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Application of probiotics and microalgae (*Chaetoceros calcitrans*) to stimulate non-specific Immune responses in white Shrimp (*Litopenaeus vannamei*) infected with *Vibrio harveyi*

Mila Ayu Ambarsari[1] and Woro Hastuti Satyantini[2]*

[1]Post Graduate Program of Marine Biotechnology, Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya 60115, Indonesia
[2]Department of Fish Health Management and Aquaculture Faculty of Fisheries and Marine, Universitas Airlangga, Surabaya 60115, Indonesia

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ABSTRACT

This study aims to determine whether the administration of probiotics and microalgae *Chaetoceros calcitrans* can improve the non-specific immune response of white shrimp after *Vibrio harveyi* bacterial infection. This research is experimental with a completely randomized design (CRD) consisting of 5 treatments and 4 replications. -C: Rearing shrimp without the administration of probiotics or microalgae *Chaetoceros calcitrans,* the 15th day was injected with PBS (*Phosphate Buffer Saline*). +C: Rearing shrimp without the provision of probiotics or microalgae *Chaetoceros calcitrans,* and on the 15th day infected with *Vibrio harveyi*. PA: Shrimp maintenance by providing Probiotics (Pro). PB: Shrimp maintenance by giving Microalgae (Mic). PC: Shrimp maintenance by providing Probiotics and Microalgae (Promic) for 14 days, and on 15th day infected with *V. harveyi.* The parameters observed included *Total Haemocyte Count* (THC), *Phagocytic activity*. Data analysis used Analysis of Variance (ANOVA) followed by Duncan's multiple range test (DMRT). The results of this study indicate that the administration of probiotics and microalgae gave significantly different results ($p<0.05$) on non-specific immune responses in white shrimp. The best research results on the treatment of probiotics and microalgae *Chaetoceros calcitrans* are able to increase the non-specific immune response in white shrimp after *Vibrio harveyi* bacterial infection THC value of 42.86×10^6 cells/mL, phagocytic activity (PA) activity by 95.50%, SR 94.75%. Probiotics (Pro), Microalgae (Mic) *Chaetoceros calcitrans* and Combination Probiotics and Microalgae (Promic) on the maintenance media can increase non-specific immune response and survival rate in white shrimp after *V. harveyi* bacterial infection.

Key words: *Probiotics, Bacillus subtilis, Bacillus mycoides, Pseudomonas diminuta, Litopenaeus vannamei*

Introduction

White Shrimp (*Litopenaeus vannamei*) was firstly imported into Indonesia in 2000 to replace the Tiger shrimp (*Penaeus monodon*) which is widely affected by diseases, especially bacteria and viruses (Marwiyah *et al.*, 2019; Kharisma *et al.*, 2020). But in the course of this effort, losses from disease attacks continued. This Vibriosis disease can cause death in shrimp 90 – 100% of the total population, one of which is by *Vibrio harve*yi bacteria. A number of methods have been applied in the effort to control

diseases such as control of the immune system through the administration of probiotics (Jefri *et al.*, 2020; Rangka and Gunarto, 2012; Sriwulan *et al.*, 2019; Latifah *et al.*, 2019) and microalgae *Chaetoceros calcitrans* that can be a substitute antibiotictoreduce pathogenic bacteria. Probiotics have a cell wall structure of lipopolysaccharides (LPS) and have antioxidant activity (Trisnawati *et al.*, 2018; Rusmarilin *et al.*, 2018; Silalahi *et al.*, 2018), whereas microalgae *Chaetoceros calcitrans* contain a content of β-1-3 glucan. Both materials can be used to enhance nonspecific immune responses. β-1-3 glucan and LPS may increase PO activity after β-1-3 glucan and LPS react with β-glucan binding protein (BGBP) or LPS binding proteins. After the bind, proPO will be reactivated into a PO enzyme that further performs its function in the process of melanization, while in microalgae that have a content of β-1-3 glucan can increase cell activating factors in hemocytes, So that it can increase the activity of PO and phagocytosis in shrimp (Smith *et al.*, 2014). The objectives of this study were to investigate the effects of probiotics and microalgae (*Chaetoceros calcitrans*) on the stimulation of non-specific immune responses in white shrimp (*Litopenaeus vannamei*) infected with *Vibrio harveyi.*

Materials and Methods

Probiotics used (*Bacillus subtilis, Bacillus mycoides, Pseudomonas diminuta*), Microalgae *Chaetoceros calcitrans*, *Vibrio harveyi* bacterial isolate. The size of aquarium used in this experimentation was 60 × 30 × 35cm^3. Whiteshrimp (*Litopenaeus vannamei*) were in healthy condition and free from *Vibrio harveyi* infection with body length 6-8cm, and weights 6-6.5g.

Research Design

This research was conducted using a Completely Randomized Design consisting of 5 treatments and 4 replications. The dosage of *Vibrio harveyi* used in this experiment was 10^6 CFU/mL Cahayati (2012), and the dosage of *Chaetoceros calcitrans* microalgae was as much as 67.50 × 10^5 cells/mL (Yeh *et al.*, 2015).

The treatments used in this study were

Negative control (-C): Shrimp rearing without the provision of probiotics or microalgae *Chaetoceros calcitrans*, then on the day 15 was injected with PBS (Phosphate Buffer Saline).

- Positive control (+C): Maintenance of shrimp without the provision of probiotics and microalgae *C. calcitrans*, and on the day 15 infected with *Vibrio harveyi*.
- PA: Shrimp maintenance by providing Probiotics (Pro), for 14 days, and on the 15th day infected with *V. harveyi.*
- PB: Shrimp maintenance by giving Microalgae (Mic), for 14 days, and on the 15th day infected with *V. harveyi.*
- PC: Shrimp maintenance by providing Probiotics and Microalgae (Promic), for 14 days, and on the 15th day infected with *V. harveyi.*

Observation of Research Parameters

Observation parameters of white shrimp immune response were consisted of Total Hemocyte Count (THC), Phagocytic activity (PA), Survival Rate (SR).

Hemolymph collection

Hemolymph for measurement of immune parameters was taken from all shrimps of each treatment unit. About 0.1 mL of hemolymphwas taken from the ventral sinus at the base of the 5th leg using a 1mL syringe after 0.1 mL of anticoagulant was added (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, pH 7.5) (Mannopo, 2014).

Total Haemocyte Count (THC)

After hemolymph collection, approximately of 100 ml of hemolymph was taken and dropped into the hemocytometer to calculate the Total Haemocyte Count (THC) under a light microscope with 400x magnification. Measurement of total haemocyte was carried out four times: at day 0 (at the beginning of the experiment, H0), at day 14 (H-14), after bacterial infection of *Vibrio harveyi* (at day 16, H-16) and at day 25 (H-25). THCs were expressed as number of cells/ml of hemolymph.

Phagocytic activity (PA)

Phagocytic activity was determined as described below. Retrieval of fresh shrimp hemolymph (20 µL), inserted into the microtube and added with 20 µL suspension of *Staphylococcus aureus* with density of 10^8 cells/mL, incubated at room temperature for 30 minutes. Furthermore, 5 µL was taken to make a smear on the preparation object glass and let it dry. The preparations were then soaked with methanol 95% for 5 minutes and rinsed with 0.85% NaCl and

dried again. Then painted using 10% Giemsa for 15 minutes and air dried. The curing preparation was then watered for about 5 minutes to remove the remaining color of Giemsa. The preparations were observed under a microscope at 400x magnification. PA values was calculated using the following formula:

AF=a/b x 100%

Where:

PA = Phagocytic Activity (%)

a = Number of cells undergoing phagocytosis (cell/mL)

b = Total number of cells observed (cell/mL)

Data analysis

Measurement data were expressed as mean ± SD. THC dan PA data then analyzed using One-way ANOVA by using statistical software SPSS-21. If significant difference were observed, the Duncan test then used to analyze which treatment caused significantly different to immune responses of white shrimpat $p<0.05$.

Water quality

Water quality parameters (temperature, dissolved oxygen, pH, Salinity, Ammonium, Nitrite, Ammonia, and Nitrate) were measured during the study are presented in. Table 3. The water quality was within the normal range based on standards for white shrimp culture.

Results and Discussion

Survival Rate

The survival rates of infected shrimp by *Vibrio harveyi* after provision of probiotic (Pro), microalgae (Mic) and probiotic+microalge are presented in Figure 1. Both at day 16 and day 25, the provision of probiotic+microalgae (Promic) treatments had a significantly different on survival rate of shrimp compared to negative control (-C), positive control(+C) and microalgae (Mic) treatment. There were not significantly different between Pro and Promic treatment. Both at H-16 and H-25, the lowest survival rates were noted at +C.

Total Haemocyte Count (THC)

The total number of hemocyte count of *Litopenaeus vannamei* at H0, H-14, H-16 and H-25 are presented in Table 1. Haemocyte play an important role in the immune system of crustacea. At H0 the THC values ranged from 4.21 × 10^6 cell/ml to 4.47x10^6sel/mL. All treatments were not significantly different at H0. Furthermore, the THC values of Pro and Mic treatments at H-14 were higher than thoese of -C, +C and Promic treatments.

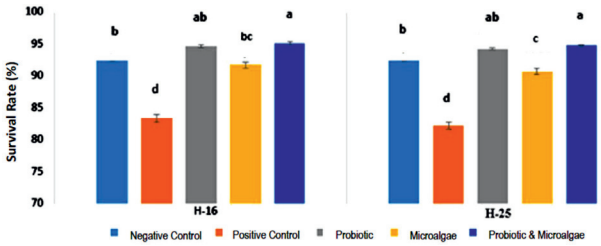

Fig. 1. Application of probiotics and microalgae on survival rate of *Litopenaeus vannamei* infected by *Vibrio harveyi*

One day after *Vibrio harveyi* infection (at H-16) the THC values of Pro and Promic were not significantly different. The lowest value of THC was noted at -C. Furthermore, 10 days after *Vibrio harveyi* infection (at H-25) the THC values of Pro, Mic and Promic were not significantly different, and their levels were higher than -C and +C.

Phagocytic activity

Phagocytosis is the process of digesting particulate matter, especially bacteria, into the cytoplasm of white blood cells (Gullian *et al*., 2014). The phago-

Table 1. Average Total Haemocyte Count of White Shrimp during the study

Treatment	Average value of haemocytes on day to- (x10^6) cell/mL			
	H-0	H-14	H-16	H-25
-C	4.21^{a} ± 0.492	4.71^{c} ± 0.512	9.16^{d} ± 3.568	21.40^{b} ± 3.290
+C	4.28^{a} ± 0.543	6.16^{b} ± 0.191	11.63^{cd} ± 2.391	13.75^{b} ± 1.946
Probiotics	4.30^{a} ± 0.486	7.96^{a} ± 1.346	18.64^{a} ± 2.122	34.41^{a} ± 8.372
Microalgae	4.27^{a} ± 0.505	7.50^{a} ± 0.493	13.81^{bc} ± 2.121	34.50^{a} ± 4.328
Probiotics and Microalgae	4.47^{a} ± 0.408	4.54^{c} ± 0.327	17.66^{ab} ± 2.673	42.86^{a} ± 9.677

Table 2. Average values of *phagocytic activity* (PA) during the study

Treatment	The Average value of PA (%) on observation			
	H-0	H-14	H-16	H-25
-C	$61.25^{a} \pm 4.788$	$67.25^{c} \pm 3.594$	$77.50^{ab} \pm 2.082$	$84.50^{c} \pm 1.000$
+C	$60.75^{a} \pm 5.620$	$68.25^{c} \pm 4.646$	$74.75^{c} \pm 2.500$	$81.00^{d} \pm 1.155$
Probiotics	$62.50^{a} \pm 2.082$	$78.50^{ab} \pm 2.380$	$83.75^{a} \pm 1.500$	$90.25^{b} \pm 1.258$
Microalgae	$62.25^{a} \pm 1.708$	$73.75^{b} \pm 0.500$	$79.75^{b} \pm 0.500$	$88.00^{b} \pm 2.160$
Probiotics and Microalgae	$63.00^{a} \pm 2.449$	$80.50^{a} \pm 4.203$	$85.00^{a} \pm 3.559$	$95.50^{a} \pm 1.732$

Table 3. The Range of Water Quality Value of Media Maintenance of Shrimp

Variable	Treatment					The Range of Normal values
	K^{-}	K^{+}	Probiotics	Microalgae	Probiotics and Microalgae	
pH	7.7	7.6	7.7	7.8	7.7	7.5 – 8.5
Salinity (ppt)	22	21	21.5	20	21	16 – 30
DO (mg/L)	6.21	5.48	6.01	6.11	6.03	>3 – 7
Temperature (°C)	28	27	29.1	26.5	28.7	27 – 32
Ammonium	0.25	0.3	0.18	0.17	0.17	0.1 – 5
Nitrit	0.17	0.2	0.15	0.15	0.1	0.1 – 1
Nitrat	0.4	0.5	0.5	0.6	0.6	>0.2 – 0.8
Ammonia	0.018	0.021	0.013	0.012	0.012	< 1

cytic activity values of all treatments starting from H-0, H-14, H-16 and H-25 are presented in Table 2. At H-0, all phagocytic activity of all treatments were not significantly different. At H-14, the highest phagocytic activity was recorded at Promic, and the lowest were observed at -C and +C. At H-16 there were not significantly different between -C, Pro and Promic, the lowest phagocytic activity was note at +C. At H-25 the highest phagocytic activity was noted at Promic and the lowest was observed at +C.

Discussion

The level of survival is an opportunity for an individual's life in a certain time. At the end of the study (H-25) the Promic treatment received ansurvival rate (SR) value of around 94.75%, the Pro treatment 94.25%, the Mic treatment 90.75%, the -C treatment 92.37%, and lowest was noted in +C treatments around 82.25%. The high observations of SR values in the Promictreatments compared to other treatments especially to Pro and Mic allegedly due to a balanced work between probiotics and microalgae.

Maximum probiotic work can function as a biological control agent that produces antibacterial molecules such as bacteriocin directly capable of inhibiting other bacteria such as *Vibrio harveyi* and actively participating in fighting infections. Probiotics are also able to inhibit the movement of other bacteria in the intestinal wall (translocation), so they can improve the function of the mucosal barrier by increasing the production of non-specific immune responses or modulating inflammation (Cerezuela *et al*., 2011). In addition, the microalgae *Chaetoceros calcitrans* has the ability to inhibit the growth of gram-negative bacteria, by increasing the permeability of bacterial membranes (Zheng *et al*., 2015).

Shrimp blood does not contain haemoglobin, so the blood is not red (Person *et al*., 2014). In shrimp the function is replaced by haemocyanin, a protein that contains Cu and can bind with oxygen. Haemocyanin functions as oxygen transport, as a buffer in shrimp blood and plays an important role in blood osmotic (Maynard, 2014). White shrimp has nonspecific immunity (innate) which can recognize and destroy foreign objects that enter the body, so that the nonspecific immune system plays an important role in the shrimp immune system. Haemocytes play an important role in the crustacean immune system. Haemocytes play a role in phagocytosis, encapsulation, degranulation and aggregation of pathogens or foreign particles (Sahoo *et al.*, 2014).

This haemocyte value can be measured and can be used as a health assessment of shrimp through the activity of the defense system against infectious

agents. This total haemocyte is very important in resistance to pathogens. From the results of data during the research on the observation of THC H0 ranged from $4.21x10^6$ cells/mL to $4.47x10^6$ cells/mL. The H-14 before *Vibrio harveyi* infection, in Promic treatment at $4.54x10^6$ sel/mL showed the lowest results compared to Pro treatment $7.96x10^6$ sel/mL, Mic treatment at $7.50x10^6$ cells/mL and positive control $6.16x10^6$ cells/mL. This is suspected that the treatment of Promic given during maintenance influence the work of competitors in the utilization of nitrogen nutrients by probiotic bacteria and microalgae, so that the number of probiotics entering the digestive tract becomes less than in the Pro treatment. At H-16 (1 day after *Vibrio harveyi* infection) where the THC value of Promic increased compared toH-14. This is because as a form of shrimp immune system reaction in response to foreign objects that enter is the pathogenic bacterium *Vibrio harveyi*. However, after 10 days after the occurrence of *Vibrio harveyi* infection (H-25) THC value of Promic, Pro and Mic treatments were not significantly different, but they were significantly different compared to positive control treatments. This is because the mechanism of action of probiotics can also provide resistance to the body against *Vibrio harveyi* attacks. Where the *Pseudomonas diminuta* bacterium contains *Lipopolysaccharide*, LGBP (*Lipopolysaccharide β-glucan Binding Protein*) will induce haemocyte degranulocytes and stimulate the activation of the ProPO system into PO, hence the opsonin factor protein will produce an increase in phagocytosis. Whereas *Chaetoceros calcitrans* Microalgae also contains *β-1,3 Glucan* from carbohydrate group which through the introduction of proteins activated by PPA, and PPA itself can be activated by *lipopolysaccharides* from microorganisms through recognition will form *β-1,3 Glucan Binding Protein* found in granulocytes so as to increase haemocytes in white shrimp. The results of the study reported by Sahoo *et al*. (2014) also stated that white shrimp treated with Probiotics and Microalgae (Promic) had higher THC values compared to other treatments. The high THC value in white shrimp is due to the high mobilization of hemolymph in the body of the shrimp so that it can increase immunity and the introduction of foreign objects that enter the shrimp body. While the low THC value greatly affects the susceptibility of shrimp to pathogens (Le Moullac *et al*. 2014).

This study showed that increased THC values in the treatment of Promic also followed by an increase in the value of phagocytic activity (PA). Determination of the value of PA is to determine the increase in shrimp endurance because in the phagocytosis process is a non-specific defense mechanism that is generally able to protect against disease (Fontaine and Lightner, 2013). The PA values of H-25, the Promic treatments showed the highest PA values of 95.50% compared to the Pro treatment 90.25% and the Mic treatment at 88 %. In the Promic treatment, the effect was significantly different compared to controls. So that the treatment of Promic can improve shrimp immune response by increasing phagocytic cells to carry out the process of phagocytosis, namely the ability to phagocyte in destroying the *Vibrio harveyi* attack. The higher of phagocytic activity value, the immune system of white shrimp is suspected to be getting better, this is because in probiotics there is an antibacterial and antimicrobial content that can inhibit the formation of nucleic acids which causes the target cell membrane to be disrupted so that *Vibrio harveyi* lysis. Whereas the *Pseudomonas* bacteria are able to produce extracellular enzymes in the form of bacteriocin, siderophore and antibiotics which can inhibit pathogenic bacteria. Furthermore Itami and Takeuchi, (2013) support the theory which states that the administration of immunostimulants can prevent disease infections in the host body and cause increased phagocyte activity of haemocytes and proPO enzymes. From the data obtained during the H-25 maintenance period that white shrimp treated with Promic can provide significant survival rate values on the immune response that can be seen from the data there is a linear increase in THC, and PA values and quality water is one of the factors that greatly influences the life of shrimp, so in this study water quality parameters become one of the considerations of the results obtained. The water quality of the maintenance media during the study was in the ideal range for the maintenance of white shrimp. So that with good water quality parameters, and an increased immune response can provide shrimp growth and good survival value, resulting in an increase in production of white shrimp culture.

Conclusion

Based on the results of the research that has been done, the following conclusions can be drawn: In the combination treatment gives a very real effect on the non-specific immune response in white shrimp. Which results in an increase in the number of

haemocytes by 42.86x10^6 cells/mL, *phagocytic activity* (PA). To obtain an effective protocol for administering *Chaetoceros calcitrans* microalgae, further research is needed to observe.

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