Bacillus NP5 Improves Growth Performance and Resistance Against Infectious Myonecrosis Virus in White Shrimp (*Litopenaeus vannamei*)

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Abstrak

Bacillus NP5 Meningkatkan Pertumbuhan dan Ketahanan Terhadap Infeksi Virus Myonecrosis pada Udang Putih (Litopenaeus vannamei)

Infectious Myonecrosis (IMN) merupakan salah satu penyakit yang sering menyerang udang vaname. Probiotik banyak digunakan pada budidaya udang karena terbukti mampu mengurangi serangan penyakit pada udang. Penelitian ini bertujuan untuk menguji pengaruh pemberian probiotik Bacillus NP5 melalui pakan terhadap kinerja pertumbuhan, respons imun, dan resistensi udang vaname terhadap infeksi Infectious Myonecrosis Virus (IMNV). Udang vaname Litopenaeus vannamei (2.41±0.07 g ekor^{-1}) diberi pakan yang disuplementasi probiotik Bacillus NP5 dengan dosis yang berbeda, 10^2 CFU.g^{-1} (A), 10^4 CFU.g^{-1} (B), 10^6 CFU.g^{-1} (C), dan kontrol tanpa suplementasi probiotik (kontrol negatif, KN; kontrol positif, KP) selama 30 hari dan dengan tiga ulangan untuk masing-masing dosis, kemudian KP, perlakuan A, B, dan C diuji tantang secara intramuskular dengan IMNV (100 µl.ekor^{-1}). Hasil penelitian ini menunjukkan bahwa udang vaname yang diberi pakan dengan suplementasi probiotik mempunyai laju pertumbuhan harian (LPH), rasio konversi pakan (RKP), dan respons imun yang lebih tinggi. Udang tersebut juga mempunyai total hemocyte count (THC) dan resistensi terhadap IMNV yang lebih tinggi dibandingkan kontrol positif. Konsentrasi probiotik 10^6 CFU.g^{-1} memberikan hasil terbaik dalam meningkatkan pertumbuhan, respon imun, dan resistensi udang vaname terhadap infeksi IMNV.

***Kata kunci**: probiotik, Bacillus NP5, Litopenaeus vannamei, pertumbuhan, IMNV*

Abstract

Infectious Myonecrosis (IMN) is one of the most prevalent white shrimp diseases. Probiotics are widely used in shrimp cultivation because they have been proven to reduce shrimp disease outbreak. This study aimed to observe the effect of oraly administered probiotic Bacillus NP5 on the white shrimp's growth performance, immune response, and resistance to Infectious Myonecrosis Virus (IMNV) infection. White shrimp Litopenaeus vannamei (2.41±0.07 g individual^{-1}) were fed with a feed supplemented with different doses of the probiotic Bacillus NP5, i.e. 10^2 CFU.g^{-1} (A), 10^4 CFU.g^{-1} (B), 10^6 CFU.g^{-1} (C), and control without any probiotic (negative control, KN; positive control, KP) for 30 days and with three replications for each dose, then KP, treatment A, B, and C were challenged intramuscularly with IMNV (100 µl.shrimp^{-1}). The results of the study showed that white shrimp fed with the supplemented probiotic had higher Daily Growth Rate (DGR), Feed Conversion Ratio (FCR), and immune response. They also had the higher Total Hemocyte Count (THC) and resistance to IMNV than the positive control. Probiotic with concentration of 10^6 CFU.g^{-1} gave the highest value on enhancing growth, immunity, and resistance of white shrimp towards IMNV infection.

***Key words:** probiotic, Bacillus NP5, Litopenaeus vannamei, growth, IMNV*

Introduction

White shrimp (*Litopenaeus vannamei*) is one of the superior fisheries export commodities. One of the problems in increasing productivity in the cultivation of white shrimp is the attacks by various diseases, especially by the viruses (Lightner, 2011). Viral diseases that often attack shrimp are Infectious Hypodermal and Hematopoietic Necrosis (IHHN), Yellow Head (YH), White Spot Syndrome (WSS), Taura Syndrome (TS), and Infectious Myonecrosis (IMN). IMN is the most prevalent white shrimp disease (Walker and Winton, 2010). It attacks white shrimp which is caused by infectious myonecrosis

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Diterima/Received: 23-10-2014
Disetujui/Accepted: 20-11-2014

virus (IMNV) and causes high mortality in brackishwater ponds in Indonesia (Senapin *et al.*, 2007). IMNV is a dsRNA virus from the Totiviridae family (Poulos *et al.*, 2006; Tang *et al.*, 2008). It is a non-envelope virus and is a 40 nm icosahedral-shaped virion (Senapin *et al.*, 2007). The cumulative mortality index of cultivated shrimp due to IMNV infections up to 70% (Poulos *et al.*, 2006).

The use of probiotics is one way to prevent disease in white shrimp. Probiotics are defined as a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance, immune system, environmental quality, and feed nutritional value (Merrifield *et al.*, 2010). Probiotics are widely used in shrimp cultivation because they have been proven to reduce the chances of shrimps being infected by diseases. In addition, probiotics are environmentally safe because they leave no residues and do not elicit resistance in the micro flora found in the water (Li *et al.*, 2009). A similar argument was delivered by Partida-Arangure *et al.* (2013) who stated that the addition of probiotic bacteria increase immunity and reduce the prevalence of WSSV infection in white shrimp.

Bacillus NP5 is a probiotic agent found in the intestines of tilapia (*Oreochromis niloticus*). *Bacillus* NP5 has been tested for its efficacy in improving the tilapia growth performance (Putra, 2010) and increase the tilapia immune response and resistance to *Streptococcus agalactiae* (Tanbiyaskur, 2011). This study was carried out to test the effect of oraly supplemented probiotic *Bacillus* NP5 at different doses on white shrimp growth, immune response, and resistance to IMNV (infectious myonecrosis virus) infection.

Materials and Methods

Probiotic preparation

The pure culture of probiotic *Bacillus* NP5 which was used was obtained from Fish Health Laboratory, Department of Aquaculture, The Faculty of Fisheries and Marine Sciences, Bogor Agricultural University. Before being fed to the shrimp, probiotic *Bacillus* NP5 was marked with rifampicin resistant markers (Rf^R) at a dose of 50 µg.mL^{-1} through spontaneous mutation.

The probiotic *Bacillus* NP5 was cultured in 25 mL sea water complete (SWC)-broth media (5g bacto peptone, 1g yeast extract, 3 mL glycerol, 750 mL sea water, and 250 mL aquadest) and incubated in a waterbath shaker (140 rpm, 29^{o}C) for 24 hours (the density produced was 10^8 cfu.mL^{-1}). After that, the bacteria cells were separated from the media by centrifuge and washed twice with sterile physiological solution (NaCl 0.85%). Serial dilution was done according to the experimental doses with densities of 10^8 cfu.mL^{-1}, 10^6 cfu.mL^{-1}, and 10^4 cfu.mL^{-1}. Culture purity was routinely checked during this study with the spread plate method.

Test-feed preparation

The base feed used was commercial feed with 35% protein to which the cultured probiotic was added at a rate 1% v/w, mixed with egg white at a dose of 2% v/w used as a binding agent (Wang, 2007). The formulations of the tested feed groups were: KN and KP: Positive and negative control groups with no probiotic. A: Probiotic at a density of 10^2 cfu.g^{-1}; B: Probiotic at a density of 10^4 cfu.g^{-1}; and C: Probiotic at a density of 10^6 cfu.g^{-1}

The feed, probiotic, and egg white were mixed manually before being mixed into the feed. The control contained only the commercial feed and egg white. The feed was air-dried for 5-10 minutes at room temperature (24-25^{o}C) and stored at 4^{o}C until used.

Experimental design

White shrimps (PL13) were obtained from PT Suri Tani Pemuka Carita, Pandeglang, Banten, Indonesia. The shrimps (2.41±0.07 g.indv^{-1}) were stocked randomly into 60x30x40 cm^3 aquariums, with sea water in 30 liter and stocked each with 10 shrimps. Each aquarium was equipped with a shelter, aeration, and covered with a net. The net was used to reduce the intensity of light entering the aquarium and preventing the shrimp from escaping. The shrimps were given probiotic-supplemented feed in various doses once a day at 11.00 Western Indonesia Time for 30 days, and they were fed with commercial feed at 08.00, 14.00, and 17.00 Western Indonesia Time.

On day 31 after the initiation of the probiotic treatment, eight shrimps in each aquarium were infected with IMNV (except for the negative control) at doses of 100 µL.shrimp^{-1} via intramuscular injection in the dorsal region between the third and fourth segments (Tang *et al.*, 2005). The mortality rates of the injected shrimp were monitored for a 14-day period. Shrimps infected by IMNV were confirmed by PCR testing.

The quality of the water in the aquarium during the study was maintained and 10% of the water was changed daily. Fecal matter and leftover feed which accumulated in the aquariums were removed by siphoning. The water quality in the

rearing medium of the shrimp during the study were: water temperature 28.4-29.0^{o}C, salinity 28.9-30.0 (g.L^{-1}), total ammonia nitrogen 0.3-0.5 mg.L^{-1}, dissolved oxygen 5.0–6.3 mg.L^{-1}, and pH 7.6-8.1.

Survival and growth observations

The number of shrimp was counted at the end of the probiotic treatment and challenge test for survival data. The average body weight of the shrimp was recorded throughout the treatment period with probiotic for daily growth rate (DGR) and feed conversion ratio (FCR) data. Survival (Lin *et al.*, 2012), daily growth rate (DGR), and feed conversion ratio (FCR) (Lin *et al.*, 2012) were calculated using the following equation:

$$\text{Survival}(\%) = \frac{\text{final number of shrimp}}{\text{initial number of shrimp}} \text{x} 100\%$$

$$DGR(\%) = \left[\sqrt[days]{\frac{final\ weight}{initial\ weight}} - 1 \right] x 100\%$$

$$\text{FCR} = \frac{\text{feed given (dry weight)}}{\text{weight gain (wet gain)}}$$

Immune parameters observations

Observations of the immune response were done at the end of the probiotic treatment and the challenge test. The immune response parameters observed in this study were total haemocyte count (THC) and differential haemocyte (DH).

The THC observation was done according to the method by Yeh and Chen (2009). An amount of 0.1 mL of haemolymph was drawn from the base of the fourth pedicle with a syringe containing 0.1 mL anti-coagulant (trisodium citrate 8.82 g; sodium chloride 19.89 g; ethylene diamine tetra-acetic acid 3.72 g; and 1000 mL aquadest). The number of haemocytes per mL was counted using a binocular microscope with a magnification of 400 times.

$$THC(cell.mL^{-1}) = Total\ haemocyte\ x \frac{1}{1x1x0,1} m^3\ x\ 2\ x 10^3\ mL$$

Differential haemocyte was calculated from haemolymph samples by making haemocyte slides. The slides glass were soaked in methanol for 5-10 minutes and air-dried. Haemolymph was dropped onto the slides and made into blood smears then air-dried. The slides were then fixed with methanol for 5-10 minutes then again air-dried. The slides were soaked in Giemsa staining solution for 15-20 minutes then flushed with running water then left to dry. The haemolymph smears were observed using a binocular microscope at 1000 times magnification and the cells were identified. The number of haemocytes was counted up to 100 cells and the percentages of the types of cells were calculated. Differential haemocyte was calculated using the following equation:

$$\text{Haemocyte type}(\%) = \frac{\text{Number of each haemocyte type}}{\text{Total haemocyte}} \text{x} 100\%$$

Bacterial quantification

The shrimp intestines were collected and homogenized into a sterile physiological solution (NaCl 0.85%) with a ratio of 1:10 (w/v) and then the serial dilution were carried out. After that, the bacteria from the shrimp intestine samples were counted using the spread plate method. The medium used was SWC-agar for total bacterial count (TBC) and SWC+Rf 50 µg.mL^{-1} for *Bacillus* NP5 count. After 24-hour incubation in an incubator at 29 ^{o}C, the number of bacteria colonies was counted in Colony Forming Units (CFU.g^{-1}).

Data and statistical analysis

Data analysis was done with statistical analysis methods at a 95% confidence interval (alpha=0.05). The experimental design used was the Complete Randomized Design with one factor including five treatments with three replications. The data analyzed using the statistical software IBM SPSS statistics version 17.0. If the result was significantly different, it was followed up with Duncan's Multiple Range Test (DMRT).

Result and Discussion

Growth

The white shrimp DGR after 30 days given probiotic ranged between 4.68-5.39%; treatment C showed the best result (5.39%) and was significantly different (P<0.05) from all other treatments (Figure 1a.). The white shrimp FCR after 30 days treated with probiotic ranged between 1.56-2.04. Treatment C also showed the best result at 1.56, and was significantly different (*P*<0.05) from all other treatments (Figure 1b.).

The white shrimp's daily growth rate (DGR) and feed conversion ratio (FCR) after probiotic supplementation for 30 days showed significantly different results between the treatments and the controls. Addition of probiotic *Bacillus* NP5 with a dose of 10^{6} CFU.g^{-1} showed the best results when

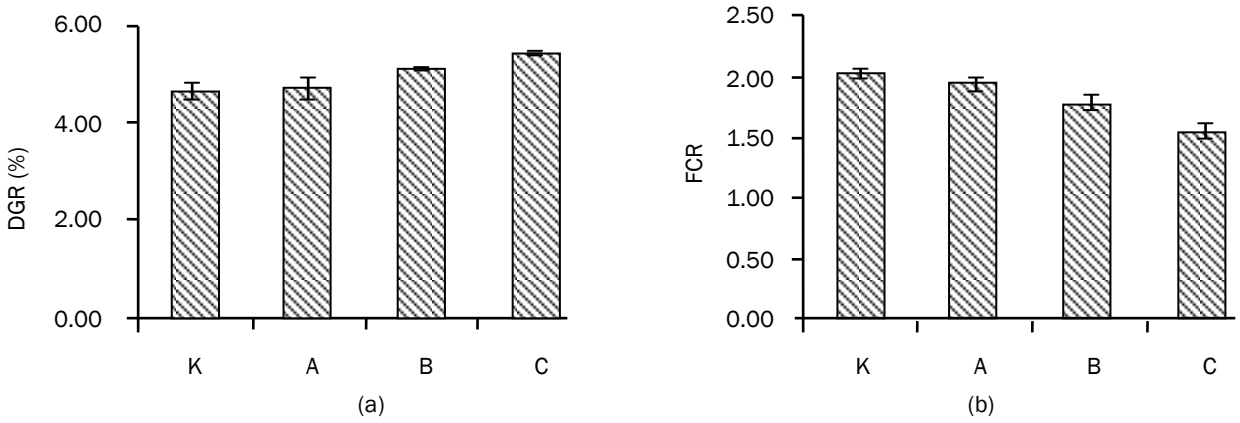

Figure 1. Daily growth rate (DGR) (a); Feed conversion ratio (b) of white shrimp after 30 days probiotic treatments
Notes: *The different letters in the chart indicate significant different results (P<0.05)
**K (control), A (probiotic 10^2 CFU.g^{-1}), B (probiotic 10^4 CFU.g^{-1}), C (probiotic 10^6 CFU.g^{-1})

compared to all other treatments. This signifies that the higher probiotic doses administered, the better white shrimp growth performance. The increasing of body weight and growth rate, and the decreasing of feed conversion rate in the white shrimp also occurred in the study where photosynthetic probiotic bacteria and *Bacillus* sp. (10^8-10^9 cfu.g^{-1} feed) (Wang, 2007), *Lactobacillus plantarum* (10^8 cfu.g^{-1} feed) (Kongnum and Hongpattarakere, 2012), and *Bacillus subtilis* (10^8 cfu.g^{-1} feed) (Zokaeifar *et al.*, 2012) were added to feed.

The administration of probiotic will influence hepatopancreas physiology associated with enzyme property of host digestive tract which will determine its digestive capabilities. The incerasing of white shrimp growth due to treatment with feed supplemented with the probiotic showed the effectiveness of the utilization of *Bacillus* NP5 by the host. The increasing of growth was assumed to be a result of digestive enzyme (amylase) activity increasing which in turn increased feed digestibility. The probiotic *Bacillus* NP5 used was probiotic bacteria isolated from the tilapia intestines and have been proven to be able to secrete amylase enzymes (Putra, 2010). The amylase enzymes played a role as exogenous enzymes which assisted the white shrimp endogenous enzymes performance in hydrolyzing feed macromolecules (carbohydrates) into simpler molecules. Administration of probiotics may increase the activity of amylase, protease, and lipase enzymes in the white shrimp intestines (Wang, 2007; Zhou *et al.*, 2009; Zokaeifar *et al.*, 2012). According to Wang (2007), improvement of the shrimp growth factor by probiotics is postulated as a result of induction of digestive enzymes such as protease and amylase which could stimulate the host natural enzyme activity. Probiotic enzymes also have a broader pH range than the shrimp enzymes; this would prolong the digestion period (Gómez and Shen, 2008). This improvement in digestion process will increase absortion of food; then it makes the feed nutrients converted into fish carcass which expressed by decreasing of FCR.

Survival

The white shrimp survival rate was divided into two phases, after 30 days probiotic treatment and after 14 days of the challenge test (Figure 2a,b). The white shrimp survival rate after 30 days given probiotic was 100% for all treatments. However, the white shrimp survival rate 14 days after being infected with IMNV through intramuscular injections was between 40.00-93.33%. The highest survival rate was shown by KN (93.33%). Treatment C showed the best result, 76.67% and was significantly different (P<0.05) from KN, KP, and treatment A.

Administration of probiotic *Bacillus* NP5 through feed in white shrimp for 30 days resulted survival rates that were not significantly different (P>0.05). After being infected with IMNV, the white shrimp given the probiotic showed a higher resistance compared to the controls. The addition of probiotic *Bacillus* NP5 with a dose of 10^6 CFU.g^{-1} via feed showed the best survival rate. Similar results were shown by Li *et al.* (2009), the shrimp survival rate significantly increased in line with the increased dose of the probiotic *Bacillus* OJ (PB) which was added to the feed. The white shrimp's increased resistance to pathogens was caused by the host's increased immune response (Li *et al.*, 2009; Zokaeifar *et al.*, 2012). The mortality of the shrimps was caused by IMNV that confirmed by PCR analysis of the shrimp which showed IMN clinical signs. The first IMN clinical sign occurred on day 3-5 after injection, in which there was a necrosis in the muscle tissue of the shrimp with white color at the

injection site then the infection spread to the tail with the wider necrosis area and the color changed to be reddish at the uropods. The infection became wider to the head then finally the shrimp dead. PCR analysis were done on three shrimp samples (sample A, B, and C). The results of the PCR analysis showed that samples A, B, and C were positively infected with IMNV (Figure 3.).

Immune Response

The white shrimp's THC after 30 days probiotic treatment ranged between 6.32-11.57x10^6 cells. mL^{-1}. Treatment C showed the best result at 11.57x10^6 cells.mL^{-1} and was significantly different ($P<0.05$) from the other treatments. After 14 days of being infected with IMNV, THC range dropped to 3.78-7.83×10^6 cells.mL^{-1}. Treatment C showed the best results at 7.83x10^6 cells.mL^{-1} and was significantly different ($P<0.05$) from all other treatments (Figure 4a,b.).

Haemocyte are a non-specific cellular response found in shrimp and other crustaceans which play a role in the immune response (Lin and Söderhäll, 2011). Based on the results obtained, administration of probiotic *Bacillus* NP5 at a dose of 10^6 CFU.g^{-1} through feed resulted in the highest THC. This showed that shrimp treated with the probiotic had a higher immune response compared to those not given the probiotic. After the challenge test, as a whole, there were the reductions in THC in all the treatments, in which treatment C showed the best result. The decrease in THC indicated that there was a white shrimp quick immune response to the infection. The decrease in the number of

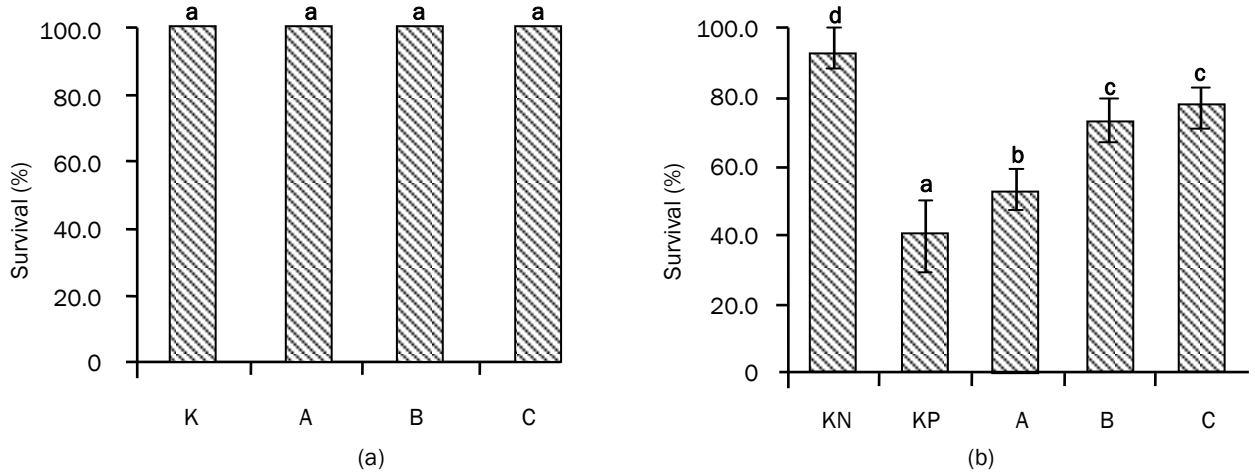

Figure 2. Survival of white shrimp after 30 days probiotic treatments (a); Survival of white shrimp after 14 days-challenge test with IMNV via injection (b). Notes: *The different letters in the chart indicate significant different results ($P<0.05$). **K (control), KN (negative control), KP (positive control), A (probiotic 10^2 CFU.g^{-1}), B (probiotic 10^4 CFU.g^{-1}), C (probiotic 10^6 CFU.g^{-1})

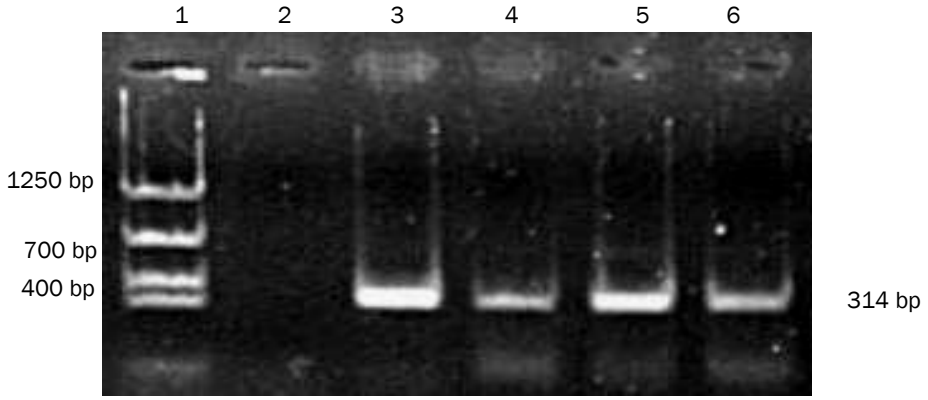

Figure 3. The result of PCR analysis of the IMNV-infected white shrimp. Notes : Lane 1: Marker ; Lane 2: negative control; Lane 3: Positive control; Lane 4: Sampel A IMNV-positive-detected; Lane 4: Sampel B IMNV-positive-detected; Lane 5: Sampel C IMNV-positive-detected. The presence of band at 314 bp shows IMNV-positive-detected of the sample.

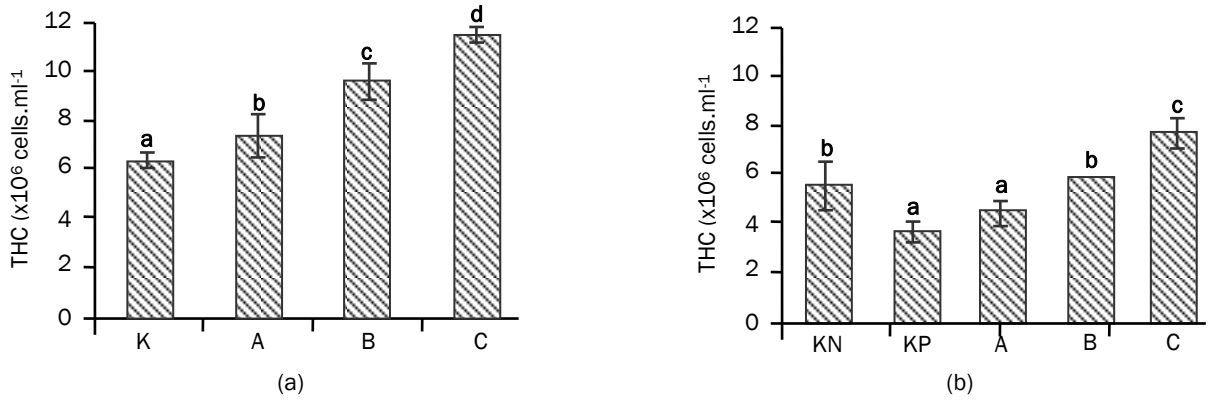

Figure 4. Total haemocyte count (THC) of white shrimp in the end of probiotic treatments (a); Total haemocyte count (THC) of white shrimp in the end of the challenge test with IMNV (b). Notes: *The different letters in the chart indicate significant different results (P<0.05). **K (control), KN (negative control), KP (positive control), A (probiotic 10^2 CFU.g^{-1}), B (probiotic 10^4 CFU.g^{-1}), C (probiotic 10^6 CFU.g^{-1})

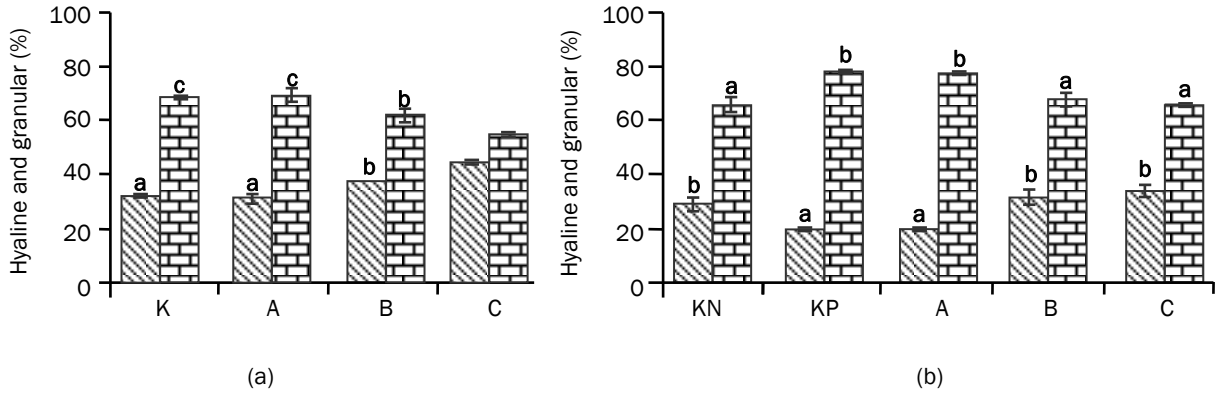

Figure 5. The percentage of hyaline and granular of white shrimp in the end of probiotic treatments (a); in the end of the challenge test with IMNV (b). Notes: *The different letters in the same pattern of the chart indicate significant different results (P<0.05).**K (control), KN (negative control), KP (positive control), A (probiotic 10^2 CFU.g^{-1}), B (probiotic 10^4 CFU.g^{-1}), C (probiotic 10^6 CFU.g^{-1})

haemocytes was an effect of the immune response mechanism such as the haemocyte infiltration in infected tissue which would result in haemocyte death due to apoptosis (Costa *et al.*, 2009; Yeh *et al.*, 2009).

The types of haemocytes observed were hyaline and granular or semigranular cells (Figure 5a,b). The white shrimp hyaline percentage after 30 days being treated with probiotic ranged between 31.00-45.00%. Treatment C showed the best result at 45.00% and was significantly different (*P*<0.05) from all other treatments. As for the percentage of the white shrimp granular cells after 30 days of probiotic treatment was between 55.00-69.00%. Treatment C showed the best result at 55.00% and was significantly different (*P*<0.05) from all other treatments. After 14 days being infected with IMNV via injection, the hyaline range dropped to 18.67-33.33%. Treatment C showed the best result at 33.33% and was significantly different (*P*<0.05) from KP and treatment A. After 14 days being infected with IMNV through injection, the granular cell range increased to 66.67-81.33%. Treatment C showed the best result at 66.67% and was significantly different (*P*<0.05) KP and treatment A.

Haemocytes consist of three types of cells based on the granules in their cytoplasm, *i.e.* hyaline, granular, and semi granular (Lin and Söderhäll, 2011). The three types of cells destroy foreign particles which enter the shrimp body through phagocytosis, encapsulation, nodule formation, and production of humoral components stored in haemocytic granules i.e. anticoagulant proteins, aglutinins, PO enzymes, antimicrobial peptides, and protease inhibitors (Jiravanichpaisal *et al.*, 2006). The infecting virus causes the host cells to modify; making them identifiable by the haemocyte as modified cells or foreign cells which

would be digested or destroyed by the haemocytes (Lin *et al.*, 2006). Probiotic administered in this study was live bacteria which have cell wall components such as peptidoglycan (PG), Lipopolysaccharides (LPS), and β-glucans (BGs); they are recognized as foreign materials and will activate several immune molecules into haemolymph, in which immune molecules are produced and stored in the granules of haemocytes (Sritunyalucksana and Söderhäll, 2000). The high concentration of granular cells in the haemolymph is related to the high phenoloxidase activity and resistance to disease. Granular cells have a role in phagocytosis, encapsulation, early recognition, melanisation and coagulation in some groups, producing and secreting antimicrobial peptides, and are involved in the cytotoxic reaction (Hauton, 2012).

Total bacteria and probiotic Bacillus NP5

The total bacterial count (TBC) quantification in the white shrimp was done at the end of the probiotic treatment. The total bacteria in the white shrimp intestines after 30 days probiotic treatment ranged between 2.30-4.43x10^{10} CFU.g^{-1}. Treatment B and C showed the higher results at 4.43x10^{10} CFU.g^{-1} and 4.33x10^{10} CFU.g^{-1}, respectively; and were significantly different (P<0.05) from the other treatments. As the total probiotic *Bacillus* NP5 in the white shrimp intestines after 30 days treatment with probiotic, treatment C showed the highest result at 4.07x10^{3} CFU.g^{-1} and was significantly different (P<0.05) from all other treatments (Figure 6a,b.).

Administration of probiotic *Bacillus* NP5 via feed with different doses has different effects on the white shrimp survival rate and immune response. Meanwhile, the total bacteria and total *Bacillus* NP5 in the intestines of shrimp treated with probiotic bacteria undergo an increase as the increasing of probiotic doses. According to Li *et al.* (2009), the elevated immune response in shrimp is probably because of the modulating micro flora in the shrimp gut, which is also related to the dose of the bacteria.

Conclusion

The administration of probiotic *Bacillus* NP5 in shrimp diet could significantly improve growth performance and disease resistance by enhancing immunity with an optimum dose of 10^{6} cfu.g^{-1}.

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