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Effectivity of prebiotic mannan oligosaccharides as the immunity enhancer and growth response on whiteleg shrimp *Litopenaeus vannamei* against white spot disease

Efektivitas prebiotik mannan oligosakarida pada respons imun dan pertumbuhan udang vaname *Litopenaeus vannamei* serta resistensi terhadap *white spot disease*

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ABSTRACT

This study aimed to evaluate the immune response and growth performance of white shrimp administered with prebiotic mannan oligosaccharides (MOS) with dosages of (0%, 0.2%, 0.4%, and 0.8% in diet) and used in the feeding trial. Shrimps (*Litopenaeus vannamei*) (the initial average weight was 3.416 ± 0.064 g) were fed at satiation, three times a day. A completely randomized design was used in the study. Shrimps were cultured at the stock density of 15 shrimps 40/L for each treatment in triplicates. After 30 days of the feeding trial, the experimental shrimps were challenged with white spot syndrome virus filtrate by intramuscular injection. The total gut bacteria, total haemocyte count (THC), phenoloxydase (PO), and respiratory burst (RB) activity were observed 4 times, before the experiment, day 30th before challenge test, day 32nd after challenge test, and day 36th the end of the experiment. The shrimp survival was observed at day 36th to evaluate the immune responses. The results showed that THC, PO activity, RB activity, growth performance, and shrimp survival administered with prebiotic 0.8% were significantly higher ($P<0.05$) than control. The administration of prebiotic with dose 0.8% was the best result and could effectively improve the immune responses and growth performance of whiteleg shrimp.

Keywords: prebiotic, whiteleg shrimp, white spot disease

ABSTRAK

Tujuan dari penelitian ini adalah untuk mengevaluasi respons imun dan performa pertumbuhan pada udang vaname yang diberi prebiotik *mannan-oligosaccharides* (MOS) dengan dosis berbeda (0%, 0,2%, 0,4%, dan 0,8%) pada pakan. Udang vaname (*Litopenaeus vannamei*) (dengan rata-rata bobot 3,41 ± 0,06 g) diberi pakan tiga kali sehari secara *at satiation*. Penelitian ini menggunakan rancangan acak lengkap. Udang dipelihara dengan kepadatan 15 ekor per 40/L pada setiap perlakuan dengan tiga kali pengulangan. Setelah 30 hari pemberian pakan, udang diuji tantang menggunakan *white spot syndrome virus* dengan diinjeksi secara intramuskular. Total bakteri usus, *total haemocyte count* (THC), aktivitas *phenoloxydase* (PO), dan aktivitas *respiratory burst* (RB) diamati 4 kali, yaitu sebelum perlakuan hari ke-30 sebelum uji tantang, hari ke-32 setelah uji tantang, dan hari ke-36 pada akhir penelitian. Hasil penelitian menunjukkan bahwa THC, aktivitas RB, aktivitas PO, performa pertumbuhan, dan kelangsungan hidup yang diberi prebiotik dengan dosis 0,8% lebih tinggi ($P<0,05$) jika dibandingkan dengan kontrol (dosis 0%). Pemberian prebiotik dengan dosis 0,8% merupakan hasil terbaik dan secara efektif mampu meningkatkan respons imun dan performa pertumbuhan pada udang vaname.

Kata kunci: prebiotik, udang vaname, *white spot disease*

INTRODUCTION

Whiteleg shrimp is one of the major aquaculture commodity in Indonesia. According to FAO, Indonesia is the 4th place in worldwide shrimp production. Nevertheless, in shrimp production, viral disease is the major problem, one of the diseases is white spot disease (WSD) which caused by white spot syndrome virus (WSSV) (Huang *et al.,* 2012). In 2014, there was an extreme decreasing of whiteleg shrimp production because of the WSSV (KKP, 2014). A prevention step towards WSSV can be done through prebiotics application to induce the immune response of whiteleg shrimp.

The application of prebiotics is a strategy to develop aquaculture industry in the past few years (Torrecillas *et al.,* 2007). Prebiotic is effectively affected in increasing the host health status associated with several bacteria species (Fuller, 2011). The identified microflora which naturally lives in whiteleg shrimp intestine was *Pseudoalteromonas* (Tzuc *et al.,* 2014). The bacteria is known actively contribute in enzymatic activity (lipase, chitinase, and amylase) in order to expedite shrimp digestion mechanism.

Mannan oligosaccharides (MOS) and β–glucan, the cell wall derivatives of *Saccharomyces cerevisiae,* were widely used as prebiotics in aquaculture industry (Huang *et al.,* 2008). *S. cerevisiae* was an easily obtained, stable, and widely available yeast (Rad *et al.,* 2013). Yeast application was potentially increased immunity because of the beneficial compounds, such as mannoprotein, chitin, and nucleotide (Rad *et al.,* 2013). This study aimed to evaluate the effectivity of MOS to induce the immune response and growth performance of whiteleg shrimp towards WSSV infection.

MATERIALS AND METHODS

The experiment period

This study was conducted in October–December 2016 at Fish Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University, and Agency for Brackishwater Aquaculture, Situbondo, East Java.

Experiment implementation

The prebiotics used in this study was mannan oligosaccharide (MOS) which was the derivatives from *S. cerevisiae* cell wall. The compounds of the cell wall are 21.61% ± 1.79 of crude protein, 22.48% ± 1.97 of β-glucan, 26.70% ± 1.43 of MOS, and starch 2.31% ± 1.12. The experimental object was specific pathogen-free whiteleg shrimp towards WSSV and IMNV through PCR test. The average body weight of the shrimp was 3.41 ± 0.06 g/ind. The whiteleg shrimp was obtained from *Unit Produksi Udang Gelung*, Agency for Brackishwater Aquaculture, Situbondo. The virus which used in challenge test was white spot syndrome virus isolate from Agency for Brackishwater Aquaculture, Situbondo.

The experimental feed used commercial feed. There were two kinds of feed, then the feeds were mixed along with the prebiotics manually using egg white (2% v/v) as binder and water (6% v/w) as a solvent. The MOS probiotics treatments were 0.2% (Tc0.2), 0.4% (Tc0.4), and 0.8% (Tc0.8), referred to Andrino (2014). For non-treatment feed was used commercial feed without any prebiotics addition on positive control (Kp) which tested using WSSV and negative control using PBS. The experimental feed was generated by mixing the commercial feed and prebiotics comply with the treatment, then dried before distributed to each treatment.

The oral MOS prebiotics application was conducted by rearing the whiteleg shrimp for 30 days in 12 aquariums sized 60×35×30 cm^3, filled with 40 L rearing media and already equipped with an aerator. The stocking density each aquarium was 15 ind/aquarium. The feeding frequency was 4 times/day, 7.00 a.m., 11.00 p.m., 15.00 p.m., and 19.00 p.m. using feeding rate 6% per day. The whiteleg shrimps were reared in the water temperature ranged from 28–29°C, pH 7.6–7.92, TAN 0.33–1.61 mg/L, salinity 33–35 g/L, and the dissolved oxygen 5.1–5.99 mg/L.

The challenge test was conducted after 30 days of rearing, precisely on the 31st day through intramuscular injection between the 3rd and 4th segment of the shrimp. The substance which injected into the Tc and Kp treatments was WSSV filtrate (100 µl/ind) in concentration 10^4 copy/mL. The Kn treatment was injected using PBS. The clinical symptom obervation was conducted for 7 days post-injection. After the challenge test, the feeding activity was continued using commercial feed without prebiotics addition. During the treatment feeding (day-0 to day-30 and day-32 to day-36), the haemolymph and intestine sampling on the Tc treatment were conducted to determine total bacteria and immunity parameter.

This study used complete randomized design

with three replication. The entire data were analyzed using variance analyze with 95% confidence range and continued using Duncan multiple range test through SPSS 16 to analyze the differences between treatments.

Parameter measurement

Immune response

The immune response of whiteleg shrimp was measured on day-0, day-30 (before the challenge test), day-32, and day-36 (after the challenge test). The immune response parameter consisted of total haemocyte count (THC), phenoloxydase activity (PO), and respiratory burst activity (RB). The THC measurement was done referred to Martin and Graves (1985) method. The haemolymph as many of 0.1 mL was taken from the first swimming leg using 1 mL syringe which already filled with 0.3 mL of anticoagulant Na-citrate 3.8%. The total amount of haemolymph cell was counted using microscope at 400× magnification.

The PO activity was measured according to dopachrome formation which generated by L–dihidroxyphenylalanine (L-DOPA). The mixture of anticoagulant and haemolymph was centrifuged as many of 1 mL (1500 rpm; 4°C) for 10 minutes. The supernatant was removed and the residual was suspended into 1 mL cacodylate-citrate buffer (0.01 M sodium cacodylate; 0.45 M sodium chloride; 0.01 M calcium chloride; 0.26 M magnesium chloride; pH 7), then centrifuged. The residual was taken and put into 200 µL cacodylate–citrate buffer. The 100 µL cell suspension was incubated with 50 µL trypsin for 10 minutes in 25–26°C, added with 50 µL L-DOPA (3 mg/mL cacodylate–citrate buffer) after 5 minutes, and then added with 800 µL cacodylate-citrate buffer. The optical density was measured using spectrophotometer in at 490 nm wavelength. The standard solution contained 100 haemocyte suspension, 50 µL cacodylate–citrate buffer (substitute the trypsin), and 50 µL L-DOPA which used to measure the background activity of PO in every tested solution.

The RB was measured referred to Liu and Chen (2004) method. A 100 µL haemolymph which already diluted in anticoagulant incubated in normal temperature for 30 minutes. Microplates was centrifuged at 700×g (gravity) for 20 minutes and then the supernatant was removed. The plasma was removed, then a 100 µL NBT was added to HBSS (Hank's balanced salt solution) at concentration 0.3% and incubated in for 2 hours in room temperature. Furthermore, the mixture was centrifuged at 700×g (gravity) for 10 minutes (supernatant was removed), after that it rinsed twice using 100 µL methanol 70% and then wind drying. Formazan was diluted by adding a 120 µL KOH 2 M and 140 µL dimethyl sulphoxide (DMSO). The optical density was measured with 630 nm wavelength using microplate reader and stated as NBT reduction in 10 µL hemolymph.

The growth performance and whiteleg shrimp resistance

The growth performance of whiteleg shrimp was measured through specific growth rate (SGR) and feed conversion ratio (FCR). The disease resistance was measured through survival rate (SR) after injected by WSSV filtrate.

RESULTS AND DISCUSSION

The immune response of whiteleg shrimp

The immune response of whiteleg shrimp, THC, PO, and RB was shown in Table 1. The highest THC value was shown at 0.8% of prebiotic treatment ($P<0.05$) compared with the other treatment. Haemocyte holds an important role as crustacean immune response against pathogenic bacteria (Rodriguez & Le Muollac, 2000). As an initial defense on invertebrate (Cerenius *et al.*, 2010), haemocyte recognized each unfamiliar particle which penetrated into the host, and then it would respond through several mechanisms, such as intracellular signaling cascade, phagocytosis, encapsulation, and nodular aggregates (Rodriguez & Le Muollac, 2000).

The decreasing of THC value after challenge test indicated that the immune system of whiteleg shrimp was functioned. Costa *et al.* (2009) and Yeh *et al.* (2009) stated that the decreasing of THC value was the effect of the immune system as a result of infiltration and haemocyte accumulation in infected tissues and haemocyte damage as result of apoptosis. The prebiotics application on whiteleg shrimp for 30 days potentially increased PO activity. The PO activity of 0.8% prebiotic treatment showed a significant difference ($P<0.05$) compared with the other treatments.

The PO activity indicated the ability to identify unfamiliar particle (Garcia-Carreno *et al.*, 2008) and the immune system was well-function (Costa *et al.*, 2009). The PO activity is one of the major immune defense in the crustacean. This process is controlled by a phenoloxydase enzyme which works as a catalyst for melanin synthesis (Song & Li, 2014). In this case, PO and THC are

Table 1. THC, PO, and RB activity of whiteleg shrimp which fed using prebiotic addition

Time	Treatment	THC (10^6 cell/mL)	PO	RB
Initial	Kp	2.96 ± 0.15[a]	0.15 ± 0.00[a]	0.23 ± 0.02[a]
	Kn	2.86 ± 0.60[a]	0.15 ± 0.00[a]	0.22 ± 0.02[a]
	Tc0.2%	2.93 ± 0.15[a]	0.15 ± 0.00[a]	0.20 ± 0.02[a]
	Tc0.4%	3.03 ± 0.35[a]	0.15 ± 0.00[a]	0.19 ± 0.01[a]
	Tc0.8%	3.06 ± 0.20[a]	0.15 ± 0.00[a]	0.21 ± 0.02[a]
Day−30	Kp	5.06 ± 0.56[a]	0.18 ± 0.01[a]	0.43 ± 0.04[a]
	Kn	5.10 ± 0.21[a]	0.18 ± 0.15[a]	0.44 ± 0.15[a]
	Tc0.2%	8.40 ± 0.20[b]	0.18 ± 0.00[a]	0.48 ± 0.05[a]
	Tc0.4%	9.66 ± 0.28[c]	0.26 ± 0.00[b]	0.65 ± 0.05[b]
	Tc0.8%	15.2 ± 0.14[d]	0.45 ± 0.01[c]	0.70 ± 0.06[b]
Day−32	Kp	1.73 ± 0.20[a]	0.17 ± 0.00[a]	0.17 ± 0.00[a]
	Kn	5.30 ± 0.70[d]	0.31 ± 0.00[d]	0.65 ± 0.13[c]
	Tc0.2%	1.70 ± 0.10[a]	0.18 ± 0.01[a]	0.29 ± 0.02[ab]
	Tc0.4%	3.43 ± 0.25[b]	0.20 ± 0.00[b]	0.30 ± 0.04[b]
	Tc0.8%	4.16 ± 0.20[c]	0.28 ± 0.01[c]	0.36 ± 0.01[b]
Day−36	Kp	1.40 ± 0.43[a]	0.16 ± 0.00[a]	0.14 ± 0.02[a]
	Kn	5.43 ± 0.15[e]	0.33 ± 0.00[e]	0.73 ± 0.01[c]
	Tc0.2%	1.90 ± 0.17[b]	0.18 ± 0.00[b]	0.30 ± 0.04[b]
	Tc0.4%	3.70 ± 0.26[c]	0.20 ± 0.00[c]	0.37 ± 0.03[b]
	Tc0.8%	4.33 ± 0.47[d]	0.28 ± 0.00[d]	0.36 ± 0.01[b]

Note: Different superscript in the same column indicates significant difference according to Duncan test at significant level of $p<0.05$.

synergizing and haemocyte produces and release PO into haemolymph in form inactive pro-enzyme, known as PO. The active proPO system, involves several molecules to conduct immune response in recognizing unfamiliar particle, intercell communication, melanin synthesis, reactant cytotoxic production, encapsulation, and nodular and capsule forming (Amparyup *et al.*, 2013).

The increasing of RB at the final rearing period showed that the prebiotics addition was potentially increased immune system of whiteleg shrimp. The MOS prebiotics which mixed with the feed affected the RB activity on whiteleg shrimp Ringo *et al.* (2010). The decreasing of RB activity after challenge test was related to the decreasing of THC, along with the immune system in a certain infected tissue. The whole RB activity occurred in haemocyte cell which conducted unfamiliar particle exclusion on phagocytosis process (Rodriguez & Le Muollac, 2000). The RB activity was an unfamiliar particle exclusion mechanism by phagocyte cell which involved degradative enzyme releasing into phagosome (oxygen- dependent killing mechanism) and produces ROIs (reactive oxygen intermediates) (Rodriguez & Le Muollac, 2000).

The growth performance and disease resistance of whiteleg shrimp

The growth performance of whiteleg shrimp was measured through several parameters, such as specific growth rate (SGR), feed conversion ratio (FCR), and survival rate (SR), which shown in Figure 1. The SGR in Tc0.4 (2.311 ± 0.170%/day) and Tc0.8 (2.610 ± 0.041%/day) treatment showed significant result ($P<0.05$) compared with the control. While the SGR in Kp and Kn treatments were 2.016 ± 0.191%/day and 2.165 ± 0.067%/day, respectively. A high value of SGR in Tc treatment was assumed caused by feed addition. It showed that MOS prebiotic inside shrimp intestine was able to increase growth performance. According to Zhang *et al.* (2012), a 0.4% MOS addition was potentially increased weight growth (WG), specific growth rate (SGR), and also potentially lengthen the microphilli of the bacteria in the shrimp intestine. Nutrition absorption would be easier when the intestine microphilli length was fairly long (Cerezuela *et al.*, 2011).

The Tc0.8 treatment had the lowest FCR value (1.80 ± 0.09), compared with the Tc0.2 and Tc0.4 which had FCR value 2.19 ± 0.39 and 2.16 ± 0.02, respectively. Meanwhile, the Kp and Kn had

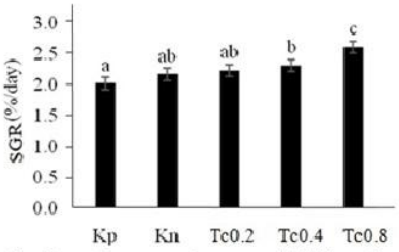

Figure 1. Spesific growth rate (SGR) of whiteleg shrimp *L. vannamei* after 30 days prebiotic feed addition; Kp (positive control), Kn (negative control), Tc0.2 (prebiotic 0.2%), Tc0.4 (prebiotic 0.4%), and Tc0.8% (prebiotic 0.8%). Different superscript at the same observation period indicated significant difference ($p<0.05$) between treatments.

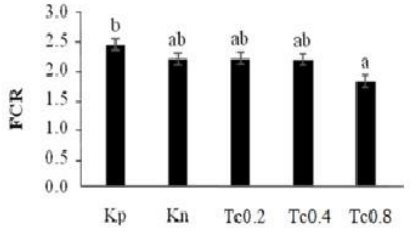

Figure 2. Feed conversion ratio (FCR) of whiteleg shrimp *L. vannamei* after 30 days prebiotic feed addition; Kp (positive control), Kn (negative control), Tc0.2 (prebiotic 0.2%), Tc0.4 (prebiotic 0.4%), and Tc0.8% (prebiotic 0.8%). Different superscript at the same observation period indicated significant difference ($p<0.05$) between treatments.

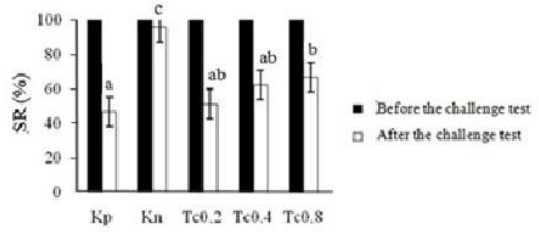

Figure 3. Survival rate (SR) of whiteleg shrimp *L. vannamei* after 30 days prebiotic feed addition; Kp (positive control), Kn (negative control), Tc0.2 (prebiotic 0.2%), Tc0.4 (prebiotic 0.4%), and Tc0.8% (prebiotic 0.8%). Different superscript at the same observation period indicated significant difference ($p<0.05$) between treatments.

higher FCR value 2.42 ± 0.41 and 2.18 ± 0.07, respectively. The higher interaction between prebiotic dosages was able to optimize feed conversion ratio on experimental shrimp. The FCR value at Tc0.8 treatment was significantly different ($P<0.05$) towards control. The other treatment did not show any differences ($P>0.05$) because the other treatments were assumed to affect growth performance indirectly through microphilli lengthen in shrimp intestine. The TEM analysis on Zhang *et al.* (2012) showed that MOS supplementation in feed significantly lengthen *L. vannamei* intestine microphilli up to 1.10–2.39 μm compared with control 0.92 μm, so that it was assumed that the experimental shrimp would absorb more nutrient than the control shrimp to support its growth.

The survival rate of whiteleg shrimp after challenge test showed Tc0.4 (62.23%) and Tc0.8 (66.67%) were significantly from control ($P<0.05$). The Tc0.2 (51.11%) showed no significant difference ($P>0.05$) compared to both Kp (46.67%) and Kn (95.55%). The high value of survival rate on Tc0.8 treatment showed excellent immune system compared to other treatments without prebiotics. The high survival rate was closely related to immune response after prebiotics addition. Febrianti *et al.* (2016) reported that WSSV infection on whiteleg shrimp potentially caused mortality up to 64.44% or survival rate only 35.55% on positive control. The immune response parameter (THC, PO, and RB) increased due to the infection. Bai *et al.* (2014) reported that 0.1% of derivative β–glucan addition could induce the immune response of whiteleg shrimp towards WSSV infection.

CONCLUSION

Feed addition using 0.8% MOS was potentially increased immune response on whiteleg shrimp challenged test using WSSV injection, characterized by total haemocyte count (THC), PO (prophenoloxydase) activity, RB (respiratory burst) activity, survival rate, growth performance (specific growth rate and feed conversion ratio).

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