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Oral Supplementation of Alginate and Spirulina as Immunostimulant of Non-Specific Immune System of *Litopenaeus vannamei*

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ABSTRACT

Litopenaeus vannamei gains a high export demand value. However, the productivity has decreased in the recent years due to several problems, such as infectious diseases. This study aimed to determine the effect of alginate and spirulina extract diet supplementation as an immunostimulant agent in increasing the non-specific immunity of *L. vannamei*. The study was conducted with four treatments, namely control, the addition of 3g of alginate in 1.0kg of feed **(**alginate 3g/kg**)**, 5mg spirulina in 1.0kg of feed **(**spirulina 5 mg/kg**)**, and 3g of alginate + 5 mg spirulina in 1kg of feed **(**alginate 3g/kg+spirulina 5mg/kg**)**. The shrimps were reared for 12 days, and the cellular immune parameters were observed on days 0, 4, 8, and 12. The parameters determined in this study were the THC **(**total hemocyte count**)**, PA **(**phagocytic activity **)**/PI **(**phagocytic index**)**, the activity of PO **(**phenoloxidase**)**, and SOD **(**superoxide dismutase**)**. Generally, the results showed that the supplementation treatments were significantly different from the control **(***P*<0.05**)**. Highest THC value was found in the alginate 3g/kg+spirulina 5mg/kg treatment on day 12, the PA value in the alginate 3g/kg + spirulina 5mg/kg treatment on day 12, while an increase in PI was shown in the alginate 3g/kg+spirulina 5mg/kg treatment on day 12. The highest PO activity was shown in the treatment of alginate 3g/kg+spirulina 5mg/kg on day 4, while the highest activity of SOD was shown by treatment spirulina 5mg/kg on day 8. It is concluded that supplementation with alginate and spirulina extract enhances all parameters of the non-specific immune system in *Litopenaeus vannamei*.

INTRODUCTION

Litopenaeus vannamei is high in productivity due to its fast growth and the capacity of rearing in high density **(Rohmin, 2017)**. White shrimp production in 2010 was 206,578 tons and increased by 411,729 tons in 2014 **(KKP, 2014)**. The production in 2015 was 786,654 tons, while the production in 2016 was around 265,000 tons **(Febriani *et al.*, 2018)**. In fact, there has been a decline in 2016 total production compared to the

previous years. Disease problems are the main cause of failure in *L. vannamei* production **(Samuria *et al.*, 2018)**.

Prevention and treatment of disease in shrimp culture is often done by using antibiotics **(Hasniar *et al.*, 2013)**. The continuous and uncontrolled use of antibiotics can lead to the more severe diseases. This leads to the resistance to the antibiotics and harm to the human health **(Kusmarwati *et al.*, 2017)**. Diseases in shrimp can be controlled by increasing the shrimp's body defense system by using spirulina **(Tayag *et al.*, 2010; Yudiati *et al.*, 2021)**, alginate **(Yudiati *et al.*, 2016; Yudiati *et al.*, 2019)** as immunostimulants **(Ismawati *et al.*, 2019)**. Immunity performs resistance to the infection of some diseases. The immune system is the protections system against foreign substances **(Artini & Veranita, 2021)**. **Kurniawan *et al.* (2018)** also stated that shrimp only have an innate immune system that rely on non-specific defensive system. Haematocytes are the first defense against infection through phagocytic activity, encapsulation, and nodule formation **(Ridlo & Pramesti, 2009; Yudiati *et al.*, 2016)**.

Based on previous research, alginate has an antibacterial activity **(Hu *et al.*, 2005; Yudiati *et al.*, 2022)**, antidiabetic, and anti-obesity **(Wan-Loy & Siew-Moi, 2016)**, as well as antitumor **(Chen *et al.*, 2017)**. According to **Lee *et al.* (2020)**, the polysaccharide extract of *Sargassum fusiforme* was evaluated as a feed additive for young shrimp *Fenneropenaeus chinensis*, against vibriosis as immune activity. Oral administration of 0.5 and 1.0% polysaccharide extract for 12 days can effectively increase vibriosis resistance and increase the immune activity of shrimp **(Rahim *et al.*, 2020)**. *Sargassum muticum* has the ability as an antioxidant and antimicrobial **(Mazumder *et al.*, 2016)**. Similar observations were noted in **Kokilam *et al.* (2017)**, where a diet using sodium alginate in *L. vannamei* increased resistance to microbes. According to **Yudiati *et al.* (2019)**, *Sargassum siliquosum* significantly increased Total Haemocyte Count (THC), thereby contributing to the enhancement of the non-specific immune response in *Litopenaeus vannamei*. It has been remarked that sodium alginate can increase the ability of shrimp to effectively interact synergistically with cellular and humoral compounds to increase the immune response in shrimp, in terms of combating invading microbes.

Haematocytes are the first defense against infection through phagocytic activity, encapsulation, and nodule formation **(Ridlo & Pramesti, 2009; Yudiati *et al.*, 2016)**. Similar to the alginate, polysaccharide from fucoidan can trigger the activity of the innate immune system of *L. vannamei*. This is indicated by the increase in proPhenol Oxidase **(**proPO**)** activity **(Sinurat *et al.*, 2016)**. According to **Yudiati *et al.* (2019)**, their research showed that *Sargassum* can increase THC, Phenol Oxidase **(**PO**)**, and Superoxide Dismutase **(**SOD**)** and can play a significant role in enhancing the non-specific immune response of *L. vannamei*.

Spirulina platensis is a cyanobacterium or blue-green filamentous algae that grows in carbohydrate-rich lakes in the scorching hot zone **(Shuba & Kifle, 2018)**. *Spirulina* cultivation in sea water has higher minerals than in fresh or brackish water. Sea

water spirulina cultivation contains higher polysaccharides, phycocyanins, and inositol **(Christwardana *et al.*, 2013)**. Spirulina in dry powder form (SDP) has been shown to enhance the resistance of *Oreochromis niloticus* to *Aeromonas hydrophila* **(Abdel-Khaliq *et al.*, 2014)**. In addition, feeding spirulina dry powder **(**SDP**)** to crustaceans increased phagocytic activity in the banana shrimp *Fenneropenaeus merguiensis* **(Chen *et al.*, 2016)**. Additionally, it increased the number of hemocytes and hemolymph protein in the white shrimp **(Macias-Sancho *et al.*, 2014)**. Significantly, this will subsequently reduce mortality caused by microorganisms' infection **(Chuchird *et al.*, 2021)**. Phycocyanin in Spirulina has been reported to stimulate antioxidant activity **(Hidayati *et al.*, 2020)**. Based on these findings, further research is warranted to investigate the potential of alginate and spirulina supplementation in enhancing the non-specific immune response of *Litopenaeus vannamei*. Therefore, the aim of this study was to evaluate the effect of alginate and/or spirulina extract supplementation, administered orally, as an immunostimulant on non-specific immune parameters—total haemocyte count (THC), adhesive and phagocytic activity (AF/IF), phenoloxidase (PO), and superoxide dismutase (SOD)—in *L. vannamei*.

MATERIALS AND METHODS

The research was carried out at the Biology Laboratory, Faculty of Fisheries and Marine Sciences, Diponegoro University, Semarang, Indonesia for shrimp rearing and assessment of THC, PA/PI. The determination of PO and SOD was carried out at Marine Science Techno Park **(**MSTP**)** Laboratory, Faculty of Fisheries and Marine Sciences, Diponegoro University, Jepara, Indonesia.

1. Sample preparation

L. vannamei samples, as many as 240, with an average weight of 10-13 grams were purchased from BBIAPL **(**Balai Budidaya Ikan Air Payau dan Laut**)** Central Java. Acclimatization was conducted for 7 days in a plastic container with 150 liters volume per 30 shrimp. The media in this study was seawater with a salinity of 25ppt, DO from 4.6-5.0 mg/L and pH from 7.2-8.1. Water quality management was managed by siphoning twice a day. The administration was done in the morning before feeding at 07.00 and in the afternoon at 16.00. These efforts were made to reduce the remaining feed that has settled at the bottom of container.

2. Experimental design

The study was conducted experimentally using a completely randomized design **(**CRD**)** with 4 treatments, namely control without alginate or spirulina supplementation, alginate 3g/kg **(**alginate supplementation at 3g per kg of feed**)**, spirulina 5mg/kg **(**5mg/kg

spirulina supplementation per kg feed**)** and alginate 3g/kg + spirulina 5mg/kg **(**supplementation of alginate + spirulina at 3g + 5mg per kg of feed**)**. Each treatment was done with 3 repetitions.

3. Feeding trial

The test feed used in this study was Feng Li Gold GR-3L brand feed **(**pellet**)**, which was added with alginate and spirulina water extract. Alginate and spirulina water extract were provided by Tropical Marine Technology Laboratory, Faculty of Fisheries and Marine Science, Diponegoro University. Both ingredients were weighed according to the dose, then dissolved with distilled water and mixed with feed by spraying. After that, the feed was dried at room temperature. The dried feed was then stored at 4^{o}C to maintain feed quality. Feeding was carried out 4 times a day at 07.00, 11.00, 16.00, and 20.00, as much as 5% of the weight of shrimp biomass **(Tahe & Suwoyo, 2011)**.

4. Sampling of shrimp haemolymph

Sampling of shrimp haemolymph was carried out on the second swimming leg at the base of the pleopod in the abdomen. The haemolymph collection technique was carried out with a 1.0mL syringe needle previously coated with an anticoagulant **(**10% sodium citrate, pH 7.2**)**. Hemolymp has been taken as much as 200-300µL, and then transferred to a sterile microtube and stored in a coolbox. Furthermore, the hemolymph samples were divided for testing the activity of THC **(**20µL**)**, PA/PI **(**20µL**)**, PO **(**100µL**)**, and SOD 40µL **(Yudiati *et al.*, 2019)**.

5. THC and PA/PI test

The THC test was carried out by inserting 20µL hemolymph into the tube, then PBS was added in a ratio of 1: 4 and homogenized. A 20 µL aliquot of the homogeneous sample was placed onto a hemocytometer and observed under a microscope at 40× magnification. For the phagocytic activity/phagocytic index (PA/PI) assay of the hemolymph, 20 µL of the sample was mixed with phosphate-buffered saline (PBS) and incubated for 20 minutes. After incubation, 20µL of the mixture was dropped onto a glass slide and allowed to air dry. The slide was then fixed by spraying with 70% alcohol and left to dry again. Subsequently, the preparation was stained with 10% Giemsa solution diluted in distilled water and rinsed with tap water. The stained samples were observed under a microscope at 100× magnification.

6. PO and SOD test

The assay of PO and SOD test, basically refer to **Beauchamp and Fridovich (1971)** and **Liu *et al.* (2004)**, respectively. The PO test was observed from the formation of dopachrome formed from L-dihydroxyphenylaline **(**L-DOPA**)**, measured by ELISA

Reader with absorbance of 490nm. The 100µL shrimp hemolymph sample was supplemented with the addition of 100µL phosphate buffer saline **(**PBS**)**, then centrifuged at 4ºC at 700g for 20 minutes. The supernatant was discarded and an addition of 100µL cacodylate citrate buffer was performed, then the supernatant was centrifuged again at the same temperature and speed for 20 minutes. The pellets obtained were treated with the addition of 100µL cacodylate buffer, then homogenized. After that, 50µL trypsin was added and incubated for 10 minutes. The sample was then supplemented with the addition of 50µL L-DOPA. The SOD test was observed from riboflavin produced by nitroblue tetraolium **(**NBT**)**. Approximately, 40µL of haemolymph was added to 360µL PBS. The sample was homogenized and centrifuged at 4ºC at a speed of 6000g for 8 minutes. The supernatant was then heated with a hot plate stirrer at a temperature of 65ºC for 15 minutes. Finally, upon the addition of 50µL NBT to 50µL of the supernatant sample, the reaction was recorded spectrometrically. The SOD activity was measured with an ELISA Reader at 630nm.

7. Analysis data

The data were analyzed statistically using Excel to determine the differences among treatments. The data that have been confirmed to be normal, homogeneous, and additive are followed by one-way analysis of variance **(**ANOVA**)**. If the treatment has a significant effect **(***P* <0.05**)**, then it was continued with Duncan to find out the differences between treatments.

RESULTS

1. Effect of alginate and/or spirulina supplementation on shrimps' THC, PA, and PI

The effects of alginate and/or Spirulina supplementation on *L. vannamei* are presented in the following figures: Fig. (1) shows the average Total Haemocyte Count (THC), Fig. (2) displays the average phagocytic activity (PA), and Fig. (3) presents the average phagocytic index (PI) over a 12-day rearing period.

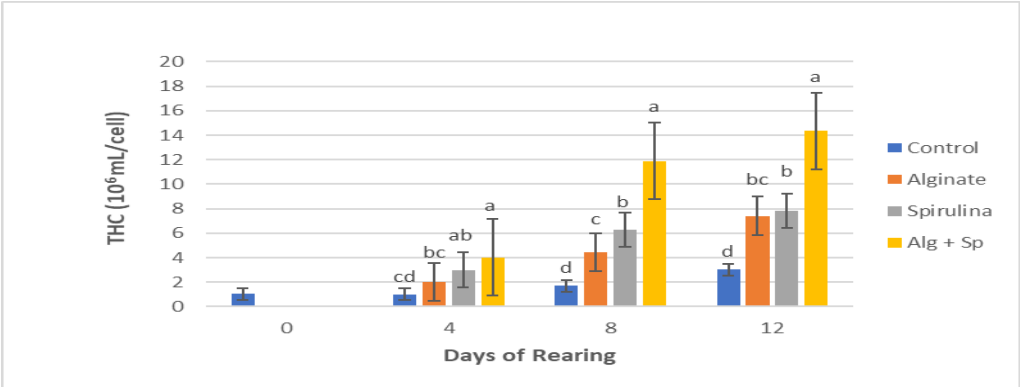

Fig. 1. THC of *L. vannamei* with different feeding regimes on 0, 4, 8, and 12 rearing days. Significant differences are indicated by different letters **(***P* < 0.05**)**

Based on THC observations that have been carried out for 0 - 12 days, it was obvious that the control group gave the lowest yield along with the rearing time **(**0.89 x 10^6 mL/cell**)**. The largest increase in THC occurred in the treatment of alginate 3g+spirulina 5 mg/kg **(**10.09 x 10^6 mL/cell**)**.

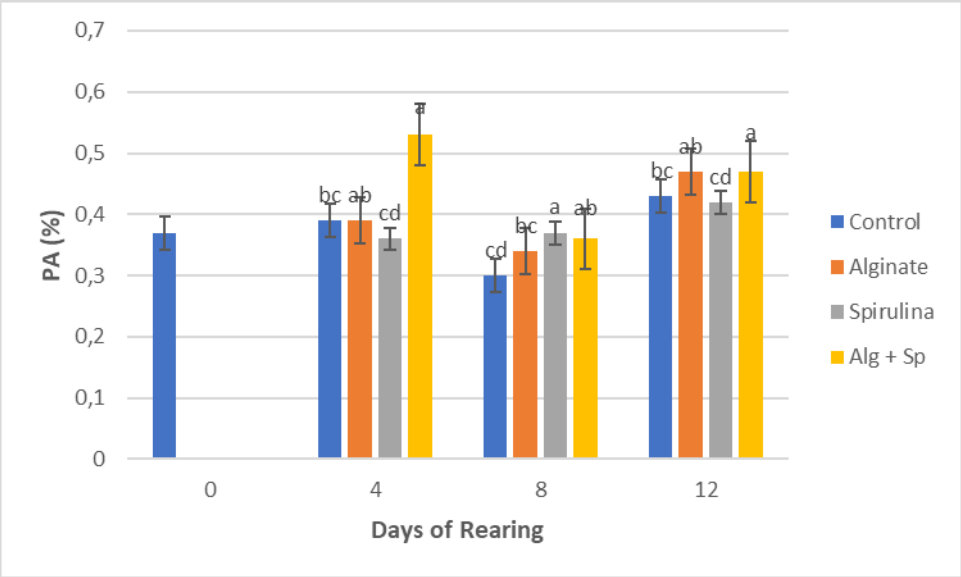

Fig. 2. PA of *L. vannamei* with different feeding regimes on 0, 4, 8, and 12 rearing days. Significant differences are indicated by different letters **(***P*< 0.05**)**

Based on the observational data of PA at 0 to 12 days of rearing, it shows that the control or without the addition of alginate and/or spirulina extract displays the lowest average results along with the rearing time, which is 0.37 x 10^6 mL/cell. The largest increase in the average of PA occurred in the alginate 3g+spirulina 5 mg/kg treatment, which is 0.47 x 10^6 mL/cell.

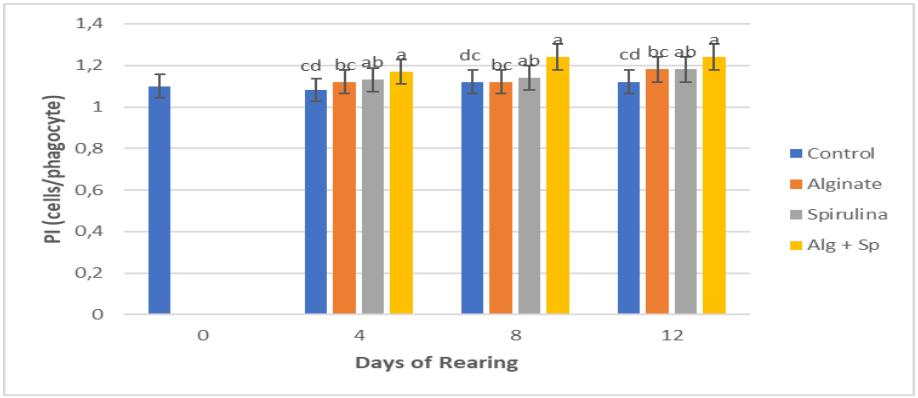

Fig. 3. PI of *L. vannamei* with different feeding regimes on 0, 4, 8, and 12 rearing days. Significant differences are indicated by different letters **(***P*< 0.05**).**

Based on the examination of the PI carried out during shrimp rearing for 0 days to 12 days, it was observed that the control or the experiment without the addition of alginate and/or spirulina extract exhibited the lowest average yield along with the rearing time, which was 0.44 x 10^6 mL/cell. The largest increase in the average phagocytic index occurred in the alginate 3g+spirulina 5 mg/kg treatment **(**1.21 x 10^6 mL/cell**)**.

2. Effect of alginate and/or spirulina extract supplementation on shrimps' PO and SOD activities

The results of alginate and/or spirulina supplementation in *L. vannamei* are presented in Fig. (4) showing the PO activity, while Fig. (5) displays the SOD activity in 12 days of rearing.

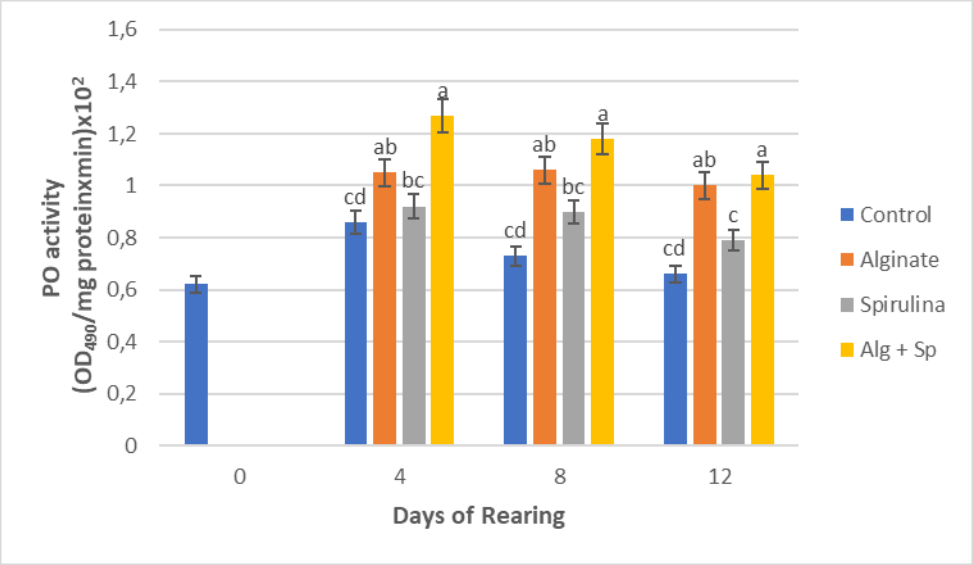

Fig. 4. PO activity of *L. vannamei* with different feeding regimes on 0, 4, 8, and 12 rearing days. Significant differences are indicated by different letters **(***P*< 0.05**).**

Based on the results, it is cristal clear that the control treatment demonstrates the lowest activity during the 12 rearing days. The highest activity of PO occurred in the alginate 3g/kg + spirulina 5mg/kg treatment on the 4th day of rearing. The decrease in PO activity occurred in the alginate 3g/kg + spirulina 5mg/kg treatment and spirulina 5mg/kg treatment at 12th rearing day. PO activity in treated shrimp was higher than the control and this value continued to increase from day 0 to day 12.

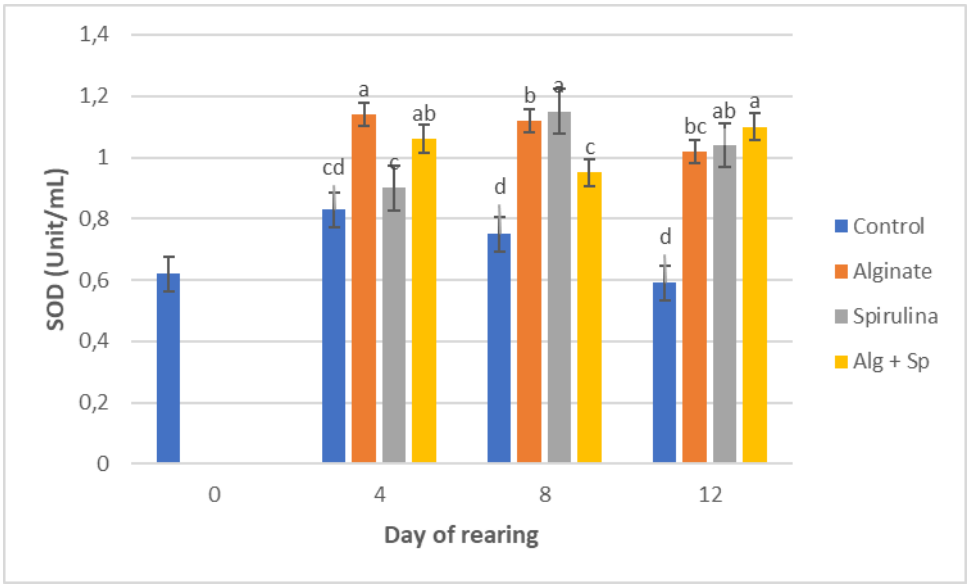

Fig. 5. SOD activity of *L. vannamei* with different feeding regimes on 0, 4, 8, and 12 days of rearing. Significant differences are indicated by different letters **(***P*< 0.05**)**.

Based on the results obtained from the 12 days of rearing period, it was apparent that the treatment without the addition of alginate and/or spirulina extract showed the lowest yield of SOD activity **(**Fig. 5**)**. The highest increase in SOD activity was in the 5mg/ kg spirulina treatment on the 8th day of rearing. The 3g/kg alginate treatment increased on the 4th day, then showed a decrease in succession. In addition, the treatment of alginate 3g/kg + spirulina 5mg/kg increased the SOD value on the 12th day of rearing.

DISCUSSION

The THC increment exhibits the response of the immune system in shrimp. The immune response as the body's defense system is correlated to the total number of hemocytes. The increase in the total number of hemocytes is related to the positive response in the shrimp body **(Famelia *et al.*, 2013; Yudiati *et al.*, 2019)**. The increase in THC showed that the administration of alginate and spirulina extract was able to stimulate shrimp's innate immune system. According to **Johansson *et al.* (2000)**, shrimp hematocytes play an important role in the innate immune response through recognition, phagocytosis, and communication between cells.

Phagocytic activity is one indication of the immune system to recognize, control, and eliminate foreign particles. The defense process through phagocytosis is divided into several processes, namely: chemotaxis, recognition, and internalization **(Bachère *et al.*, 1995)**. According to **Smith and Sullivan (2003)**, hemocytes perform inflammatory-type reactions such as phagocytosis, hemocyte clumping, production of oxygen-reactive metabolites, and release of microbicidal proteins. Increased body defense can be seen by

increasing the activity of phagocytic cells and hemocytes. This phagocytic cell function is specified in carrying out the phagocytosis of foreign objects that enter the shrimp body. Phagocytosis is a non-specific defense mechanism to protect against disease. Hemocytes are known as a very important factor in the non-specific cellular defense system. To find out that hemocytes are a cellular defense of the body, their potential can be detected through the phagocytic activity which increases during infection **(Setyawan *et al.*, 2021)**.

Spirulina has the main component of the cell wall, which contains peptidoglycan and lipopolysaccharide **(Sze, 1993)**. **Alifuddin (2002)** reported that 1,3-glucan and lipopolysaccharide **(**LPS**)** can stimulate cellular defense response activity in terms of activating phagocytic, melanization, encapsulation, nodulation, and coagulation activities. The data from the observation of the PI demonstrated that there was a difference between the control and the treatment. The value of the PI in the treated shrimp was higher than the control, and this value continued to increase from day 0 to 12 **(**Fig. 3**)**. Cells that play a major role in this phagocytic process are hyaline cells. Administration of sodium alginate extract increases resistance to infectious diseases, not because of an increase in specific immune responses, but by increasing non-specific defense mechanism **(Sakai, 1999)** or in other words alginate can stimulate the *L. vannamei* resistance system. Initially, when bacteria enter the shrimp's body, it will be immediately recognized by the plasma and bound. The semi-granular and granular cells then respond by releasing an activated proPO system, which includes the cell-adhesive and opsonic protein, peroxectin. Furthermore, it can stimulate phagocytosis by hyaline cells or encapsulation by semi-granular cells **(Söderhäll & Cerenius, 1998)**. This shows that alginate and spirulina extract have an effect on the phagocytic index of *L. vannamei*. Phagocytosis and its index show how much the shrimp's immune system reacts to microorganisms that infect its body. A high phagocytic index value illustrates that the organism has the ability to produce more phagocytic cells in hemocytes, so that upon the exposure to pathogenic microorganisms, hemocyte cells are ready to perform phagocytosis **(Widanarni *et al.*, 2016)**.

Alginate is rich in polysaccharides, derived from mannuronic and guluronic acid **(Yudiati & Isnansetyo, 2017)**. This raise of PO activity is due to stimulation by lipopolysaccharides that activate proPO **(Ramadhani *et al.*, 2017)**. Meanwhile, in the control treatment, without the addition of supplementation, the PO activity value continued to decrease successively. Eventually, this response will result in the death of shrimp, this is because pathogens will easily multiply and damage shrimp continuously due to the weak shrimp immune response **(Lesmanawati *et al.*, 2017)**. Alginate and/or spirulina supplementation showed an increase in PO activity. The polysaccharides in alginate have the ability to stimulate phagocytosis to kill microbes **(Bi *et al.*, 2017)**. Meanwhile, spirulina containing lipopolysaccharide **(**LPS**)** is able to increase shrimp

immunity by stimulating hemocyte cells against pathogens **(Lin *et al.*, 2010)**. Components of polysaccharides and lipopolysaccharides **(**LPS**)** in supplementation can activate prophenoloxidase activating enzyme **(**PPA**)** to form PO.

The value of PO activity in alginate 3g/kg and spirulina 5mg/kg experienced an unstable increase. On the 12th day of rearing, the PO activity of alginate 3g/kg + spirulina 5mg/kg treatment also decreased successively. However, the decrease in PO activity does not necessarily have a negative impact on the shrimp. **Widanarni *et al.* (2016)** reported that the decrease in PO activity was as a part of the adaptation of the shrimp immune system to fight pathogens. Decrease in PO value is a form of adaptation of shrimp in addition of symbiotics as supplementation or immunostimulants.

The treatment with alginate and/or spirulina supplements demonstrate a difference and an increase in SOD activity compared to the control treatment. This response is part of hemocytes activity that produce reactive oxygen species **(**ROS**)**. This is influenced by the presence of important content in Spirulina supplementation given in the treatment of this study. These protein, phenolic compound, phycocyanin form the spirulina water extract **(Hidayati *et al.*, 2020; Yudiati *et al.*, 2021)** are able to produce enzymes with SOD activity as free radical traps to minimize tissue damage in shrimp **(Kilawati *et al.*, 2021)**. Alginate and spirulina has been reported to increase bioactivity including immunity in fish and crustaceans, as well as exhibiting an antioxidant activity **(Yudiati *et al.*, 2018b)**. Phenolic compounds from alginate and spirulina water extract have a high antioxidant activity in addition to displaying the activities to reduce free radicals with hydroxyl groups on phenol compounds. In this regard, these compounds can assist antioxidant activity in inhibiting and preventing the oxidation reactions made by free radicals **(Yudiati *et al.*, 2018a)**. The use of alginate at 2g/kg as a supplement in *L. vannamei* showed an increase in SOD activity. This is indicated by the production of superoxide anions that are not excessive, so that it can trigger the increase in SOD activity **(Yudiati *et al.*, 2019)**. Furthermore, spirulina can increase glutathione S-transferase **(**GST**)** which has an antioxidant activity to induce the SOD system. As this happens, the SOD enzyme will slow down the performance of oxygen which produces radical reactions **(Tayag *et al.*, 2010)**. The content of α-tocopherol in spirulina has also been reported to protect cell membranes and organelles of the vannamei shrimp from damage that can be caused by free radicals **(Radhakrishnan *et al.*, 2014)**.

The formation of the SOD enzyme is influenced by the attack of pathogens or stressors that interfere with shrimp immunity. The infection will stimulate the formation of reactive oxygen species **(**ROS**)**. Furthermore, ROS will balance antioxidants to fight pathogens and protect cells in shrimp **(Gómez-Anduro *et al.*, 2012)**. SOD is an antioxidant enzyme for the degradation of intracellular free radicals. Moreover, the

decrease in SOD activity in alginate and/or spirulina supplements on day 14 of rearing is the reaction of the shrimp immune system against pathogens via the ROS synthesis process. This was explained by **Ramadhani *et al.* (2017)** who postulated that the decreased SOD value due to the administration of symbiotics was part of the response of shrimp that were neither infected nor under stress.

CONCLUSION

Alginate supplementation and/or spirulina extract stimulate the *L. vannamei* innate immune system effectively. This combined formulation of alginate and spirulina extract managed to improve THC and PA/PI non-specific immunity, as well as PO and SOD activities.

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