Original article

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Immune responses and resistance of white shrimp *Litopenaeus vannamei* administered *Bacillus* sp. NP5 and honey against white spot syndrome virus infection

Respons imun dan resistansi udang vaname *Litopenaeus vannamei* yang diberi *Bacillus* sp. NP5 dan madu terhadap infeksi *white spot syndrome virus*

Widanarni[1*], Dewi Rahmi[1], Muhamad Gustilatov[1], Sukenda[1], Diah Ayu Satyari Utami[2]

[1]Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University, Bogor, West Java, Indonesia 16880

[2]Department of Aquaculture, Marine and Fisheries Polytechnic of Jembrana, Jembrana, Bali, Indonesia 82218

*Corresponding author: widanarni@apps.ipb.ac.id

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ABSTRACT

White spot disease caused by white spot syndrome virus (WSSV) is the most serious viral disease and has major impact on the decline in the production of white shrimp *Litopenaeus vannamei* farming. The improvement of immune responses through the application of probiotic, prebiotic, and synbiotic are expected to be the alternative efforts to prevent the disease attack. This study aimed to evaluate the effects of the administration of *Bacillus* sp. NP5, honey, and a combination of those two (synbiotic) in enhancing immune responses and resistance of white shrimp to WSSV infection. This study consisted of five treatments and three replications, i.e. positive control (feeding without probiotic, prebiotic, and synbiotic then challenged by WSSV), negative control (feeding without probiotic, prebiotic, synbiotic, then injected with phosphate buffer saline), pro (feeding with the administration of *Bacillus* sp. NP5 then challenged by WSSV), pre (feeding with the administration of honey then challenged by WSSV), and syn (feeding with the administration of synbiotic then challenged by WSSV). White shrimp with an average weight of 1.8 ± 0.06 g/shrimp were reared at a density of 15 shrimps per aquarium for eight weeks, then challenged by WSSV at a lethal dose 50% (LD_{50}) of 10^{-4} with a volume of 0.1 mL per shrimp. The results of this study showed that synbiotic treatment indicating higher results on immune responses and resistance of white shrimp compared to control, probiotic, and prebiotic treatments.

Keywords: *Bacillus* sp. NP5, honey, immune responses, white shrimp, WSSV

ABSTRAK

White spot disease yang disebabkan oleh *white spot syndrome virus* (WSSV) merupakan penyakit viral yang berdampak besar terhadap penurunan produksi budidaya udang vaname *Litopenaeus vannamei*. Perbaikan respons imun melalui aplikasi probiotik, prebiotik, dan sinbiotik dapat menjadi salah satu alternatif untuk pencegahan serangan penyakit tersebut. Penelitian ini bertujuan mengevaluasi pengaruh pemberian *Bacillus* sp. NP5, madu dan gabungan keduanya (sinbiotik) dalam meningkatkan respons imun dan resistansi udang vaname terhadap infeksi WSSV. Penelitian ini terdiri dari lima perlakuan dan tiga ulangan yaitu kontrol positif (pemberian pakan tanpa probiotik, prebiotik, dan sinbiotik kemudian diuji tantang dengan WSSV), kontrol negatif (pemberian pakan tanpa probiotik, prebiotik, dan sinbiotik kemudian diinjeksi *phosphate buffer saline*), pro (pemberian pakan dengan penambahan *Bacillus* sp. NP5 kemudian diuji tantang dengan WSSV), pre (pemberian pakan dengan penambahan madu kemudian diuji tantang dengan WSSV), dan syn (pemberian pakan dengan penambahan sinbiotik kemudian diuji tantang dengan WSSV). Udang vaname dengan bobot rata-rata 1.8 ± 0.06 g/ekor dipelihara dengan kepadatan 15 ekor per akuarium selama delapan minggu, kemudian diuji tantang dengan WSSV pada *lethal dose* 50% (LD_{50}) 10^{-4} dengan volume 0.1 mL per ekor. Hasil penelitian ini menunjukkan perlakuan prebiotik menunjukkan hasil yang lebih tinggi pada respons imun dan resistansi udang vaname dibanding perlakuan kontrol, probiotik, dan prebiotik.

Kata kunci: *Bacillus* sp. NP5, madu, respons imun, udang vaname, WSSV

INTRODUCTION

White spot disease caused by *white spot syndrome virus* (WSSV) infection is the most serious viral disease in white shrimp (*Litopenaeus vannamei*) which led to decreased production of white shrimp. Lightner (2011) reported that shrimp infected by WSSV would experience 100% of mortality in three to ten days allowing economic loss in the shrimp culture business. Hulten *et al*. (2001) stated that WSSV is the virus from the genus *Whispovirus* and the family *Nimaviridae*. This virus is an enveloped rod-shaped DNA virus, has virion contains nucleocapsid with a diameter of 65–70 nm and a length of 300–350 nm, and has a rapid transmission.

The application of probiotic, prebiotic, and synbiotic is one of the economic friendly alternative efforts to prevent disease in fish and shrimp culture. Probiotic is living microorganism that can provide beneficial effects to the host, improve the balance of microbes in the gastrointestinal tract, feed efficiency, and environment quality (Pandiyan *et al.*, 2013; Banerjee & Ray, 2017). Some previous studies reported that probiotic can increase survival, immune response, and shrimp resistance against pathogen infection (Kewcharoen & Srisapoome, 2019; Amoah *et al*., 2019). Probiotics used in aquaculture generally come from the genus *Lactococcus*, *Bacillus*, dan *Lactobacillus* (Huynh *et al*., 2017). Probiotic used in this study was *Bacillus* sp. NP5 isolated from the gastrointestinal tract of tilapia (Putra & Widanarni, 2015) and resulted in a positive impact in growth performance and immune response of tilapia, common carp, catfish, and white shrimp (Widanarni *et al*., 2014; Widanarni & Tanbiyaskur, 2015; Agung *et al.*, 2015; Tanbiyaskur *et al.*, 2015; Utami *et al.*, 2015a, b; Djauhari *et al.*, 2016; Tamamdusturi *et al.*, 2016; Putra & Romdhonah, 2019).

The role of probiotic bacteria can be increased by prebiotic application (Hoseinifar *et al.*, 2014). Prebiotic is a food ingredient that can not be digested by the host, but it can increase growth, nutrient utilization, and health status of the host by inducing the growth of beneficial microorganisms in the gastrointestinal tract (Ringø & Song, 2015). Some prebiotics such as inulin and mannanoligosacharide (MOS) has been used to increase shrimp immunity and decrease the prevalence of WSSV (Luna-González *et al.*, 2012; Zhang *et al.*, 2012). Prebiotic used in this study was honey. Karimah *et al*. (2011) explained that honey is fulfilled the criteria of probiotic including being resistant to gastric acid and digestive enzymes, stimulating the growth of beneficial bacteria selectively, and inducing bacteria fermentation activity. The application of honey as a prebiotic has been proven to increase growth, survival, feed efficiency, and increase the immune response of the shrimp against *Vibrio parahaemolyticus* (Fuandila *et al.,* 2019) and WSSV infection (Widanarni *et al.*, 2019). The result of the study by El-Asely *et al*. (2014) showed that the application of honey bee pollen in Nile tilapia has been proven to improve growth rate, immune response, and protection against *Aeromonas hydrophila* infection.

Synbiotic is defined as a mixture of probiotic and prebiotic (Song *et al.*, 2014; Akhter *et al.*, 2015) that beneficially affect the host by improving survival, increasing growth rate, and stimulating probiotic bacteria metabolism through metabolic synergy (Zhang *et al*., 2012). Some prebiotics, indigenous probiotics, synbiotic, and organic acid has been used as feed supplements to improve white shrimp health (Li *et al.*, 2018). *Bacillus* sp. NP5 as a probiotic and some prebiotics such as sweet potato extract and mannanoligosacharide (MOS) has been tested to produce synbiotics and has been proven to increase growth and immune response of tilapia, channel catfish, catfish, common carp, and white shrimp (Munaeni *et al.*, 2014; Agung *et al.*, 2015; Zubaidah *et al.*, 2015; Tamamdusturi *et al.*, 2016; Djauhari *et al.*, 2017; Putra & Romdhonah, 2019). The effects of *Bacillus* sp. NP5 as probiotic and honey as prebiotic, or the mixture to induce white shrimp growth and immune response against WSSV infection is unknown yet, therefore, this study is needed to be done. Hence, the aim of this study was to evaluate the immune response and the resistance of white shrimp administered *Bacillus* sp. NP5, honey, and synbiotic against WSSV infection.

MATERIALS AND METHOD

Experimental design

This study used five treatments and three replications, such as positive and negative controls (shrimp were not fed with probiotic, prebiotic, and synbiotic), pro (shrimp were fed with *Bacillus* sp. NP5 as probiotic), pre (shrimp were fed with honey as prebiotic), and syn (shrimp were fed with synbiotic). The shrimp in the positive control, probiotic, prebiotic, and

synbiotic treatments were injected by WSSV filtrate suspension in the challenge test, while the shrimp in the negative control were injected by *phosphate buffer saline* (PBS).

Experimental medium

The aquarium used in this study sized of 60 × 30 × 30 cm^3. There were 15 aquariums equipped with aeration, top filter, and shelter. The aquariums were washed with detergent and rinsed with water. The aquariums were then disinfected using 30 mg/L of chlorine solution for 24 hours. That process was followed by rinsing aquariums with clean water and draining aquariums to evaporate the residue of chlorine solution. Before being used, the aquariums were covered by black plastic, then the aquariums were filled out with 25 g/L marine water.

Experimental animals

White shrimp used in this study derived from the hatchery unit of PT. Suri Tani Pemuka, Indramayu, Jawa Barat. Before the treatments, the shrimp in postlarvae (PL) 9 stadia were reared in fiber containers sized of 200 × 100 × 40 cm^3 for acclimatization. During white shrimp rearing, the shrimp were fed with *Artemia* nauplii and commercial feed five times a day through *ad satiation* method.

Experimental diets

The feed used in this study was commercial feed containing 40% protein. The diet with probiotic was prepared by adding the probiotic into the feed at a dose of 1% (v/w) with a bacterial density of 10^8 CFU/mL and 2% egg white as a binder (Putra *et al.*, 2015). The prebiotic used in this study was honey obtained from the beekeeper in Depok, West Java. Honey was diluted in PBS at a ratio of 1:1 (v/v), then 2% egg white (v/w) was added. The diet with prebiotic was prepared by adding the mixture of prebiotic and egg white into the feed at a dose of 0.5% (v/w). The diet with synbiotic was prepared by adding 1% probiotic (v/w), 0.5% prebiotic (v/w), and 2% egg white into the feed. The control diet was prepared by adding egg white at a dose of 2% (v/w). The prepared diet was air-dried to reduce the humidity of the experimental diet.

Shrimp rearing

Shrimp with an average weight of 1.8 ± 0.06 g/shrimp were stocked into experimental aquariums with a stocking density of 15 shrimps per aquarium. The white shrimps were reared for eight weeks fed with experimental diets according to each treatment. The shrimp were fed five times a day at 07.00, 11.00, 15.00, 18.00, and 21.00 through *ad satiation* method. During the treatments, the water quality was maintained in the optimal condition through daily siphoning, water replacement at a level of 50% from initial water volume every two days, and cleaning the top filter every week. The ranges of water quality parameters in all treatments were 5.7–6.8 mg/L of dissolved oxygen (DO), 29–30°C of water temperature, 7.2–7.5 of pH, 0.042–0.65 mg/L of total ammonia nitrogen (TAN), 0.25–0.87 mg/L of nitrite, and 0.30–0.77 mg/L of nitrate.

Challenge test

The challenge test was done by using WSSV filtrate. The WSSV positive confirmed shrimp were obtained from Loka Pemeriksaan Penyakit Ikan dan Lingkungan (LP2IL) Serang, Banten. The WSSV filtrate was prepared according to a procedure explained by Ramos-Carreño *et al*. (2014). The infected white shrimp's organ (gills and flesh) at a weight of 2 g was crushed and diluted in TN buffer (200 mM of Tris-HCl, 400 mM of NaCl, pH 7.4) with a ratio of sample and TN buffer was 1:4. The diluted sample was centrifuged using a centrifuge at a speed of 6000 rpm and a temperature of 4 °C for 15 minutes. The supernatant was then taken and was re-centrifuged at a speed of 12000 rpm and a temperature of 4°C for 30 minutes. The supernatant was filtered using a syringe filter with a mesh size of 0.45 µm. This virus filtrate could directly be used or could be stored in a freezer at a temperature of -80°C as WSSV filtrate stock. The filtrate virus was used for Koch's postulate test subjected to white shrimp to increase the virulence of the virus. The lethal dose 50% (LD_{50}) test was done using the virus filtrate at dilution levels of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-5} to determine the dose used for the challenge test (Widanarni *et al.*, 2019). In this study, the LD_{50} obtained was 10^{-4}. The challenge test was done through the injection of WSSV filtrate into white shrimp using 1 mL syringe (positive control, probiotic, prebiotic, and synbiotic treatments). The shrimp were injected WSSV filtrate (0.1 mL virus suspension per shrimp) in the dorsal body part between 3rd and 4th segments, while shrimp in the negative control treatment were injected by PBS.

Experimental parameters

Total hemocyte count

The sample was taken after the feeding trial and the challenge test. The sampling of hemolymph was carried out using a syringe at a volume of 1 mL filled with anticoagulant (30 mM trisodium citrate, 0.34 sodium chloride, 10 mM EDTA, 0.12 M glucose, pH 7.55) to take hemolymph from the 5th periopode at a volume of 0.1 mL. The hemolymph was dropped in a hemocytometer and the total hemocyte count (THC) was counted using a microscope with a magnification of 100 x (Wang & Chen, 2006).

Phagocytic activity

The hemolymph at a volume of 100 µL was mixed with 25 µL the suspension of *Staphylococcus aureus* (10^7 CFU/mL) and was incubated for 20 minutes. The mixture of those solutions was taken as a sample for the blood film. The blood film was processed to fixation using methanol 100% for five minutes and was drained. It was soaked in the Giemsa solution for 15 minutes. The blood film was washed using running water and was drained again. The blood film was then observed under a microscope at a magnification of 400 x to observe phagocytic activity (PA) based on the percentage of phagocytic cells that showed a phagocytosis process (Anderson & Siwicki, 1995).

Respiratory burst

The respiratory burst activity (RB) was measured based on superoxide anion production. The mixture of hemolymph and anticoagulant at a volume of 100 µL was incubated for 30 minutes at room temperature. This mixture was then centrifuged using a centrifuge at a speed of 3000 rpm for 20 minutes and the supernatant was disposed. The pellet was added with 100 µL of nitro blue tetrazolium (0.3% of NBT solution) and was incubated for two hours at room temperature. Therefore, NBT suspension was centrifuged at a speed of 3000 rpm for 10 minutes and the supernatant was disposed. Absolute methanol at a volume of 100 µL was added and was re-centrifuged at a speed of 3000 rpm for 10 minutes. The pellet was washed using methanol 70%. The process was followed by adding 120 µL of KOH and 140 µL of dimethyl sulfonyl oxide (DMSO) and the sample was added into microplate titer. The RB activity was measured using a microplate reader at a wavelength of 630 nm. Blanco used was KOH and DMSO as a standard value. The respiratory burst activity (RB) is expressed as an NBT reduction per 10 µL of hemolymph (Cheng *et al*., 2004).

Phenoloxidase activity

Phenoloxidase activity (PO) was measured based on the formation of dopachrome produced by L-dihyroxyphenyl alanine (L-DOPA). The mixture of 0.2 mL hemolymph and 0.8 mL anticoagulant was centrifuged using a centrifuge at a speed of 1500 rpm for 10 minutes at a temperature of 4 °C. The supernatant was disposed, while the pellet was slowly re-suspended in 1 mL of cacodylate-citrate buffer (0.01 M of sodium cacodylate, 0.45 M of sodium chloride, 0.10 M of trisodium citrate, pH 7.0). The mixture was then re-centrifuged at a speed of 1500 rpm for 10 minutes at a temperature of 4°C. The supernatant was disposed and the pellet was added with 200 µL cacodylate-citrate buffer. The produced suspension at a volume of 100 µL was added with 50 µL trypsin (1 mg/mL cacodylate buffer) as an activator and was incubated for 10 minutes at a temperature range of 25–26°C. This suspension was then added with 50 µL L-DOPA (3 mg/mL cacodylate buffer) and was left for 5 minutes, followed by the addition of 800 µL cacodylate buffer. Optical density (OD) was measured using a microplate reader with a wavelength of 492 nm. The PO is expressed by the formation of dopachrome in 50 µL of hemolymph (Liu & Chen, 2004).

Survival

Survival is the ratio between the number of alive white shrimp at the end of the treatment and the initial number of white shrimp. The survival of white shrimp was calculated using this following formula:

$$SR\ (\%) = \frac{Nt}{No} \times 100$$

Note: = survival (%)
Nt = the number of alive white shrimp at the end of the treatment (shrimp)
No = the initial number of white shrimp (shrimp)

Data analysis

The data obtained were analyzed through analysis of variance (ANOVA) using the SPSS version 22.0 software with a 95% confidence interval. Duncan's test was carried out if the results were significantly different ($P < 0.05$) among treatments.

RESULTS AND DISCUSSIONS

Results

Total hemocyte count

The THC values of white shrimp after the feeding trial, three days after the challenge test, and five days after the challenge test are presented in Figure 1. The THC values on probiotic, prebiotic, and synbiotic showed better results and were significantly different (P<0.05) from the controls. The highest THC value after the feeding trial was obtained in synbiotic treatment (8.47 ± 0.15×10^6 cells/mL) and was not significantly different (P>0.05) from prebiotic treatment (8.29 ± 0.18×10^6 cells/mL), followed by probiotic treatment (7.50 ± 0,22 ×10^6 cells/mL), while the lowest THC value was obtained in the control treatment (5.30 ± 0.19×10^5 cells/mL). Three days after the challenge test, the THC value on synbiotic treatment was 4.92 ± 0.06×10^6 cells/mL and was not significantly different (P>0.05) from prebiotic treatment (4.55 ± 0.31×10^6 cells/mL), but it was significantly different (P<0.05) from probiotic (4.17 ± 0.20×10^6 cells/mL) and the positive control treatment (3.10 ± 0.17×10^6 cells/mL). The observation of THC value on five days after the challenge test showed the lowest THC value was obtained in the positive control treatment (0.28 ± 0.12×10^6 cells/mL) and was significantly different (P<0.05) from prebiotic, probiotic, and synbiotic with THC values of 3.74 ± 0.17×10^6 cells/mL, 3.20 ± 0.16×10^{6}cells/mL, and 3.69 ± 0.12×10^6 cells/mL, respectively.

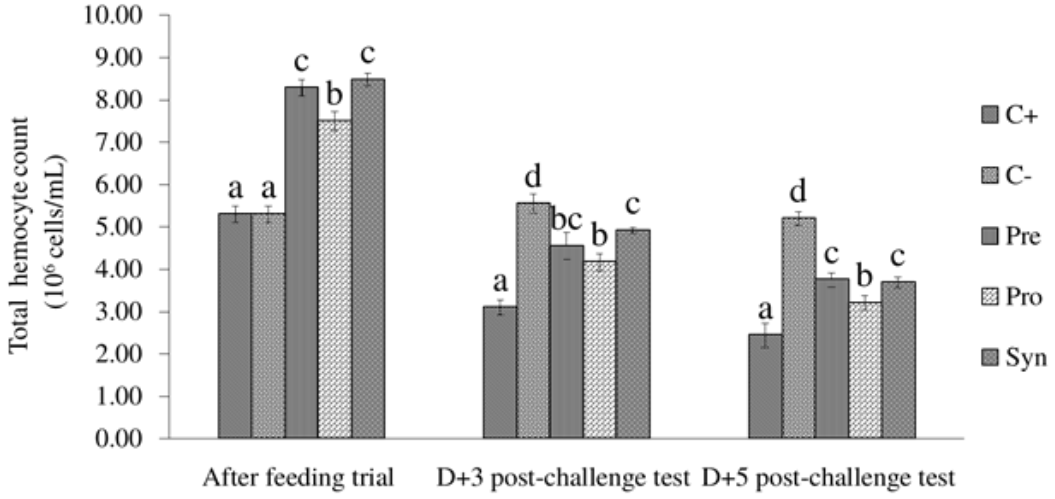

Figure 1. Total hemocyte count of white shrimp after the feeding trial, three days after the challenge test, and five days after the challenge test with white spot syndrome virus. Different letters on bars on each observation period show a significant different result (P<0.05). C+: positive control; C-: negative control; Pre: prebiotic; Pro: probiotic; Syn: synbiotic

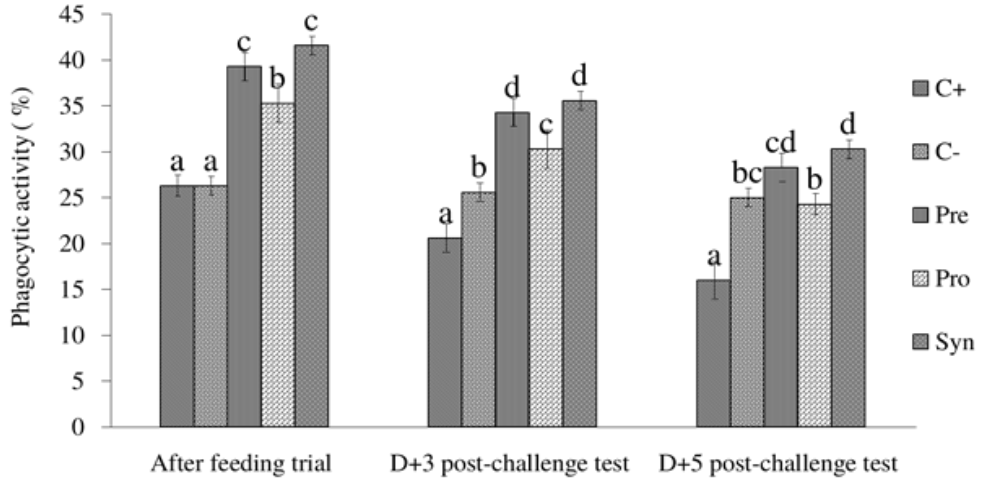

Figure 2. The phagocytic activity of white shrimp after the feeding trial, three days after the challenge test, and five days after the challenge test with white spot syndrome virus. Different letters on bars on each observation period show a significant difference result (P<0.05). C+: positive control; C-: negative control; Pre: prebiotic; Pro: probiotic; Syn: symbiotic.

Phagocytic activity

The measurement result of PA after the feeding trial, three days after the challenge test, and five days after the challenge test is presented in Figure 2. After the feeding trial, the highest PA value was obtained in synbiotic treatment (43.6 ± 2.08%) and was significantly different (P<0.05) from the control treatments (26.3 ± 1.52%) which showed the lowest PA values. The PA value on three days after the challenge test in synbiotic (35.6 ± 31.15%) and prebiotic (34.6 ± 1.27%) treatments were significantly different (P<0.05) from positive (20.6 ± 1.24%) and negative (25.6 ± 2.51%) control treatments. On five days after the challenge test, synbiotic treatment had the highest PA value (30.3 ± 2.08%), while the lowest PA value was obtained in the positive control treatment (16 ± 1.00%).

Respiratory burst

The RB values after the feeding trial, three days after the challenge test, and five days after the challenge test are presented in Figure 3. The RB values after the feeding trial showed insignificant different results (P>0.05) among prebiotic, probiotic, and synbiotic treatments with values of 0.745 ± 0.044, 0.692 ± 0.031, and 0.745 ± 0.035, respectively, but those were significantly different (P<0.05) from the control treatment (0.372 ± 0.004). On three days after the challenge test, the highest RB value was obtained in synbiotic treatment (0.583 ± 0.041) and it was significantly different (P<0.05) from the positive control treatment (0.262 ± 0.024). The observation on five days after the challenge test showed the highest RB value obtained in synbiotic treatment

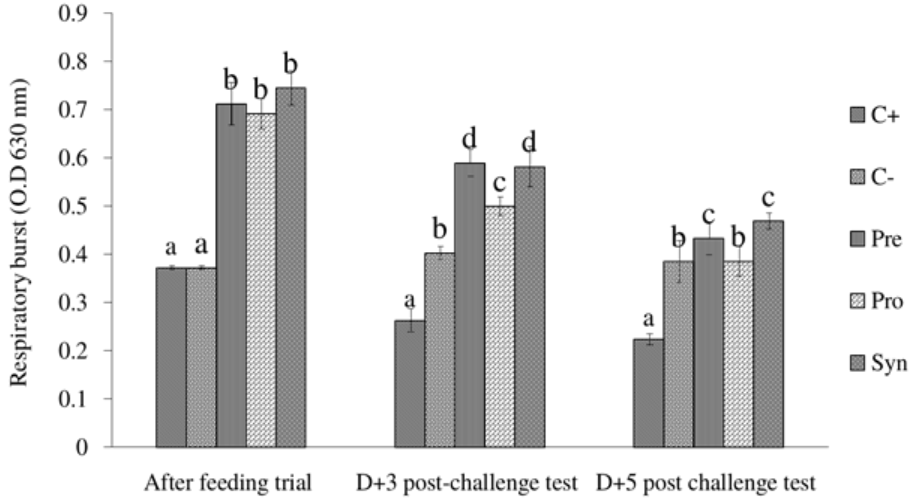

Figure 3. The respiratory burst of white shrimp after the feeding trial, three days after the challenge test, and five days after the challenge test with white spot syndrome virus. Different letters on bars on each observation period show a significant difference result (P<0.05). C+: positive control; C-: negative control; Pre: prebiotic; Pro: probiotic; Syn: synbiotic.

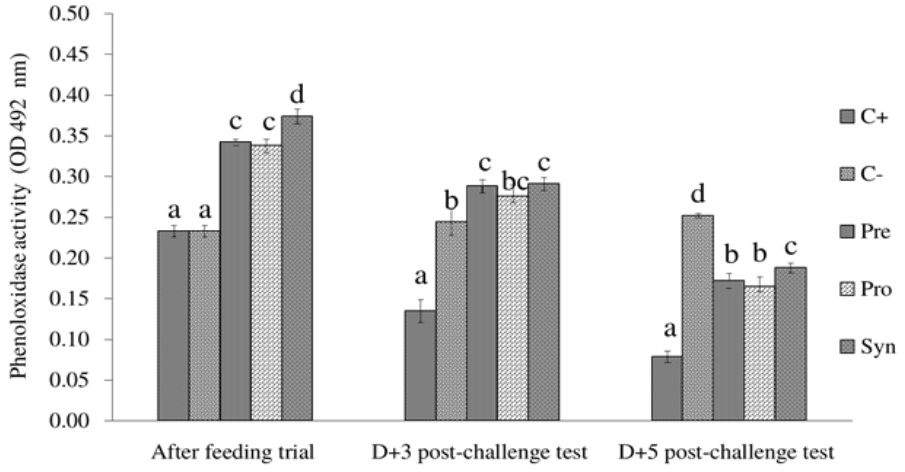

Figure 4. The phenoloxidase activity of white shrimp after the feeding trial, three days after the challenge test, five days after the challenge test with white spot syndrome virus. Different letters on bars on each observation period show a significant difference result (P<0.05). C+: positive control; C-: negative control; Pre: prebiotic; Pro: probiotic; Syn: synbiotic.

(0.469 ± 0.016) and the lowest value was on the positive control (0.224 ± 0.008).

Phenoloxidase activity

The measurement results of PO in white shrimp after the feeding trial, three days after the challenge test, and five days after the challenge test is presented in Figure 4. After the feeding trial, the highest PO value was obtained in synbiotic treatment (0.373 ± 0.009) and it was significantly different (P<0.05) from the control treatments (0.233 ± 0.007), prebiotic (0.342 ± 0.004), and probiotic (0.338 ± 0.007). On three days after the challenge test, the PO value in prebiotic treatment (0.257 ± 0.008) was not significantly different (P>0.05) from synbiotic treatment (0.274 ± 0.008), but it was significantly different (P<0.05) from the positive control (0.135 ± 0.04). On five days after the challenge test, the positive control treatment showed the lowest PO value (0.079 ± 0.007), while the highest PO value was obtained in synbiotic treatment (0.188 ± 0.009).

Survival

The shrimp mortality after the challenge test was observed daily for 5 days and survival values are presented in Figure 5. The highest survival values of white shrimp after the challenge test with WSSV were shown on prebiotic (68.89 ± 3.84%) and synbiotic (68.89 ± 3.84%) treatments, followed by probiotic treatment (62.2 ± 3.14%) which were significantly different (P<0.05) from the positive control treatment (35.5 ± 3.85%).

Discussions

Hemocytes are cells that play a central role in the crustacean immune system. According to Johansson *et al*. (2000), hemocytes act in crustacean immune response as foreign particle identifiers, conducting a phagocytic activity, cytotoxicity, encapsulation, the healing process, and acting as prophenoloxidase (proPO) activators. Increased shrimp body defense is in line with the increased phagocytic activity in hemocyte cells. There are three types of hemocyte cells in the crustacean. These types are divided based on the granule cytoplasm existence, namely hyaline, semi granular, and granular cells. Hyaline cells have a ratio that nucleus is more than cytoplasm with less submicron granules and a size of 6–13 µm. Semi granular cells have a ratio that nucleus is less than cytoplasm with submicron, micron, and refractile granules. Semi granular cells have a size of 20 µm. These cells are known as active cells in encapsulation due to responding to the foreign particles. Granular cells have a ratio that nucleus is less than cytoplasm, contain soft granules, and have a function as the proPO system activator. Hyaline cells are responsible to perform phagocytosis in shrimp immunity, while semi granular and granular cells are responsible for proPO system (Giulianini *et al*., 2007).

The result showed that the administration of honey, *Bacillus* sp. NP5, and synbiotic produced higher THC values than control. The THC value on synbiotic treatment had the highest value on each observation period. This was in line with

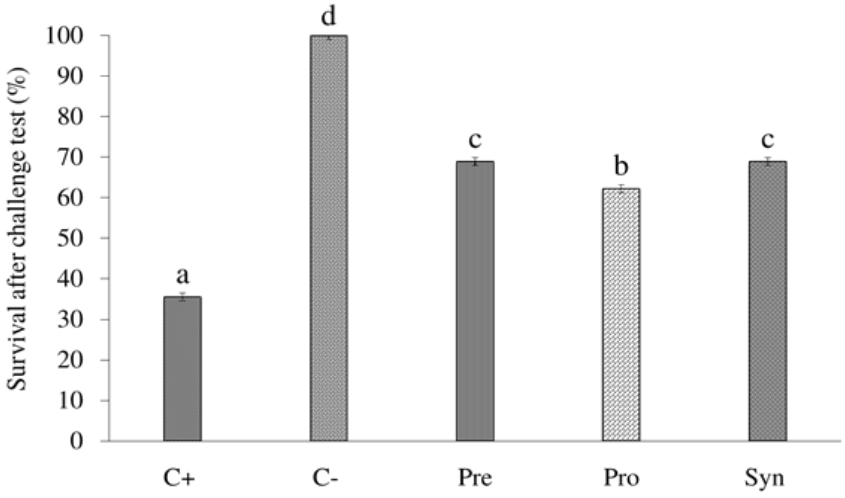

Figure 5. The survival of white shrimp administered *Bacillus* sp. NP5, honey, and synbiotic after challenged with white spot syndrome virus. Different letters on each bar show a significant different result (P<0.05). C+: positive control; C-: negative control; Pre: prebiotic; Pro: probiotic; Syn: synbiotic.

the statement of Hamsah *et al*. (2019), who stated that the administration of *Pseudoalteromonas piscicida* and mannanoligosaccharides could enhance THC in white shrimp. The same result was also reported by Ziaeenezhad and Sharifpour (2016) that the synbiotic application (commercial and non-commercial) could significantly enhance the total hemocytes after the application of those materials for 2 months. Higher THC values on probiotic, prebiotic, and synbiotic than control indicated that the role of probiotic, prebiotic, and synbiotic to enhance the shrimp immune response. This was caused by *Bacillus* sp. NP5 and honey were immunogenic substances and were recognized as foreign particles by the shrimp body, so those were capable to induce the shrimp immune response through the hemocyte cell production enhancement. After the challenge test, THC values decreased on all treatments, but THC values on prebiotic, probiotic, and synbiotic treatments were still higher than the positive control. Decreased THC also happened in white shrimp infected with infectious myonecrosis virus (IMNV) and *Vibrio harveyi* (Nurhayati *et al*., 2015). This decreased total hemocyte count was the impact of the shrimp body defense mechanism such as the hemocyte migration to the infected tissues, the hemocyte cell mortality due to apoptosis, and cell aggregation in the defense system (Xian *et al*., 2016). The hemocyte cell migration from hemolymph circulation indicates that the shrimp body defense works to attack the pathogen infection. Moreover, decreased THC was suspected due to the occurrence of apoptotic cells as a primitive defense mechanism of the host to prevent the replication of the virus in the host cells (Yeh *et al*., 2009). The result of the study by Wongprasert *et al*. (2003) found the same condition in which 20% of hemocyte cells and hematopoietic tissues of tiger shrimp experienced an apoptosis process after injected with WSSV.

The phagocytic activity (PA) is a function of the non-specific immune response, which becomes the initial defense mechanism against the microorganism attack. Along with THC, PA values on honey, *Bacillus* sp. NP5, and synbiotic were better than control with the highest value found in synbiotic treatment. The same result was found in the study of Widanarni *et al*. (2016b) demonstrated that PA in white shrimp administered synbiotic increased and it was significantly different from control. Arisa *et al*. (2015) also reported that PA in white shrimp was higher in synbiotic treatment than probiotic, prebiotic, and control on each observation period. Rodriguez and Moullac (2000) state that high PA indicates that an organism could produce more phagocytic cells, therefore when there was a pathogenic microorganism exposure, blood cells were ready to perform a phagocytic process. On three until five days after the challenge test, PA decreased. The decreased PA was in line with the decreased THC after the challenge test. A previous study stated that the decreased phagocytic activity value after the challenge test was caused by the phagocytic cells disruption and lysis after attacking the entered virus (Widanarni *et al*., 2016b).

Respiratory burst activity (RB) is a shrimp immune parameter related to the phagocytic process and a common reaction in the shrimp cellular defense system. Hyaline cells play a role in phagocytosis in which particles or microorganisms will be internalized by hemocyte cells (phagocytes) then forming a digestive vacuole called phagosome. The destruction processes of foreign particles by releasing the degradative enzymes in phagosome and producing reactive oxygen intermediate (ROI) were known as respiratory burst (Rodriguez and Mullac, 2000). The reactive oxygen intermediate (ROI) which is first formed during the phagocytic process is superoxide anion (O_2^-). Other ROIs will be produced, namely hydrogen peroxide (H_2O_2), hydroxyl radicals (OH-), and singlet oxygen (O_2). Respiratory burst (RB) is produced by the phagocytic cells to attack the pathogen invasion during the phagocytosis process (Jiang *et al*., 2013). Generally, RB decreased after the challenge test with WSSV. According to Febrianti *et al*. (2016), this decrease was suspected due to the occurrence of biological stress in the form of pathogenic infection which could cause the decreased ROI value and total hemocytes, therefore occurred a decreased product from the phagocytosis process. Moreover, decreased RB after the challenge test with WSSV was suspected related to the decreased THC on the post-infection period due to the migration of hemocyte cells specifically hyaline cells into the infection site to perform a phagocytosis process and initiate a defensive response. Decreased total hemocytes can cause a decrease in the production of some ROIs. The RB value after the challenge test

showed the highest value in synbiotic treatment among other treatments. This condition indicated that synbiotic could enhance the white shrimp immune system. This result followed the result of the study by Yunarty *et al*. (2016) who reported that white shrimp administered microencapsulated synbiotic had a higher RB value than control after challenged with WSSV and *V. harveyi*.

As an aquatic organism that has no specific immune response, PO is an important part of the humoral non-specific immune response in crustacean to recognize the foreign particles entered into the shrimp body and play a role as the body defense against pathogens (Huang *et al*., 2010). This activity is performed by semigranular or granular cells. The PO values in white shrimp after administered with probiotic, prebiotic, and synbiotic showed a better result than control with the highest PO obtained in synbiotic treatment. The result of this study followed the study by Huynh *et al*. (2018) who reported that the addition of galactooligosaccharide (GOS) and *Lactobacillus plantarum* could enhance PO in white shrimp. High PO caused the white shrimp to be better in recognizing foreign particles. After challenged by WSSV, PO value was decreased compared to PO value after the feeding trial. This decreased PO value was suspected due to semigranular or granular cells were also decreased as the shrimp body defense function worked after the occurrence of the pathogenic infection. Other studies also mentioned that PO was closely related to THC. The PO and the THC are commonly synergetic as hemocytes produce and release PO into hemolymph in the form of an inactive pro-enzyme called proPO, therefore in normal conditions, an increase in total hemocytes will be followed by an increase in PO product or *vice versa* (Smith *et al*., 2003). The highest PO value after the challenge test was obtained in synbiotic treatment. This condition followed the result of the study by Widanarni *et al*. (2016a) who reported that the synbiotic treatment with various doses on white shrimp produced a higher PO than positive control on the observation after the challenge test with IMNV.

Survival is a successive standard in the rearing of aquatic organisms on a certain period. Based on the result of this study, the addition of honey, *Bacillus* sp. NP5, and synbiotic has been proven to produce a higher survival of white shrimp than control after challenged with WSSV. The experimental shrimp in synbiotic and prebiotic treatments produced higher survival values than those of probiotic and positive control. This result followed the study by Li *et al*. (2009) showed that the addition of synbiotic in the form of a combination between prebiotic and probiotic on shrimp has been significantly proven to enhance the shrimp survival after challenged with WSSV. High survival of white shrimp in synbiotic treatment indicated that *Bacillus* sp. NP5 and honey could work synergically as a synbiotic to enhance the immune response of white shrimp against the pathogenic attack. This synergistic was related to the nutritional supplements that reacted from the combination of probiotic and prebiotic to become synbiotic (Kumar *et al*., 2017). High survival of white shrimp in prebiotic treatment was suspected as honey could stimulate the proliferation of the normal microflora in the white shrimp digestive system. The same result was also found on synbiotic treatment because prebiotic added along with probiotic induced the probiotic proliferation as beneficial bacteria in the host digestive tract (Widanarni *et al*., 2012). The use of synbiotic can give a higher benefit than the single application of probiotic and prebiotic (Merrifield *et al.*, 2010; Boonanuntanasarn *et al.*, 2015). Das *et al.* (2017) explained that prebiotic could only be utilized by the beneficial bacteria in the digestive system and could not be utilized by the pathogenic bacteria living in the digestive system. Synbiotic potentially enhances the total bacteria, the bacterial conformity, and the diversity of bacteria in the shrimp digestive tract (Huynh *et al*., 2019). The proliferation stimulation of normal microflora and beneficial bacteria in the host digestive tract will impact the host immune response enhancement which then produces a higher survival than the positive control. Enhanced immune response after the administration of synbiotic was also caused by the migration of normal flora contained in the synbiotic through the intestine wall which multiplied and produced an antigen to stimulate the shrimp immune system (Okey *et al*., 2018). Prebiotic contained in the synbiotic was metabolized by the target probiotic in the host digestive tract and produced some metabolite compounds such as short-chained fatty acids (SCFAs), amino acids, and polyamines which could enhance the host health (Huynh *et al*., 2017).

The use of a material given through the feed is necessarily considered its availability and contribution to the total production cost in the aquaculture business. The economical evaluation of the application of probiotic, prebiotic,

and synbiotic can be done by observing the zootechnical performance of the target species after the application of probiotic, prebiotic, and synbiotic (Azevedo *et al*., 2015). Based on the results of this study, the zootechnical performance of white shrimp was presented by survival which was obtained better results in prebiotic and synbiotic treatments compared to probiotic and control. This condition indicated that the application of prebiotic was more optimum in the cost efficiency a s w hite s hrimp s urvival – on prebiotic treatment was similar to synbiotic treatment, therefore it was unnecessary to provide an additional cost to produce probiotic. However, a study about a long-term impact on the application of these materials to the production performance should be performed, so an economic analysis from the use of these materials can be obtained to apply an industrial-scale production.

CONCLUSION

The application of probiotic, prebiotic, and the combination of both materials could enhance the immune response and the resistance of the white shrimp against the WSSV infection. The best result was obtained in the synbiotic treatment.

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