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Effect of Micro-encapsulated Synbiotic at Different Frequencies for Luminous Vibriosis Control in White Shrimp (*Litopenaeus vannamei*)

WAODE MUNAENI, MUNTI YUHANA*, AND WIDANARNI

Department of Aquaculture, Faculty of Fisheries and Marine Science, Institut Pertanian Bogor Campus Darmaga, Bogor 16680, Indonesia

The aim of this study was to evaluate the effect of micro-encapsulated synbiotic application at different frequencies for luminous disease control in white shrimp (*Litopenaeus vannamei*). The luminous disease is caused by *Vibrio harveyi*. In this experiment, a synbiotic which was a combination of the probiotic *Bacillus* sp. NP5 Rf^R and the oligosaccharides from sweet potato (*Ipomea batatas* L.) jago variety was applied. The synbiotic was encapsulated by spray drying method. The *in vivo* experiment was conducted by supplementing the shrimp's diet with the micro-encapsulated synbiotic for 40 d. Treatments included the administration micro-encapsulated synbiotic at different frequencies i.e. once a week (A), twice a week (B), daily (C), and without micro-encapsulated synbiotic (control treatment). The control treatment consisted of positive (K+) and negative (K-) controls. After 30 d period, all of the shrimp were challenged by intramuscular injection of pathogenic *V. harveyi* Rf^R at a cell concentration of 10^6 CFU mL^{-1} except the negative control. The treatment C resulted in significantly higher survival rate (SR), specific growth rate (SGR), and immune responses than those of the controls, whereas the feed conversion ratio (FCR) was lower than the controls. In addition, the population of *Bacillus* sp. NP5 Rf^R and total bacterial count (TBC) in the intestines increased, whereas the population of *V. harveyi* Rf^R and the total vibrio count (TVC) were lower compared to the controls.

Key words: frequencies, *Litopenaeus vannamei,* micro-encapsulated, synbiotic, *Vibrio harveyi*

Penelitian ini dilakukan untuk mengkaji efek pemberian mikrokapsul sinbiotik dengan frekuensi berbeda pada udang vaname (*Litopenaeus vannamei*) yang diinfeksi *Vibrio harveyi*. Sinbiotik yang digunakan merupakan kombinasi dari probiotik *Bacillus* sp. NP5 Rf^R dan oligosakarida dari ubi jalar (*Ipomea batatas* L.) varietas jago yang dienkapsulasi dengan metode *spray drying*. Percobaan dilakukan selama 40 hari dengan menambahkan mikrokapsul sinbiotik pada pakan. Perlakuan meliputi pemberian mikrokapsul sinbiotik dengan frekuensi satu kali seminggu (A), dua kali seminggu (B), setiap hari (C), dan tanpa mikrokapsul sinbiotik (kontrol). Perlakuan kontrol terdiri dari kontrol positif (K+) dan kontrol negatif (K-). Setelah 30 hari periode percobaan, semua udang diuji tantang dengan *Vibrio harveyi* Rf^R konsentrasi sel 10^6 CFU ml^{-1} kecuali kontrol negatif. Hasil penelitian menunjukkan bahwa, perlakuan C memberikan nilai *Survival Rate* (SR), *Specific Growth Rate* (SGR), dan respon imun yang lebih tinggi serta *Feed Conversion Ratio* (FCR) yang lebih rendah dibanding kontrol. Perlakuan juga mampu meningkatkan populasi *Bacillus* sp. NP5 Rf^R dan *total bacterial count* (TBC) di usus serta mampu mengurangi *V. harveyi* Rf^R *Count* dan *total vibrio count* (TVC) dibanding perlakuan kontrol.

Kata kunci : frekuensi, *Litopenaeus vannamei*, mikrokapsul, sinbiotik, *Vibrio harveyi*

White shrimp (*Litopenaeus vannamei*) has currently become one of the major aquacultural commodities in South East Asian countries including Indonesia. The application of intensive cultivation to increase shrimp production has resulted in deterioration of water quality which influences disease susceptibility. One of the important diseases in shrimp is vibriosis (Chiu *et al*. 2007). This disease which is mainly caused by luminous *Vibrio harveyi* (Phuoc *et al*. 2009) can cause mass mortality from shrimp larvae (Chrisolite *et al*. 2008) including the nauplius, zoea, mysis and post larvae stadia to adult shrimp in the fattening ponds (Soto-Rodriguez *et al*. 2012).

One of the biological control strategies to improve growth and disease resistance in aquaculture organisms is synbiotic application. A synbiotic is a nutritional supplement which is combination of probiotics and prebiotics (Cerezuela *et al*. 2011). Probiotics are living microbial cells which are beneficial to the host and improve the quality of the environment as well as the host's immune responses against diseases (Newaj-Fyzul *et al*. 2014). Prebiotics are food ingredients that cannot be digested by the host but can be selectively metabolized by bacteria (Ringo *et al*. 2010). The application of probiotics and prebiotics simultaneously has proven to improve the survival rate and to enhance the immune system in

*Corresponding author; Phone: +6281320006175; email: yhnmymy@yahoo.co.id

shrimp (Li *et al*. 2009), koi carp (*Cyprinus carpio* koi) (Lin *et al*. 2012), and Nile tilapia (*Oreochromis niloticus*) (Putra 2010).

Application of fresh culture synbiotics has some drawbacks such as a limited storage period because the probiotic cells are easily degraded by unfriendly enviromental conditions. One method to protect the probiotic bacteria cells is micro-encapsulation. There are several techniques used in micro-encapsulation of probiotics including spray-drying, extrusion, emulsion, and phase separation (Kailasapathy 2002; Mu˜noz-Celayaa *et al*. 2012). Spray drying is relatively simple compared to other methods (Jyothi *et al*. 2010). The genus *Bacillus* which is resistant to high temperatures and high pressures (Lin *et al*. 2012) was used in this study and oligosaccharides from sweet potato (*Ipomea batatas* L.) starch was used as the source of the prebiotic (Marlis, 2008). A previous study showed that the application of 2% micro-encapsulated synbiotic made from *Bacillus* sp. NP5 combined with oligosaccharides from sweet potato (*I. batatas* L.) has improved the survival rate, immune system, and disease resistance in white shrimp (*L. vannamei*) infected by *V. harveyi* (Zubaidah *et al*. 2014).

The effectiveness of synbiotics was influenced by several factors such as the host species, dosage, duration or frequency of administration, and the type of probiotic and prebiotic administered (Merrifield *et al*. 2010; Nayak 2010; Cerezuela *et al*. 2011). Different synbiotic administration frequencies may affect the induction of the host's immune response (Nayak 2010). Previous research has showed that the application of fresh culture synbiotic at different frequencies resulted in different effects on the immune response of white shrimp (*L. vannamei*) (Oktaviana *et al*. 2014). Based on the facts above, this study was aimed to evaluate the effect of micro-encapsulated synbiotic application in different frequencies for luminous disease control on white shrimp (*L. vannamei*).

MATERIALS AND METHODS

The Preparation of the Prebiotic and Probiotic Bacteria. The prebiotic used in this study is a product of sweet potato (*I. batatas* L.) var. jago flour extraction. The preparation of the prebiotic made from sweet potato (*I. batatas* L.) flour refers to the method by Marlis (2008). Fresh sweet potatoes (*I. batatas* L.) from the jago variety were peeled and cut into ± 1 mm thick slices. Then the sweet potato slices were dried in an oven at 55 °C for 5 h. The dried slices were then grinded using a willey mill then flour sifted through a size 60 mesh. Five hundred grams of the sweet potato flour was then mixed with water at a ratio of 1:1 (w/v) then steamed for 30 min at 100 °C. After steaming, the dough was made into flour again and was extracted for its oligosaccharides based on the method by Muchtadi (1989). The extraction used 70% ethanol and the mixture was thickened in a vacuum evaporator at 40 °C. The concentration of the oligosaccharide used was 5% (Marlis 2008).

The probiotic bacteria used was *Bacillus sp.* NP5 which was isolated from Nile tilapia (*O. niloticus*) (Putra 2010) and had been made resistant using 50 µg ml^{-1} of the antibiotic rifampicin (rifampicin 0.25 g, 9.5 ml absolute ethanol, 0.5 ml *aqua bidestilata*) (*Bacillus* sp. NP5 Rf^R). The *Bacillus* sp. NP5 Rf^R cells were cultured in an SWC-agar medium (Sea Water Complete: 5 g bactopeptone, 1 g yeast extract, 3 mL glycerol, 750 mL sea water, 250 mL aquadest, 20 g bactoagar) and incubated for 24 hours at 29 °C. The cells were inoculated to the liquid SWC medium and incubated in waterbath shaker (JULABO SW-20C) for 18 hours at 29 °C at a speed of 140 rpm. The gel suspension was transfered to a 25 mL corning tube and was centrifuged at a speed of 5.000 rpm for 10 min. The cells were then rinsed with 25 mL of PBS (Phosphate Buffered Saline: 8 g NaCl, 0.2 g KH2PO4, 1.5 g Na2HPO4, 0.2 g KCl, 1000 mL *aquadest*), homogenized, and centrifuged at a speed of 5,000 rpm for 10 min. The pellets produced were homogenized in 25 mL PBS.

The Micro-encapsulation of the Synbiotic. One percent of *Bacillus* sp. NP5 Rf^R cells at a concentration of 10^8 CFU ml^{-1} 2% of prebiotic (Mahious *et al*. 2006) were mixed with a maltodextrin coating agent and milk whey until homogenous using a mixer. The ratio of synbiotic, whey and maltodextrin was 1:1:1 (v/v/w). Then this mixture was micro-encapsulated using the spray dryer method (Mini bunchi 190) at an inlet temperature of 150 °C and an outlet temperature of 70 °C.

Research Design. The 2.6 ± 0.3 g of white shrimp larvae were obtained from Balai Budidaya Air Payau (BBAP-The Brackish Water Cultivation Research Station), Situbondo, East Java. The larvae were allowed to adapt for 2 weeks in 20 holding tanks sized 60 x 40 x 40 cm^3 (30 liter volume) at a density of 10 shrimp/tank. During the keeping period, siphoning was done daily and 50% of the water was replaced

afterwards. The water quality parameters were maintained at temperatures of 25-26 °C, pH 7.7-8.0, salinity 32-35 g L^{-1}, DO 7.3-79 ppm and TAN 0.5-0.9 mg L^{-1}.

The test feed was Gold Coin 932p commercial pellet with a protein content of 31.90%, fat 7.55%, and BETN 36.85%. The dosage of the micro-encapsulated synbiotic was 2% (Zubaidah *et al*. 2014) and the mixture was sprayed with 2% egg white as a binder. The control was also sprayed with the binder. Feed was given 4 times a day starting at 07.00, 11.00, 15.00, and 19.00 Western Indonesia Meantime *at satiation* with a Feeding Rate (FR) of 10% of the shrimp's biomass. The treatment consisted of feeding the micro-encapsulated synbiotic once a week (A), twice a week (B), every day (C), and without any micro-encapsulated synbiotic for the positive control (K+) and negative control (K-). The feeding was done for 30 d, and then all the shrimp were injected with *V. harveyi* Rf^R on day 31 except for the negative controls which were injected with *PBS*. The shrimp were then kept for a further 9 d.

Observation Parameters. Survival, Growth, and Feed Conversion Rates. The Specific Growth Rate (SGR) and Feed Conversion Rate (FCR) were calculated from day 0 to day 30, the Survival Rate (SR) was calculated from the start till the end of the study using this equation: SR (%) = [(Nt/No) x 100%]; SGR (%) = [100 x (ln We - ln Ws)/t]; FCR = [(F) / (Bt - Bo)] (Zokaeifar *et al.* 2012).

Where: *Nt* is the number of live shrimps at the end of the study (individuals), *No* is the number of live shrimps at the beginning of the study (individuals), *We* is the average weight of the shrimps at the end of the study (grams), *Ws* is the average weight of the shrimps at the beginning of the study (grams), *t* is the duration of the study (days), *F* is the amount of feed, *Bt* is the shrimp biomass at the end of the study (grams), *Bo* is the biomass of the shrimp at the beginning of the study (grams).

Immune Response. The blood sampling was done according to the method by Chiu *et al*. (2007). An amount of 0.1 mL shrimp haemolymph was drawn from the base of the first swimmeret using a 1 mL syringe which had been filled with 0.9 mL anti-coagulant (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA). The mixture of haemolymph and anti-coagulant was observed and the number of cells counted using a haemocytometer and a microscope at a 400x magnification.

The phenoloxidase activity measurement was done based on the formation of dopachrome produced by the L-DOPA. The standard solution containing 100 µL haemocyte suspension, 50 µL cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.01 M calcium chloride, 0.26 magnesium chloride) and 50 µL L-DOPA (3 mg LDOPA, 1 mL cacodylate buffer) was used to measure the PO background activity in the test solution. The measurement of the Optical Density (DO) was done using a spectrophotometer with a wavelength of 492 nm (Chiu *et al*. 2007). The observation of the immune response was done on day 0 (before treatment), day 30 (before the challenge test), day 32 (1 day post challenge test), day 36 (4 ds post challenge test), and day 40 (9 ds after challenge test).

Bacterial Population in the Intestines. The intestines from 2 shrimps from each treatment were weighed and 0.01 g was taken from each sample and homogenized with 0.99 mL PBS. This mixture was then spread onto specific TCBS (Thiosulphate Citrate Bile-Salt Sucrose) agar media with the antibiotic rifampicin for the *V. harveyi* Rf^R Count and TCBS without the antibiotic for the Total vibrio count (TVC). The *Bacillus* sp. NP5 Rf^R Count was done by spreading the mixture onto SWC agar media with antibiotics and for the Total bacterial count (TBC) the mixture was spread onto SWC agar media without antibiotics. The media were then incubated for 24 h at 35°C. The observations of the bacteria population in the intestines were done on days 0, 30, 32, 36, and 40.

Data Analysis. The data were analyzed using Microsoft Excel 2010 and tested using ANOVA (Analysis of Variants); if it had a significant effect, it was continued with a Duncan test using the SPSS (Statistical Program Software System) version 16.

RESULTS

The *L. vannamei* survival rate (SR) before the challenge test was 100%. After the challenge test, the highest SR was shown by treatment C at 63.33 ± 5.77 % and this was significantly different ($p < 0.05$) from treatment A, K+ and K-, but was not significantly different ($p > 0.05$) from treatment B at 56.66 ± 5.77% (Fig 1). The highest SGR was shown by treatment C at 3.35 ± 0.25% which was significantly different from treatment A, K+ and K-, but was not significantly different from treatment B (Fig 2a). The lowest FCR was shown by treatment C at 1.63 ± 0.03 which was significantly different ($p < 0.05$) from all other

treatments (Fig 2b).

THC (Fig 3a) and PO (Fig 3b) in the *L. vannamei* increased after 30 days of treatment and decreased after day 32 and increased again at day 36 and day 40. The highest THC and PO during the study were shown by treatment C. The THC for treatment C during the duration of the study was, respectively, $1.99 \pm 0.32 \times 10^7$ cells ml^{-1}, $5.25 \pm 0.91 \times 10^7$ cells ml^{-1}, $4.65 \pm 0.51 \times 10^7$ cells ml^{-1}, $5.07 \pm 0.70 \times 10^7$ cells ml^{-1}, and $5.99 \pm 0.46 \times 10^7$ cells ml^{-1}. The PO for treatment C during the duration of the study was, respectively, 0.28 ± 0.05, 0.56 ± 0.10, 0.49 ± 0.14, 0.44 ± 0.10, $0.66 \pm$

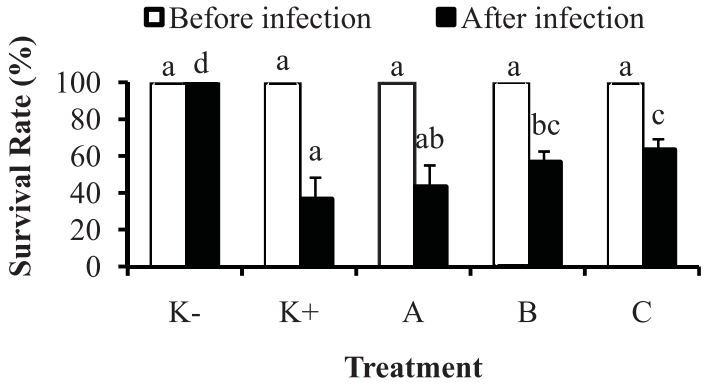

Fig 1 Survival Rate (SR) of *L. vannamei*. Different letters over each treatment bar in same colours (mean ± SD) indicated significant difference (Duncan; $P < 0.05$). Negative control (K-); positive control (K+); supplementation of micro-encapsulated synbiotic diet at once a week (A); twice a week (B); daily (C).

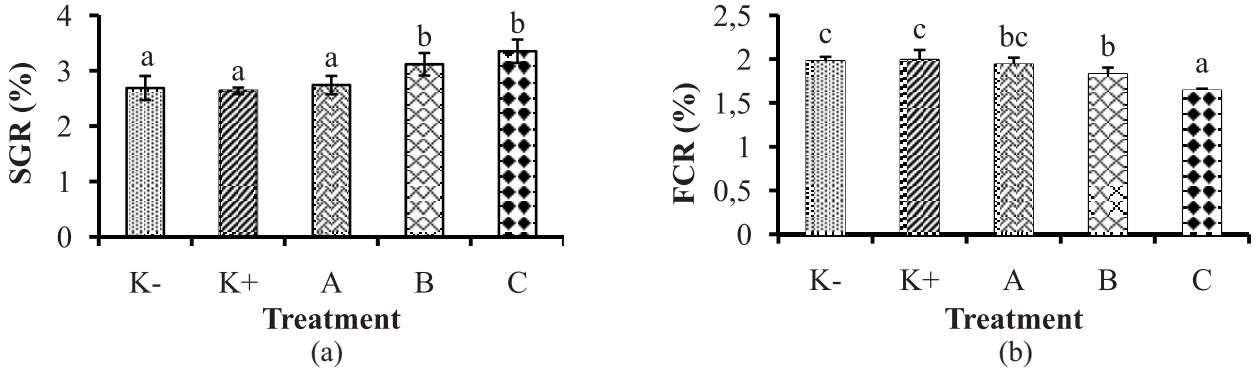

Fig 2 Spesific growth rate (SGR) (a); and feed conversion ratio (FCR) (b) of *L. vannamei*. Different letters over each treatment bar (mean ± SD) indicated significant difference (Duncan; $P < 0.05$). Negative control (K-); positive control (K+); supplementation of micro-encapsulated synbiotic diet at once a week (A); twice a week (B); daily (C).

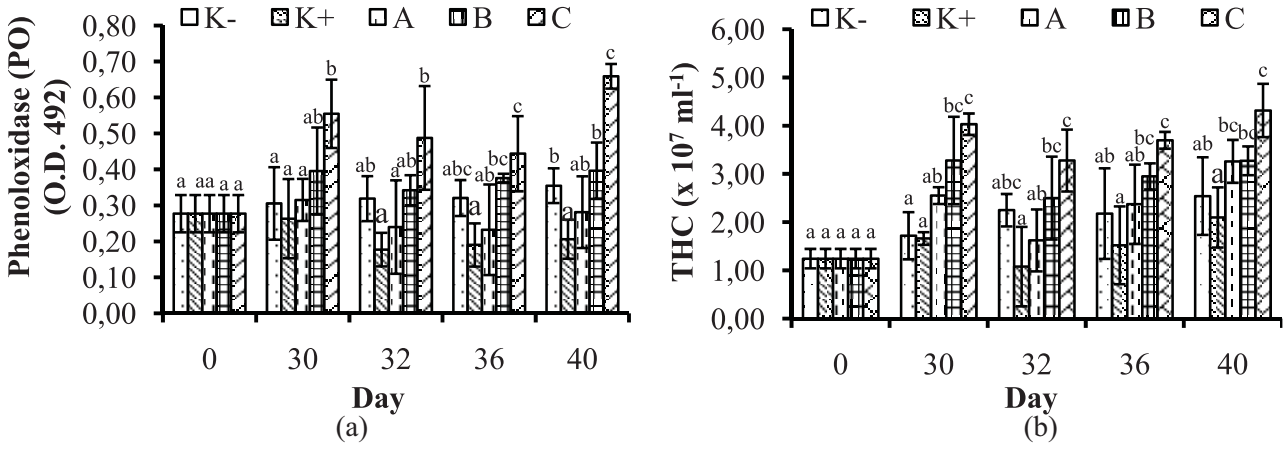

Fig 3 Total haemocyte count (THC) (a) and Phenoloxidase (PO) (b) of *L. vannamei*. Different letters over each treatment bar in same day (mean ± SD) indicated significant difference (Duncan; $P < 0.05$). Negative control (K-); positive control (K+); supplementation of micro-encapsulated synbiotic diet at once a week (A); twice a week (B); daily (C).

0.03.

After 30 days of treatment, the *Bacillus* sp. NP5 Rf^R Count (Fig 4a) and TBC (Fig 4b) increased; the highest was showed by treatment C (7.29 ± 0.02 and 8.82 ± 0.04) log 10 CFU g^{-1}. These numbers decreased after day 32, then increased again at day 36 and day 40. One day after the challenge test (day 32), the highest *V. harveyi* Rf^R count (Fig 4c) and TVC (Fig 4d) were shown by treatment A (6.49 ± 0.10 and 6.88 ± 0.24) log 10 CFU g^{-1} which were significantly different from the other treatments. After day 36 and 40, the *V. harveyi* Rf^R count and TVC for treatments C and B showed a significant decrease compared to the other treatments.

DISCUSSION

The high SGR and low FCR for treatments C and B in this study are suggested to be an effect of the increased *Bacillus* sp. NP5 Rf^R (Fig 4a) in the shrimp's intestines. The *Bacillus* sp. Np5 Rf^R are believed to have the ability to secrete exogenous enzymes which can catalyze macro molecules in feed into simpler molecules, making it easier for the host to digest the feed. Other studies have also reported that the shrimp's increased growth is believed to be caused by the increased digestive enzymes which were induced by probiotic bacteria (Wang 2007) such as protease and amylase which can stimulate and increase the host's digestion rate (Zhang *et al*. 2010). The probiotic bacteria *Bacillus* sp. NP5 which had been isolated from Nile tilapia (*O. niloticus*) are amylolytic bacteria which have an important role in digestion (Putra 2010).

Besides resulting in a higher growth performance, treatments C and B can also improve the white shrimp's immune response and resistance in comparison to the controls. This can be seen in the increased THC (Fig 3a) and PO (Fig 3b). Haemocytes have an important role in the immunity system; they remove foreign particles in the haemocoel through phagocytosis, encapsulation, and nodular aggregation (Rodriquez and Le Muollac 2000, Smith *et al.* 2003, Kakoolaki *et*

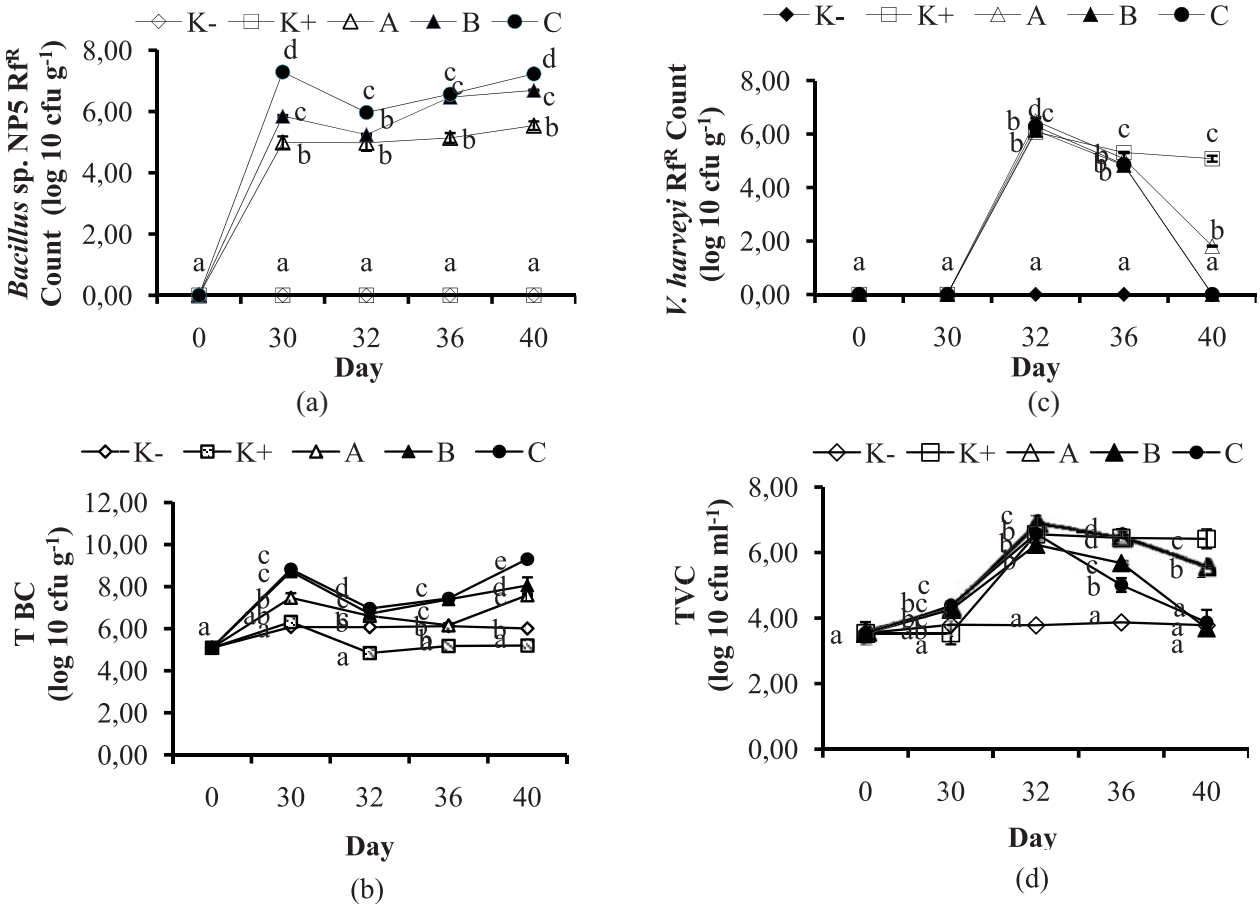

Fig 4 *Bacillus* NP5 sp. Rf^R *Count* (a); Total bacterial count (TBC) (b); *V. harveyi* Rf^R *Count* (c); and Total vibrio count (TVC) (d) of *L. vannamei*. Different letters over each treatment line in same day (mean ± SD) indicated significant difference (Duncan; P < 0.05). Negative control (K-); positive control (K+); supplementation of micro-encapsulated synbiotic diet at once a week (A); twice a week (B); daily (C).

al. 2010, Dantas-Lima *et al. 2012, Hao et al. 2014*). The supplementation of probiotics can increase the THC which in turn will improve the immune response during times of duress due to pathogenic infections in shrimp (Chiu *et al*. 2007). One day after the challenge test, the THC in all the treatments decreased. The decrease in the number of haemocytes is an effect of the shrimp's immune system mechanisms such as haemocyte infiltration to infected tissues or haemocyte death due to apoptosis (Costa *et al.* 2009).

The phenoloxidase (PO) enzyme activity is also very important against the microbial infections (Vargas-Albores and Yepiz-Plascencia 2000, Chiu *et al*. 2007; Cerenius *et al*. 2010; *Hao et al. 2014*). PO in the haemolymph functions as an inactive pro-enzyme or also known as proPO. Transformation from proPO to PO involves several reactions in the proPO activing system (Rodriquez and Le Moullac 2000). The ProPO system can be activated by several microbial polysaccharides and specific pattern recognition proteins (PRPs) such as LPS-and-β 1, 3-glucan-binding protein (LGBP) and peptidoglycan-binding proteins (PGBP) (Wang *et al*. 2007). Treatments C and B in this study were able to increase the PO value before the challenge test compared to the controls. The decrease of PO could cause lowered immunity in shrimps (Cerenius *et al*. 2010; *Hao et al.* 2014).

The high immune response in treatments C and B sumable as the effect of the increase population of *Bacillus* sp. NP5 Rf^R (Fig 4a) and TBC (Fig 4b) in the shrimp's intestines. The probiotic can directly improve immunity by passing through the epithelial cells in the intestines and having direct interaction lymphoid tissues then activating the immune response (immunostimulant). The indirect way is through contact between the epithelial cells in the intestines and GALT (gut associated limphoid tissue) which activates the cytokines, enabling communication among cells to activate an immune response (immunoregulator) (Nayak 2010).

The decrease in *V. harveyi* Rf^R count and TVC in treatments C and B in this study are believed to be influenced by the administration of the micro-encapsulated synbiotic. Probiotic bacteria can produce antibacterial molecules such as bacteriocin which directly inhibit other bacteria, actively fight infection, inhibit the movement of other bacteria on the intestinal wall (translocation), improve the mucosal barrier by increasing production of non-specific immune responses (Cerezuela *et al*. 2011; Cerezuela *et al*. 2013). Application of prebiotics could also inhibit the growth of pathogens and improve the immune system (Mahious *et al*. 2006).

In conclusion, the administration of micro-encapsulated synbiotic treatment C (supplementation of micro-encapsulated synbiotic diet at daily) is able to produce a low feed conversion ratio (FCR) value and can increase the survival rate (SR), specific growth rate (SGR), and immune response and resistance to *V. harveyi* in white shrimp (*L. vannamei*) compared to the controls. In addition, treatment C could also increase the population of *Bacillus* sp. NP5 Rf^R and the total bacterial count (TBC) and decrease *V. harveyi* Rf^R and total vibrio count (TVC) in the shrimp's intestines compared to the controls.

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