Supplementation of Carrageenan (*Kappaphycus alvarezii*) for Shrimp Diet to Improve Immune Response and Gene Expression of White Shrimp (*Litopenaeus vannamei*)

Ichoun Brawici Dhewang[1], Ervia Yudiati*[1], Subagiyo[1], Rabia Alghazeer[2]

[1]*Department of Marine Sciences, Faculty of Fisheries and Marine Sciences, Universitas Diponegoro*
Jl. Jacub Rais, Tembalang Semarang, Jawa Tengah 50275, Indonesia
[2]*Department of Chemistry, Faculty of Sciences, University of Tripoli*
Tariq Sayyidi al Misri Road, Tripoli, Libya
Email: eyudiati@gmail.com

Abstract

White shrimp (Litopenaeus vannamei) is one of the leading commodities in aquaculture. In recent years, the shrimp farming industry worldwide has suffered heavy losses due to disease. Increasing the immune system in shrimp using immunostimulants that are more environmentally friendly such as carrageenan from Kappaphycus alvarezii, seems promising. The purpose of this study was to determine the increase in immune response and gene expression in White shrimp after carrageenan supplementary diet treatment. This research was using a laboratory-scale experimental method with a factorial Completely Randomized Design (CRD) which was analyzed using One Way ANOVA. The treatments were negative control (without carrageenan addition), 5 g.kg^{-1}, 10 g.kg^{-1}, 15 g.kg^{-1}, and 20 g.kg^{-1} carrageenan supplemented feed. All treatments were replicated three times. 180 L. vannamei with average weight of 6.5±0.66 g is used for research object Total Haemocyte count (THC), Phenoloxidase (PO), Superoxide Dismutase (SOD) activity, and Phagocytic activity/Index (PA/PI) were examined after feeding the White shrimp with diets supplemented with carrageenan in time series sampling. Immune-related gene expression (Lipopolisaccharide Glucan Binding Protein/ LGBP, Pro Pehnol Oxidase/ ProPO, and Lectin Type C/ Lectin) was evaluated by quantitative Real-Time PCR (qRT-PCR) at the end of experiment. Results indicated that the immune parameters directly increased according to the doses of carrageenan and time. The 20 g.kg^{-1} carrageenan treatments gave better results. three immune-related genes expression i.e LGBP, Lectin, and proPO were upregulated. Therefore, carrageenan supplementation of shrimp feed can improve innate immunity as well as the expression of immune-related genes.

***Keywords**: carrageenan supplementary diet, Kappaphycus alvarezii, Immune Response, Gene Expression*

Introduction

White shrimp (*Litopenaeus vannamei*) is a leading and popular commodity in aquaculture. White shrimp cultivation has experienced rapid development in almost all parts of the world (Su *et al*., 2020) including in the Asian region (Wang *et al*., 2020). The white shrimp farming industry is one of the most dynamic aquaculture activities and a fast-growing food production system, especially in developing countries, and also has the potential for sustainable production (Vijayan, 2019).

Data shows that in recent years, the world's shrimp farming industry has experienced an increase in production (FAO, 2020), on the other hand, shrimp farming worldwide has also experienced huge losses caused by disease (Ngo *et al*., 2020). Several diseases that often attack shrimp include Acute Hepatopancreatic Necrosis Disease (AHPND), White Spot Syndrome Virus (WSSV), Infectious Myonecrosis Virus (IMNV), and Taura Syndrome Virus (TSV) which is capable of causing the death of shrimp reaching 80-100% (Walker and House, 2004; Oseko, 2006; Thitamadee *et al*., 2016) besides that the death of shrimp can also be caused by environmental pollutants, namely heavy metals copper (Cu) which are usually used as algaecides in the cultivation process (Qian *et al*., 2020).

The handling of disease outbreaks in aquaculture businesses has focused on the use of chemical therapy in cultivation media and antibiotics (Chen *et al*., 2020). As a consequence, the presence of chemical residues in aquaculture waters can also reduce the quality of the surrounding environment. Meanwhile, the use of antibiotics that is carried out continuously will pose a risk of increasing bacterial resistance to antibiotics within a certain period, so the use of antibiotic doses will always increase from time to time (Chaturvedi *et al*., 2021).

*) Corresponding author
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Shrimp is a group of invertebrates that are known to not have a perfect specific defense system so disease control through vaccine development cannot be carried out. Shrimp rely on the non-specific immune system (innate immunity) in dealing with various pathogen attacks in their environment (Martinez, 2003; Vazquez *et al*., 2009; Nie *et al*., 2020). One alternative that can be done is to increase the immune system in shrimp using immunostimulants which are considered more environmentally friendly (Bricknell and Dalmo 2005; Alday and Smith 2008; Govind *et al*. 2012; Yudiati *et al*., 2019; Lee *et al*., 2020; Azhar and Yudiati, 2023).

Immunostimulants are substances (drugs or nutrients) that can increase the ability of the immune system to fight infection and disease, by stimulating natural and adaptive defense mechanisms thereby increasing the activity of components of the immune system (Patil *et al*., 2012; Venkatalakshmi *et al.*, 2016). Immunostimulants can be divided into several groups based on their sources, namely bacteria, animal derivatives, immunostimulant nutritional factors, hormones/cytokinins, and algae derivatives, for example, products derived from seaweed (Sakai, 1999; Vijayaram, *et al*., 2022).

Seaweed, including *Kappaphycus alvarezii* can be used as an immunostimulant because it is a source of bioactive compounds. The cell walls of marine algae are rich in sulfated polysaccharides (SPs) such as red algae carrageenan and have many beneficial bioactive compounds as anti-coagulants, antivirals, antioxidants, anticancer and immune modulation activation (Azhar & Yudiati., 2023). Febriani & Nuryati., 2013; Wijesekara *et al*., 2011; Yudiati *et al*., 2019).

Carrageenan is a hydrocolloid compound extracted from red seaweed species, *Kappaphycus alvarezii*, *Chondrus crispus*, and *Eucheuma spinosum* (Azevedo *et al*., 2015; Bouanati *et al*., 2020). Carrageenan is an immunostimulant because it consists of sulfated linear gallstones in which α-(1,3)-galactose alternates with β-(1,4,3,6)-anhydrous-D-galactose (Yeh and Chen, 2008). Previous research the addition of carrageenan to the Shrimp diet ensured greater resistance to the White Spot Syndrome Virus (WSSV) without causing any production losses related to growth performance parameters or stress in the animals (Mariot *et al*., 2021)

Nusa Lembongan Island, Bali is one of the best producers of *Kappaphycus alvarezii* seaweed in Indonesia. Research on different treatments for carrageenan extraction from seaweed cultivation in Nusa Lembongan and the effect of the carrageenan-supplemented diet on the immune system and gene expression in *L. vannamei* farming is important but has never been done. The objective of this research is to determine the increase in cellular (Total Haemocyte Counts, Phagocytosis Activity, Phagocytosis Index), and humoral immune response (Phenol Oxidase and Superoxide Dismutase). The LGBP, Lectin, and proPO immune-related genes are also assessed quantitatively after carrageenan supplementary at 0, 5, 10, 15, and 20 $g.kg^{-1}$ in the diet by oral administration of *L. vannamei*.

Materials and Methods

This research was using a laboratory experimental method that examines all the independent variables which might have an effect, while the variables that are not relevant to the research problem are kept to a minimum. Experimental research can provide "reasons why" explanations. Causal relationships can be identified because research makes it possible to treat research objects (Thorson *et al*., 2012). This study used a completely randomized design (CRD) with three repetitions for five different treatments. The treatments were negative control (without carrageenan addition), 5 $g.kg^{-1}$ carrageenan supplemented feed (carrageenan 5g), 10 $g.kg^{-1}$ carrageenan supplemented feed (carrageenan 10g), 15 $g.kg^{-1}$ carrageenan supplemented feed (carrageenan 15g), and 20 $g.kg^{-1}$ carrageenan supplemented feed (carrageenan 20g).

Production of carrageenan supplemented feed

Prior to the extraction, dry *Kappaphycus alvarezii* from Nusa Lembongan, Bali was cleaned up with tap water to remove debris and epiphytes. The production of the carrageenan-supplemented feed was carried out by Febriani and Nuryati's (2013) method. The 20 g dried seaweed was cut into small pieces (± 1 cm), then soaked in 0.15% NaOH solution. The sample was soaked and heated at 80ºC for 2 hours with a ratio of seaweed and water (1w:20 v) and then filtered. The filtrate was then mixed with 1.25% KCl for 30 min. The carrageenan precipitate wa collected and placed in the oven at 60-80ºC until dry (48 h). After finishing in the oven, the carrageenan is crushed into carrageenan flour.

Amounts of 5 g, 10 g, 15 g, and 20 g of carrageenan from NaOH extraction were diluted in 100 mL of water and then mixed well with 1 kg of commercial shrimp food for each weight treatment. The mixes were dried at room temperature for 48 hours. The carrageenan-supplemented shrimp foods were then stored in a closed container and placed in the refrigerator.

Preparation and rearing of shrimp

The number 180 *L. vannamei* with a weighted average is 6.5±0.66 g from Mangkang traditional pond is used as our research object. The shrimp cultivation media are 6 plastic tanks (volume: 140 L). There are 30 shrimp in every tank. The shrimps were acclimatized for a week before being given feed treatment. The treatment tanks were siphoned routinely to keep the water quality. The feeding times were administered at 06.00 a.m., 11.00 a.m., 04.00 p.m., and 09.00 p.m. The amount of the feeds is 4% of the total shrimp biomass for every treatment (Yudiati *et al*., 2016).

Haemolymph sampling

The shrimp's haemolymph sampling was carried out by Yudiati *et al*. (2016) method. An amount of 0.5 ml of haemolymph was collected from the pleopod base of the first abdominal segment near the genital pore from each three shrimp are used as a repetition for observation. The haemolymph was collected using a 1-ml sterile syringe fitted with a 25-gauge needle. Before this, the syringe was coated with an anticoagulant solution (10% sodium citrate). The haemolymph samples were then saved in a cool box to avoid damage.

Total Haemocyte Count (THC)

The THC observations were done every five days, starting from before treatment (T0). The amount of haemocyte was counted using the THC method by Córdova-Campa *et al*. (2002). Calculation of THC used in hemolymph samples with dilution was carried out by adding 60 µl PBS to 20 µl hemolymph. Haemocyte was counted with a haemocytometer under a light microscope (Olympus, Japan) with 100X magnification. The haemocyte total was then counted using Córdova-Campa *et al*. (2002) formula:

$$THC = \frac{total\ haemocyte}{25}\ x\ 250\ x\ dilution\ x\ 10^3\ sel.ml^{-1}$$

Phagocytosis Activity (PA) and Phagocytosis Index (PI)

The Phagocytosis Activity (PA) and Phagocytosis Index (PI) assessment methods were following Chotigeat *et al*. (2004). 20 µl of haemolymph stock was mixed with 20 µl formalin killed *Bacillus subtilis* at 10^8 densities. The mixture was dripped on an object glass and then fixed with 2.5% glutaraldehyde for 5 minutes. NaCl 0.855% was then added to remove the cells that didn't stick to the object's glass. The samples were stained with 10% Giemsa for 20 minutes. The Phagocytosis Activities were counted with Chotigeat *et al*. (2004) formulas:

$$AF\ (\%) = \frac{total\ active\ cells}{total\ observated\ cells}\ x\ 100\ \%$$

$$IF = \frac{total\ eaten\ cells}{total\ active\ cells}$$

Prophenoloxidase

Phenoloxidase (PO) activity was measured by observing dopachrome formation produced from L-*dihydroxyphenylalanine* (L-DOPA). 100 µl Phosphate Buffer Saline (PBS) was added to the 100 µl haemolymph sample. The mixture was homogenized using a vortex and then centrifuged at 700 g, 4°C for 20 minutes. The pellet was added with 100 µl cacodylate buffer (0,01 M sodium cacodylate; 0,45 M sodium chloride; 0,01 M calcium chloride; 0,26 M magnesium chloride; pH 7). The mixture was then centrifuged again to get the new pellet. The pellet was added with 100 µl cacodylate buffer and 100 µl trypsin, homogenized, suspended, and incubated for 10 minutes. 50 µl L-DOPA was added to the cacodylate buffer and trypsin mixture. The PO activity was measured using an ELISA Reader (Thermo Scientific/Multiskan FC, USA) at 490 nm absorbance (Yudiati *et al.,* 2019).

Super Oxidase Dismutase (SOD)

The Super Oxidase Dismutase (SOD) activity was measured by observing the riboflavin formation produced by Nitroblue Tetrazolium (NBT). The amount of 360 µl Phosphate Buffer Saline (PBS) was added to a 40 µl haemolymph sample. The mixture was homogenized using a vortex, then centrifuged at 6.000 g, 4°C for 7 min. The supernatant was heated at 65°C for 5 min. 50 µl NBT was then added to the supernatant. The mixture was then measured using ELISA Reader (Thermo Scientific/Multiskan FC, USA) at 630 nm absorbance to know the SOD activity (Yudiati *et al.,* 2019).

RNA extraction

The expression of immune-related genes in shrimp was carried out by Yudiati *et al*. (2016) method. Haemolymph samples were extracted using a High Pure Viral- mRNA Extraction Kit (Roche, Germany) on the procedure by the manufacturer to get the total RNA. The total RNA was then synthesized using AMV Reverse Transcriptase Kit (Promega Corp) at 42 C from 30 min to get 5 min.cDNA, followed by transcriptase enzyme inactivated at 94 C for 5 min. the expression of LGBP, Lectin and Toll were analyzed with Sybr Green (Kapa biosystems) with 7500 Fast Real-Time PCR (applied Bio-system) using the specific primers (Tabel 1.). The qPCR condition was pre-denaturation (94 C for 7 min), denaturation (95°C for 30 s, 45 cycles), annealing (60°C for 30 s, 45 cycles),

extension (68°C for 50 s, 45 cycles) and melting curve at 95°C after 30 min (Yudiati *et al*., 2016). The amplified recorded data was analyzed by comparative methods by Livak and Schmittgen (2001) which be normalized using b-actin (primer Lvbac-F and Lvbac-R) as an internal control to determine the gene expression level.

Data analysis

One Way ANOVA was used to compare the effect of carrageenan supplementation with several doses on increasing the immune system in white shrimp. Duncan's test was used to test comparisons between pairs of carrageenan dose treatments and to determine the significance between dose treatments. Data normalization and homogeneity of variance were checked before ANOVA analysis. If the data is not normal, Arcsine is used to transform data. ANOVA was performed with a significance level of 5% ($p< 0.05$) (Kim, 2017). One Way ANOVA statistical analysis was performed using SPSS computer software version `16.

Results and Discussion

Total Haemocyte Count (THC)

The Total Haemocyte Count (THC) test results of 15 days of rearing with five different concentrations of supplementation carrageenan in shrimp are shown in Figure 1. The fifth days (T1) observation indicated ($p>0.05$) there was no significant difference between treatments ($p>0.05$). This is caused by the process of adaptation of white shrimp to a new environment so the administration of carrageenan as an immunostimulant has not had much impact on increasing the immune response of shrimp. Similar results also reported by Yudiati *et al*. (2016); Azhar and Yudiati (2023). Immunostimulant compounds in carrageenan can stimulate the production of THC in shrimp after it has accumulated. This is reinforced by research data of Yeh and Chan (2008); Chen *et al*. (2014) showing that feeding with carrageenan supplementation stimulates the increase of THC in shrimp.

Carrageenan-supplemented feed on day 10 (T2) and day 15 (T3) showed a significant difference (P=0.016) between each treatment and the control. Based on the shrimp THC data during observations, it was shown that the longer treatment with carrageenan-supplemented feed gave higher THC values. After feeding with carrageenan supplemented, there was an increase in the number of hemocytes in white shrimp, compared to shrimp that were not given carrageenan-supplemented feed (control treatment). The increase in hemocytes occurred from the tenth day (T2) until the end of the study, namely the fifteenth day (T3). This increase in the number of shrimp hemocytes indicates that carrageenan extract can increase the immune response in shrimp. This indicates that carrageenan-supplemented feed can stimulate the formation of hemocyte cells which are then released into the hemolymph of shrimp to help maintain resistance to the pathogen (Febrian *et al*., 2013).

Treatment with a dose of 20 $g.kg^{-1}$carrageenan during observation showed a higher THC increase compared to the treatment of 5 $g.kg^{-1}$, 10 $g.kg^{-1}$, and 15 $g.kg^{-1}$. This indicates that the administration of carrageenan as an immunostimulant can stimulate the formation of haemocyte cells. This increase in total haemocytes indicates that there is an increase in the body's defence reaction due to the presence of foreign particles that enter the shrimp's body. Foreign particles that enter the shrimp body will be recognized by haemocyte cell receptors to produce cellular responses such as intercellular signaling cascade,

Table 1. qRT-PCR primers of three immune-related genes of *Litopenaeus vannamei* used in this study

Gane	Primer	sequence (5'-3')	Accession number	Reference
Lectin	Lectin V-F	TTTGTAAACAACAGGCAGTTCCAC	EF583939.1	(Zhang *et al*., 2009)
	Lectin V-R	CTGTCTTTCATCAGAATGCTACCTC		
proPo	proPO-F	TTCAACGGTAGACCCGTGATTCTTC	AY723296.1	(Wang *et al*., 2007)
	proPO-R	TCTTGCCGGGTTTAAGGTGAACAGT		
LGBP	Liva LGBP qPCR F	CGG CAA CCA GTA CGG AGG ACC		(Cheng *et al*., 2005)
	Liva LGBP qPCR R	GTG GAA ATC ATC GGC GAA GGA G		
β-actin (internal control)	Lvbac-F	CCTCCACCATGAAGATCAAGATCAT		(Sun *et al*., 2007)
	Lvbac-R	CACTTCCTGTGAACAA TTGATGGTC	AF300705.2	

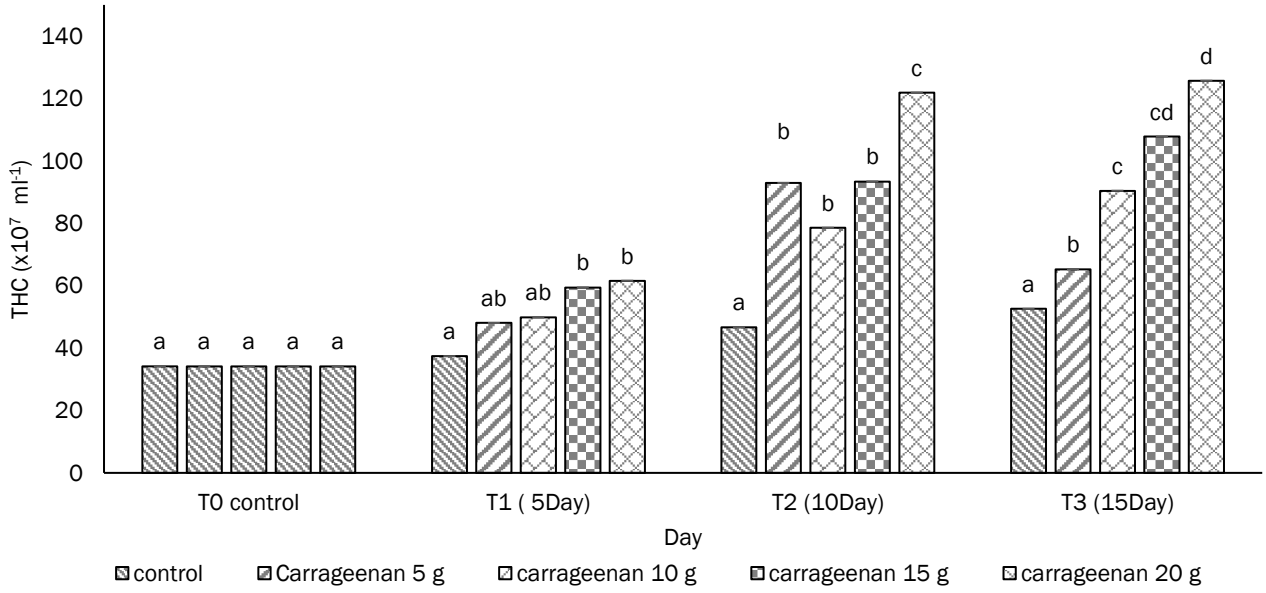

Figure 1. Total Haemocyte Count (THC) in shrimp after being given carrageenan-supplemented feed for 15 days with each observation distance of 5 days.

phagocytosis, encapsulation, and nodular aggregation (Suleman *et al*., 2019; Rodriguez and Moullac, 2000).

Hemocytes in shrimp are as important as red and white blood cells in fish. The importance of total hemocyte count (THC) in crustaceans in pathogen resistance, if there is a decrease in THC, an acute infection can occur in crustacean biota and can cause high mortality rates in *L. vannamei* culture (Novriadi *et al*., 2021). Increased THC will increase the ability of crustaceans to phagocytize pathogens because many hemocyte cells are produced to perform these functions, such as granular and semi-granular cells. The increase in THC also increases the power of granular cells to carry out phenoloxidase activity so that shrimp can survive the pathogen's attack. Shrimp haemocytes have an important role in the immune response through recognition, phagocytosis, melanization, cytotoxicity and communication between cells (Ridlo and Pramesti, 2009). So that one parameter of the ability of a substance or compound to stimulate the nonspecific defense system of shrimp is an increase in the number of hemocytes (Azhar and Yudiati, 2023). The results of the study of THC in vannamei shrimp showed that feeding supplemented with carrageenan as an immunostimulant could stimulate an increase in THC in *L. vannamei*.

Phagocytic Activity (PA) and Phagocytic Index (PI)

PA is the ability of non-specific immune cell responses in shrimp to ingest (phagocytosis) disease agents that enter the shrimp's body (Suleman *et al*., 2019) while PI is the number of disease agents ingested (phagocytosis) by 100 macrophages (Ren *et al.,*2003). The PA value from the treatment of several concentrations of carrageenan supplementation in shrimp can be seen in Figure 2.

The results of the observation of phagocytic activity (PA) in this study showed that on the fifth day (T1) and tenth day (T2) the values were not significantly different (P=0.021) between treatments. The fluctuations of different data in terms of days of rearing was vary. Yudiati *et al*. (2016; 2019) reported that the best cellular compound with alginate reached at 15th day. Other researcher, Setyawan *et al*. (2018) denoted that the peak cellular compounds reached at 4th day. Therefore, this needs to be explored. While the results of observation on the fifteenth day (T3) showed different results. (T3) had a significantly different response value of phagocytic activity between control and carrageenan supplemented feed treatment. Based on the data on the phagocytic activity of *L. vannamei* during observations, it was shown that the treatment with carrageenan-supplemented feed with a higher dose of carrageenan concentration could increase the higher PA value. In addition, feeding with carrageenan supplemented for a long time can provide a high PA value. Several studies have stated that the function of the polysaccharide structure in immunomodulation activity can stimulate respiratory bursts in the process of phagocytosis, a process that plays an important role in killing microbes (Castro *et al*., 2006; Chan *et al*., 2014; Yudiati *et al*., 2016).

Observation of phagocytic index (PI) (Fig. 3) up to day 5 showed a significant difference (p=0.023) which indicates that the provision of carrageenan in feed can increase the index of phagocytic cells to assist shrimp in stimulating the immune system in shrimp. A high phagocytic index value illustrates that the organism has the ability to produce more phagocytic cells in haemocytes so that when exposure to pathogenic microorganisms occurs in shrimp, hemocyte cells are ready to carry out phagocytosis (Widanarni *et al*., 2016). Whereas based on shrimp PI data during the study showed that carrageenan supplemented feed treatment on day 10 to day 15 showed a decrease in the production of phagocytic cells in shrimp (*P*=0.404). According to Schmidt *et al*. (1993) and Castro *et al*. (2006) certain types of carrageenan seem to damage macrophage function, this could be an indication of the cause of the decrease in PI from day 10 to day 15 in this study.

Phenol oxidase (PO) enzyme activity

Phenol Oxidase plays a role in the non-specific defence of invertebrates (one of which is *L. vannamei*) which plays an important role (Amparyup *et al*., 2013; Li and Xianng, 2013). The increase in the number of PO represents an increase in the immune system in shrimp (Yudiati *et al*., 2016). PO values obtained from shrimp blood that has been treated with carrageenan-supplemented feed with different concentrations can be seen in Figure 4.

There was an increase in the average value of Phenol Oxidase (PO) enzyme activity at T1 when compared to T0 (control), but there was a decrease in PO values from day 10 (T2) and day 15 (T3). At T1, the higher the treatment dose, the higher the PO value obtained, where the average value of the 15 g concentration treatment produces a higher PO value compared to other concentration treatments.

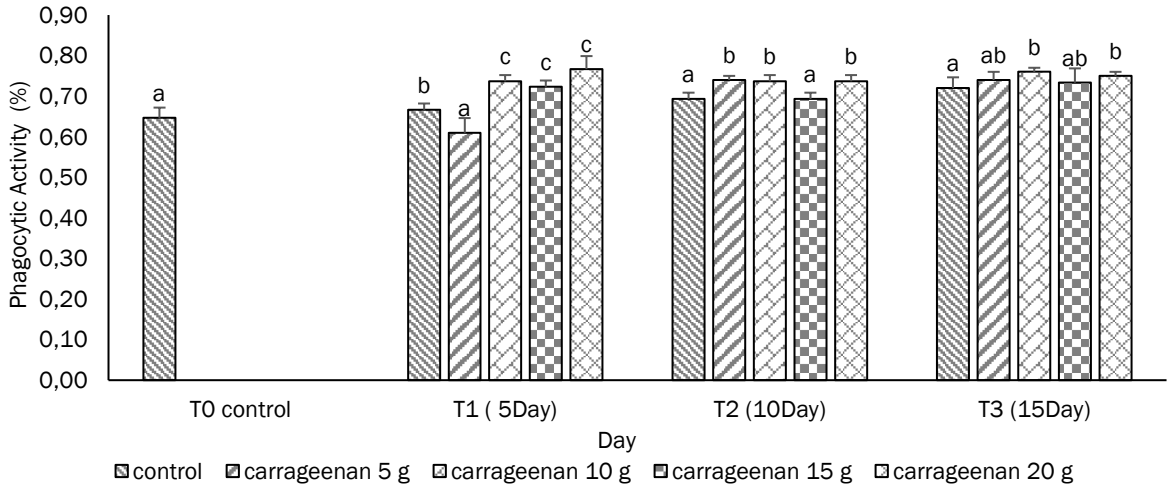

Figure 2. Phagocytosis activity in shrimp after being given carrageenan-supplemented feed for 15 days with each observation distance of 5 days.

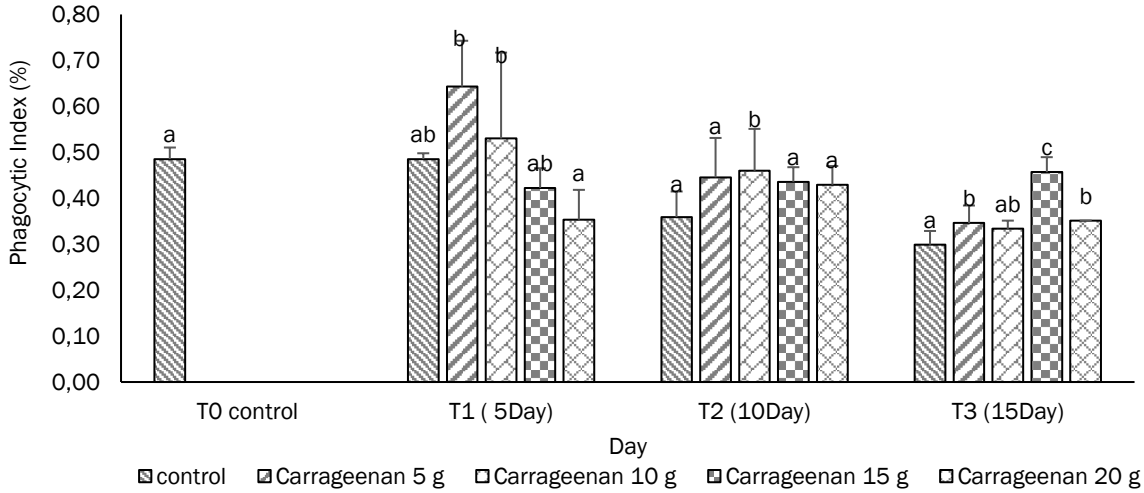

Figure 3. Phagocytosis Index in shrimp after being given carrageenan-supplemented feed for 15 days with each observation distance of 5 days.

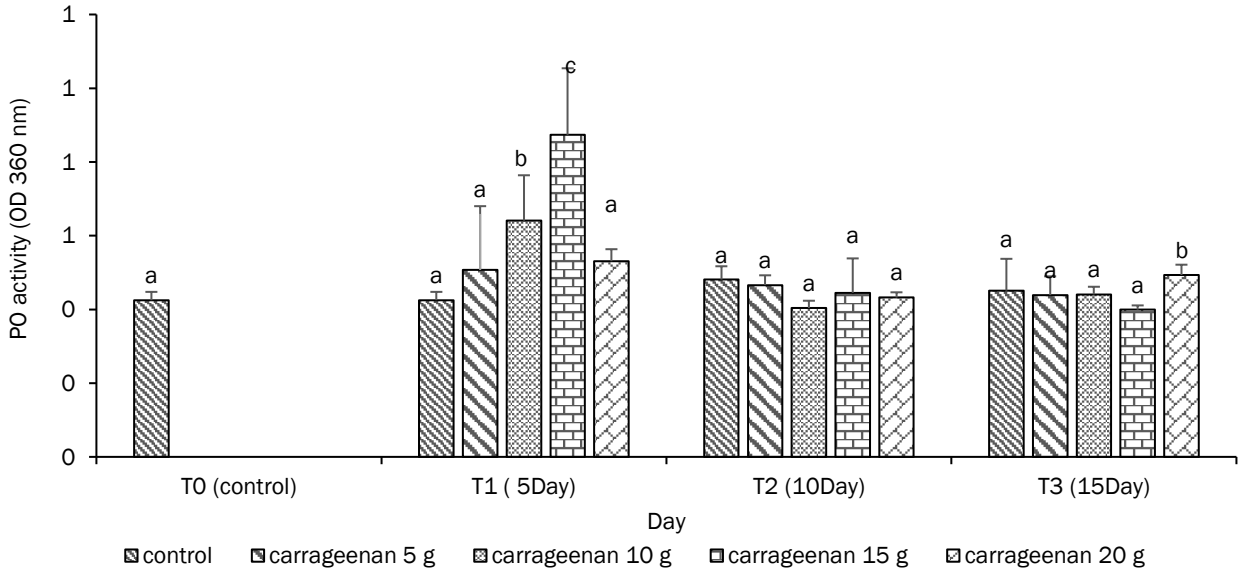

Figure 4. Phenol oxidase activity on shrimp after being given carrageenan-supplemented feed for 15 days with each observation distance of 5 days.

The similar facts were experienced by Febrian *et al*. (2013), a dose of 15 g.kg^{-1} carrageenan could significantly increase the PO value, but there was a difference where in that study the significant increase in PO lasted until the end of the study whereas this study only increased on day 5 (T1). This shows that on the fifth day of this study an increase in the activity of the phenol oxidase enzyme in shrimp after being given carrageenan extract. Supported by the results of a study by Chang *et al*. (2014) it was found that by giving carrageenan feed at a dose of 1g, 3g, and 5g, a dose of 5 g had a better value for increasing PO activity.

The enzyme phenol oxidase (PO) is present in the hemolymph as an inactive pro-enzyme called proPO. In crustaceans, proPO functions in foreign body recognition and mechanization. The transformation of proPO into PO involves several reactions known as the proPO activating system which activates beta-glucan, bacterial cell wall, and LPS (Amparyup *et al*., 2013). After binding, proPO will be activated to become a PO enzyme which will then carry out its function in the process of foreign body recognition and mechanization.

At 10th and 15th-day observation indicates a decrease in the activity of the phenol oxidase enzyme after feeding with carrageenan. The decrease in the activity of the phenol oxidase enzyme does not necessarily have a negative impact on shrimp, according to Widanarni, *et al*. (2016) the decrease in the activity of the phenol oxidase enzyme is an adaptation of the shrimp immune system to fight pathogens.

Superoxide Dismutase (SOD) Activity

Superoxide Dismutase (SOD) is an antioxidant enzyme that has a very strong function and is the body's defence against free radicals (Vasile *et al*., 2018). Antioxidant's function is to stabilize free radicals by complementing electron deficiencies and inhibiting the chain reaction of free radical formation. Increasing SOD in shrimp aims to reduce cellular superoxide explosion during defence against pathogenic infection and to protect shrimp cells from damage (Kilawati *et al*,.2021). The results of giving carrageenan-supplemented feed on SOD activity in Vannamei shrimp are shown in Figure 6.

Superoxide Dismutase (SOD) activity (Fig. 5) on the 5th-day carrageenan-supplemented feed observation showed a significantly different value (p=0.004). The significant value means there is increasing in SOD activity with carrageenan treatment. The 20 g.kg^{-1} supplementation showed a higher value than the control, 5g.kg^{-1} treatment, 10g.kg^{-1} treatment, and 15g.kg^{-1} treatment. This shows that at the beginning of the study, the increase in dose levels from the treatment was directly proportional to the increase in superoxide dismutase activity in *L. vannamei.*

Observations on the 10th and 15th days showed that the values were not significantly different (p=0,338), among treatments. This shows a decrease in SOD activity on day 10 and day 15 after feeding with carrageenan supplemented, which is directly proportional to the results of the analysis of the Phenol Oxidase Enzyme. The 20 g.kg^{-1} carrageenan

supplementation was the most effective in terms of increasing the activity of the enzyme superoxide dismutase in *L. vannamei*. The decrease in the amount of SOD after giving carrageenan-supplemented feed indicated that the shrimp did not experience infection or stress from being given carrageenan-supplemented feed (immunostimulants), this is supported by research conducted by Ramadhani (2017).

LGBP, Lectin, and proPO Gene Expression

Lectin, LGBP and ProPO genes which are related to shrimp immunity are important to observe. The results of observing the effect of feeding carrageenan supplemented with several different doses on the expression of the LGBP, lectin, and ProPO genes are shown in Figure 6.

Based on figure 6, it can be seen that gene expression on day 15 of giving carrageenan as an immunostimulant to white shrimp showed an increase in the activity of the LGBP, Lectin and ProPO genes. The increased activity of these three genes can be an indicator that giving carrageenan is effective. Increasing the activity of lectin genes will help shrimp in detecting pathogens which in turn provide signals that trigger the immune system against

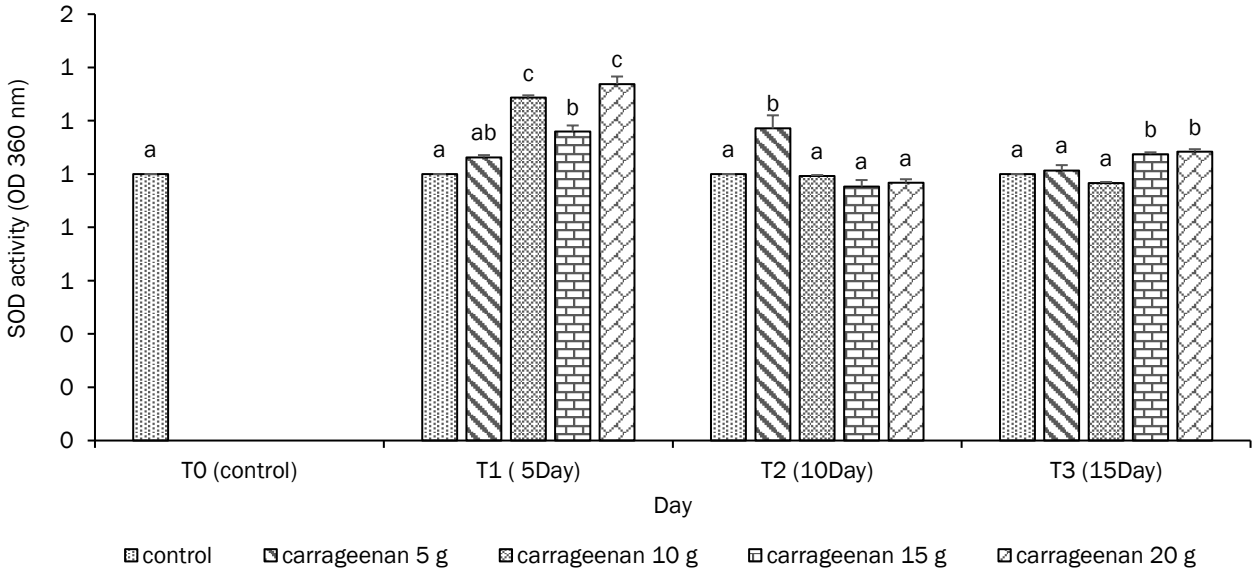

Figure 5. Superoxide Dismutase (SOD) Activity of shrimp after being given carrageenan-supplemented feed for 15 days with 5 days each observation distance.

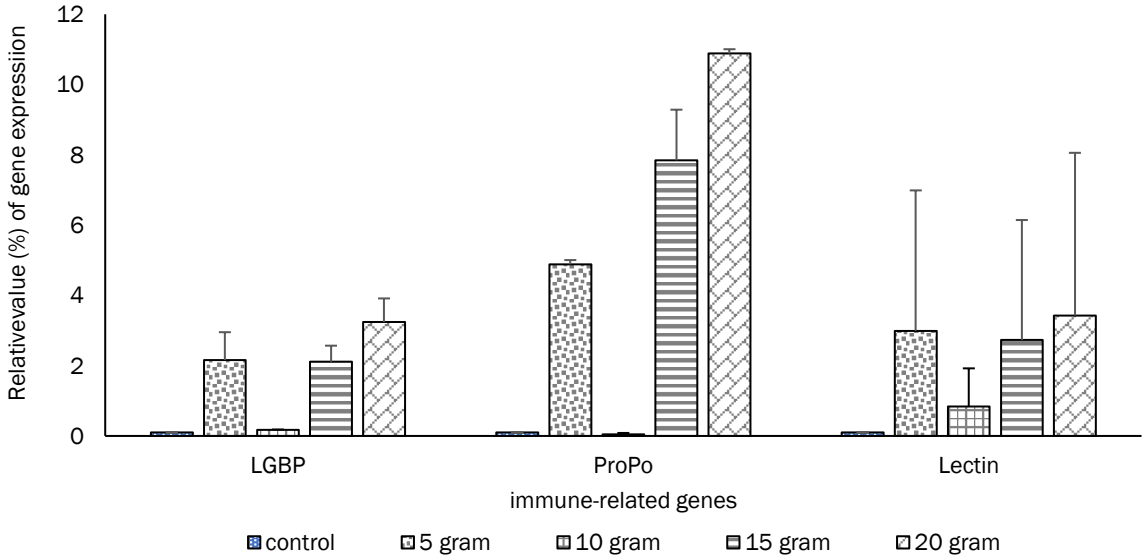

Figure 6. Relative Expression of LGBP, Lectin and proPO genes from *L. vannamei* after being given carrageenan-supplemented feed.

pathogens (Wang and Wang, 2013). Recognition of the special pattern belonging to PAMP (pathogen-associated molecular pattern) by PRR can activate ProPO to stimulate the process of melanization activation, cytotoxic reactions, encapsulation, cell adhesion, and phagocytosis in observed shrimp (Johansson and Soderhell, 1989; Amparyup *et al*., 2013). The expression of this immune related genes is in line with the results from the observations of THC, PA, PI and PO.

LGBP and Lectins genes are known to have an important role in non-specific defence systems in invertebrates based on biochemical and molecular analysis studies on several species of crustaceans, one of which is white shrimp. Based on research by Figueroa *et al*. (2014), LGBP gene taken from the hepatopancreas *L. vannamei* and challenged with Necrotizing Hepatopancreatitis (NHP) bacteria experienced an increase in regulation on the 6th day, after infection and a decrease in regulation after the 24th day. This shows that LGBP plays a role in the pathogen recognition system which in turn provides certain signals that trigger an immune response against pathogens. Whereas C-type lectin is a PRR that is found in almost all metazoans and its main role depends on the carbohydrate recognition site. Members of the C-type lectin family are present in abundance in shrimp and have diverse biological functions in the nonspecific immune system including phagocytosis, melanization, respiratory burst, agglutination, antibacterial, and antiviral responses (Wang and Wang, 2013).

Conclusion

The carrageenan supplementation in feed significantly increases the shrimp immune system with an indicator of increasing the amount of THC at the most effective dose of 20 $g.kg^{-1}$. An increase also occurred in phagocytosis activity with an effective dose of 20 $g.kg^{-1}$and an increase in the phagocytosis index with an effective dose of 5 $g.kg^{-1}$. The increment of phenoloxidase (PO) enzyme activity and superoxide dismutase (SOD) activity is reached at doses of 15 $g.kg^{-1}$for PO and 20 $g.kg^{-1}$ for SOD which had better effectiveness. Meanwhile, based on gene expression, it was concluded the transcript RNA was upregulated, and effectively in increasing the activity of the lectin, LGBP and proPO genes at a dose of 20$g.kg^{-1}$. Carrageenan supplementation of shrimp feed can improve innate immunity as well as the expression of immune-related genes in the 15 days of feeding treatment.

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