration of microencapsulated synbiotic 0.5% (A), 1% (B), 2% (C), without administration of microencapsulated synbiotic as positive control (D) and negative control (E). Different superscript letters on the same observation period indicate significant different results (p<0.05). **Note:** : ▭= After feeding trial, ▭ = After the challenge test

improve the health status and suppresed the presence of other unbeneficial intestinal bacterial cells inthe host's intestinal system.

The presumptive Vibrio cells count (PVC) decreased afterthe shrimps supplemented by synbiotic. The PVC counts of A and B treatments were significantly lower; different (P<0.05) from C, D and E treatments. The lowest PVC value was found in A and the highest PVC value was found in E. This effect suspected due to the role of competitiveness of probiotic cells, according to Cerezuela *et al.* (2011). Probiotic cells provided some benefits by producing antibacterial molecules in the form of bacteriocin which can directly inhibit other unbeneficial bacterial cells or viruses and actively participate against the infection.

The high *Bacillus* NP5 Rf^R cells population in B (dosage 1%) indicated that *Bacillus* NP5 Rf^R could survive in the intestinal tracts and reached the target organs. It also showed that probiotic cells haveresulted beneficial effects on shrimp, because hindering the movement of other bacterial cells in the intestinal wall (translocation), improving the function of mucosal barrier characterized by increasing of non-specific immune responses production or in the modulating the inflammation (Cerezuela *et al.*, 2011). Moreover, prebiotic is a non-digested food which is beneficial to the host by selectively stimulating the growth of certain bacteria in the intestine (Mahious *et al.*, 2006).

The total population of *V. harveyi* Rf^R cells after the challenge test increased, along with the increasing of PVC. Bacterial cells which entered through water and food will influence the intestinal microflora, so that they result in the fluctuation of the intestinal bacterial cells in aquatic animals as their micro environments (Austin, 2006). The microbial population fluctuation may caused increasing of *V. harveyi* Rf^R cells population after the challenge test, especially in the challenge test process that was done by immersion.

The increasing of TBC, PVC and *V. harveyi* Rf^R values after the challenge test allegedly caused by the administration of microencapsulated synbiotic, according to Chiu *et al.* (2007) who stated that the addition of probiotic bacterial cells after a certain period in shrimp increased bacterial population in the intestinal tract.

The specific growth rate (SGR) and the feed conversion ratio (FCR) of shrimp after the feeding trial are presented in figure 4. In this study, it appeared that SGR of A, B and C treatments showed the better results compared to those of controls, and the lowest FCR was found in B (1%). This was caused by the pressence of probiotic cells and prebiotic in the shrimp intestinal tract. According to Daniels *et al.* (2010) who used synbiotic containing *Bacillus* spp. cells and mannan oligosaccharides has also resulted beneficial performances; in which it could improve growth, feed conversion, immune response and disease resistance on lobster *Homarus gammarus* L. In addition, Zhang *et al.* (2012) stated that the addition of 0.4% MOS could improve growth performance; increasing the weight (WG) and specific growth rate (SGR); i.e by the values of 308% and by 2.51%, respectivelly.

The shrimps challenged by WSSV through injection and *V. harveyi* through immersion showed the survival rate that continued to decline. According to Phuoc *et al.* (2009) that co-infection of WSSV and *V. harveyi* caused mortality up to 80% and this pattern that continued to rise in their experiment. The co-infected shrimps showed the mortality pattern and susceptibility to disease which continue to rise and further weakening the shrimp conditions due to WSSV infection. Furthermore, the weakened

shrimps would be susceptible to secondary infection caused by pathogenic *Vibrio* cells which is commonly found in the marine environment (Selvin and Lipton, 2003).

Conclusions

Administration microencapsulated synbiotic to shrimp at dosages of 0.5%, 1% and 2% have showed improvement in the immune system parameters and growth performances of shrimp after feeding trial. Survival rate (SR) values of A and B treatments were higher than positive control. However, the administration of microencapsulated synbiotic has not been able to prevent a disease caused by heavy co-infection of WSSV and *V.harveyi*, which was shown by its low survival rate (SR) and mortality pattern which continued to rise.

References

Akrami, R., Razeghi-Mansour, M., Chitsaz, H., Ziaee, R, & Ahmadi, R., 2012. Effect of Dietary Mannan-oligosaccharide (MOS) on Growth Performance, Survival, Body Composition and Some Hematological Parameters of Carp Juvenile *(cyprinus carpio). J. Animal Sci Advances.* 2: 879–885. doi : 10.1007/s10695-011-9570-4

Anal, A.K & Singh, H. 2007. Recent Advances in Microencapsulation of Probiotics for Industrial Applications and Targeted Delivery. *Trends in Food Sci.Technol.* 18: 240-251. doi : 10.10 16/j.tifs.2007.01.004

Austin, B. 2006. The Bacterial Microflora of Fish. *Sci. World J.*. 6:931–945. doi : 10.1100/tsw. 2002.137

Cerezuela, R., Meseguer, J. & Esteban, M.A. 2011. Current Knowledge in Synbiotic use for Fish Aquaculture: A Review. *J. Aquaculture Res. Dev.* 8:1-7. doi: 10.4172/2155-9546.S1-008

Chiu, C.H., Guu, Y.K., Liu, C.H., Pan, T.M. & Cheng, W. 2007. Immune Responses and Gene Expression in White Shrimp, *Litopenaeus vannamei,* Induced by *Lactobacillus plantarum*. *Fish and Shellfish Immunol..* 23: 364-377. doi : 10.1016/j.fsi.2006.11.010.

Costa, A.M., Buglione, C.C., Bezerra, F.L., Martins, P.C.C. & Barracco, M.A. 2009. Immune Assessement of Farm-Reared *Penaeus vannamei* Shrimp Naturally Infected by IMNV in NE Brazil. *Aquaculture.* 291: 141-146. doi: 10.1016/j.aquaculture.2009.03.013.

Daniels, C.L., Merrifield, D.L., Boothoryd, D.P., Davies, S.J., Factor, J.R. & Arnold, K.E. 2010. Effect of Dietary *Bacillus* spp. and Mannan-Oligosaccharides (MOS) on European Lobster (*Homarus gammarus* l) Larvae Growth Performance, Gut Morphology and Gut Microbiota. *Aquaculture* 304: 49-57. doi : 10.1016/j.aquaculture.2010.03.018.

De Castro-Cislaghi, F.P., Silva, C.D.R.E., Fritzen-Freire, C.B., Lourenz, C.G. & Sant Anna, E.S. 2012. *Bifidobacterium* bb-12 Microencapsulated by Spray Drying with Whey: Survival Under Simulated Gastrointestinal Conditions, Tolerance to Nacl, and Viability During Storage. *J. Food Engineering*. 113: 186-193. doi : 10.1016/j.jfoodeng.2012.06.006.

Freire, C.B.F., Prudencio, S.E., Amboni, R.D.M.C., Pinto, S.S., Murakami, A.N.N. & Murakami, F.S. 2012. Microencapsulation of *Bifidobacteria* by Spray Drying in the Presence of Prebiotics. *Food Res. Int.*. 45: 306-312. doi : 10.106/j. foodress.2011.09.020.

Geraylou, Z., Souffreau, C., Rurangwa, E., Meester, L.D., Courtin, C.M., Delcour, J.A., Buyse, J. & Ollevier, F. 2013. Effects of Dietary Arabinoxylan-Oligosaccharides (AXOS) and Endogenous Probiotics on the Growth Performance, Non-Specific Immunity and Gut Microbiota of Juvenile Siberian Sturgeon *(Acipenser baerii). Fish and Shellfish Immunol..* 35: 766-775. doi : 10.106/j.fsi.2013.06.014.

Li, J., Tan, B. & Mai, K. 2009. Dietary Probiotic *Bacillus* OJ and Somaltooligosaccharides Influence the Intestine Microbial Populations, Immune Responses and Resistance to White Spot Syndrome Virus in Shrimp (*Litopenaeus vannamei*). *Aquaculture* 291: 35-40. doi: 10. 106/j.aquaculture.2009.03.005.

Liu, C.H & Chen, C.J. 2004. Effect of Ammonia on the Immune Response of White Shrimp *Litopenaeus Vannamei* and its Susceptibility to *Vibrio alginolyticus*. *Fish and Shellfish Immunology* 16: 321-334. doi: S1050-464 8(03)00113-X.

Mahious, A.S., Gatesoupe., Hervi, M., Metailler, R. & Ollevier. 2006. Effect of Dietary Inulin and Oligosaccharides as Prebiotics for Weaning Turbot, *Psetta maxima* (Linnaeu, C. 1758). *Aquaculture Int.* 14: 219-229. doi: 10.1007 /s10499-005-9003-4

Munaeni, W., Yuhana, M. & Widanarni. 2014. Effect of Micro-Encapsulated Synbiotic at Different

Frequencies for Luminous Vibriosis Control in White Shrimp *(Litopenaeus vanamei). Microbiol. Indonesia* 8: 73-80. doi: 010.5454 /mi.8.2.5

Partida-Arangure, B.O., Luna-González, A., Fierro-Coronado, J.A., Flores-Miranda, M.C. & González-Ocampo, H.A. 2012. Effect of Inulin and Probiotic Bacteria on Growth, Survival, Immune Response, and Prevalence of White Spot Syndrome Virus (WSSV) in *Litopenaeus vannamei* Cultured Under Laboratory Conditions. *African J. Biotechnol.* 12 : 3366-3375. doi: 10.5897/AJB12.1569.

Phuoc L.H., Corteel, M., Thanh, N.C., Nauwynck, H., Pensaert, M., Alday-Sanz, V., den Broeck, W.Y., Sorgeloos, P. & Bossier, P. 2009. Effect of Dosage and Challenge Routes of *Vibrio* spp. on Co-Infection with White Spot Syndrome Virus in *Penaeus vannamei*. *Aquaculture* 290:61-68. doi: 10.106/j.aquaculture.2009.02.004.

Putra, A.N & Widanarni. 2015. Screening of Amylolitic Bacteria as Candidate of Probiotics in Tilapia *(Oreochromis* sp*)*. *Res. J. Microbiol.*. 10:1–13. doi: 10.3923/jm.2015.1.13.

Ringo, E., Olsen, R.E., Gifstad, T.O., Dalmo, R.A., Amlund, H. & Hemre, G.I. 2010. Prebiotics in Aquaculture: A Review. *Aquaculture Nutrition.* 16:117-136. doi : 10.1111/j.1365-2095.2009 .00731.x

Rodriguez L. & Le Moullac, G. 2000. State of the Art Immunological Tools and Health Control of Penaeid Shrimp. *Aquaculture* 191: 109-119. doi: S0044-8486(00)00421-X.

Sanchez-Martinez, J.G., Guzman, G.A. & Ruiz, H.M. 2007. White Spot Syndrome in Cultured Shrimp : A Review. *Aquaculture Res.* 38:1339–1354. doi: 10.1111/j.1365-2109.2007.01827.x

Sang, F.M., Fotedar, R. & Filer, K. 2011. Effect of Dietary Mannan-Oligosaccharidae on The Survival, Growth, Immunity and Digestive Enzyme Activity of Freshwater Crayfish, *Cherax destructor* Clark (1936). *Aquaculture Nutrition* 17: 629-635. doi: 10.1111/j.1365-2095.20 10.00812.x.

Selvin, J. & Lipton, A.P. 2003. *Vibrio alginolyticus* Associated with White Spot Disease of *Penaeus monodon*. *Disease of Aquatic Organisms*. 57: 147-150. doi: http://eprints.cmfri.org.in/6063.

Singh S.K., Tiwari, V.K., Chadha, N.K., Prakash, C., Sukham, M., Das, P., Mandal, S.C. & Chanu, T.I. 2013. Effect of *Bacillus circulans* and Fructooligosaccharide Supplementation on Growth and Haemato-Immunological Function of *Labeo rohita* (Hamilton, 1822) Fingerlings Exposed To Sub-Lethal Nitrite Stress. *The Israeli J. Aquaculture–Bamidgeh*. 64:1-11.

Smith, V.J., Brown, J.H. & Hauton, C. 2003. Immunostimulation in Crustacean: does it Really Against Infection. *Fish and Shellfish Immunol.* 15: 71-90. doi: 10.1016/S1050-46 48(02)00140-7.

Teixeira-Lopes, M.A., Vieira-Girao, P.R.N., Freire, J.E.C., Rocha, I.R.C.B., Costa, F.H.F. & Radis-Baptista, G. 2011. Natural Co-Infectious Hypodermal and Hematopietic Necrosis Virus (IHHNV) and Infectious Mynecrosis Virus (IMNV) in *Litopenaeus vannamei* in Brazil. *Aquaculture* 312: 212-216. doi: 10.1016/j.aquaculture. 2011.01.005.

Wang, Y.B. 2007. Effect of Probiotics on Growth Performance and Digestive Enzyme Activity of The Shrimp *Penaeus vannamei*. *Aquaculture*. 269:259–264. Doi : 10.1016/j.aquaculture.20 07.05.035.

Weinbreck, F., Bodnar, I. & Marco, M.L. 2010. Can Encapsulation Lengthen the Shelf-Life of Probiotic Bacteria in Dry Products. *Int. J. Food Microbiol.* 136:364-367. doi: 10.1016/j.ijfood micro.2009.11.004

Widodo, Soeparno & Wahyuni, E. 2003. Bioenkapsulasi Probiotik (*Lactobacillus casei*) dengan Pollard dan Tepung Terigu serta Pengaruhnya terhadap Viabilitas dan Laju Pengasaman [Bioencapsulation of Prebiotics (*Lactobacillus casei*) with Pollard and Wheat Flour and Its Roles for the Acidification Rate]. 14:98-106.

Zhang, J., Yongjian, L.A., Lixia, T., Huijun, Y., Guiying, L. & Donghui, X. 2012. Effects of Dietary Mannan-Oligosaccharide on Growth Performance, Gut Morphology and Stress Tolerance of Juvenile Pacific White Shrimp, *Litopenaeus vannamei*. *Fish Shellfish Immunol.* 33:1027-1032. doi:10.1016/j.fsi.2012.05.001

Zubaidah, A., Yuhana, M. & Widanarni. 2015. Encapsulated Synbiotic Dietary Supplementation at Different Dosages to Prevent Vibriosis in White Shrimp, *Litopenaeus vannamei*. *Hayati Journal of Biosciences.* 22: 163-168. doi : 10.1016/j.hjb.2015.10.007.