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Immunostimulant from Marine Algae to Increase Performance of Vanamei Shrimp (*Littopenaeus vannamei*)

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Abstract

Aquatic environment such as contaminated waters can cause immune deficiency of aquatic organisms such as White Spot Syndrome Virus (WSSV) viral disease resulted in mass death of white shrimp. It is necessary to develop preventive activities by means of immunostimulant administration to improve the vanamei shrimp immune system using marine algae. The aims of this research are to analyse the effect of immunostimulant of marine algae extract on non-specific immune response of vanamei shrimp in terms of total haemocytes cell, haemocytes cell differentiation, superoxide dismutase enzyme, and respiratory burst. Experimental method was used during the research. Immunostimulant was obtained from seaweed extract of *Sargassum polycystum, Padina australis, Eucheuma cottonii*, and *Gracillaria vrrucosa* against vanamei shrimp that challenged with *White Spot Syndrome Virus* (WSSV). Observed parameters were Total Haemocyte Count (THC), Differential Hemocyte Cell (DHC) include hyaline cell, granule cell and semi granule cell, *Superoxide dismutase* (SOD) activity, and *Respiratory Brust* (RB). The result showed that freeze dried marine algae extract has significant effect to increase the immune system of *L. vannamei* challenged with White Spot Syndrome Virus (WSSV). The best treatment is E (immunostimulant extract *Eucheuma cottonii*) and F (immunostimulant extract *Gracillaria verrucossa*) that causing positive affect on the parameter i.e., Total Haemocyte Count (THC), hyaline cells, granule cells and semi granule cells, Superoxyde dismutase (SOD) activity, and Respiratory Brust (RB).

Keywords: Freeze extract; Health organism; Seaweed; Marine drugs; Microbiology

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Introduction

White spot syndrome virus (WSSV) first appearing in the 1990s in Taiwan, has spread rapidly to shrimp-farming areas all over the world and has been reported in Indonesia [1-4]. WSSV remains one of the most harmful pathogens because of its high virulence, shrimp mortality can reach 100% within 2 to 10 days of infection, which causes great economic losses to the industry [5,6]. The WSSV virus attacks the body of shrimp which starts an intracytoplasmic attack and enters the host cell, then at a higher attack rate Deoxyribonucleic Acid (DNA) virus enter the host DNA and take over the process of transcription and translation according to the process in the DNA virus. In the transcription and translation of the WSSV gene, a non-structural protein called ICP11 protein is requested, most importantly it is suitable for WSSV infection [7]. Shrimp that loses WSSV will become weak, lose their appetite and usually swim to the edge of the embankment before finally dying. Morphological viruses protected by WSSV will oppose the characteristics of female infections in carapace sections with a diameter of 1.5-2.8 mm [8,9].

Efforts to overcome WSSV using chemicals can endanger the health of consumers because residues from the chemicals used will accumulate periodically in the body of the shrimp. So, it is necessary to use other alternatives in the prevention of this virus such as the use of natural ingredients as immunostimulants, to enhance the immune system of shrimp so that they are more resistant to disease [10,11]. One of the natural ingredients that can be used as immunostimulants is seaweed which is known to be quite potential by having a lot of bioactive content in it and could act as antiviral and antimicrobial activity [12-14]. High

antioxidant content in seaweed, making this species potentially used as immunostimulant to prevent WSSV virus in shrimp. This study aimed to determine the effect of immunostimulant application of seaweed extract *Sargassum polycystum, Padina australis, Euchema cottonii, Gracilaria verrucose* and to change in immune response of vanamei shrimp (*Litopenaeus vannamei*) before and after WSSV infection.

Material and methods

Material

The material in this research is about the study and analysis of the immunostimulants application from seaweed extract of *Euchema cottonii, Gracilaria verrucose, Padina australis*, and *Sargassum polycystum* against vanamei shrimp that challenged with *White Spot Syndrome Virus* (WSSV). Observed parameters was Total Haemocyte Count (THC), Differential Haemocyte Cell (DHC) include hyaline cell, granule cell and semi granule cell, *Superoxyde dismutase* (SOD) activity, and *Respiratory Brust* (RB).

Experimental setup

The method used is an experimental method, by conducting a direct Completely Randomize Design trial design and providing treatment according to the experimental design. The immunostimulants are coming from marine algae or commonly known as seaweed. The seaweed extracted from *S. polycystum*, *P. australis*, *E.cottonii*, and *G. verrucose*. Seaweed extraction process is carried out using a methanol solvent in a ratio of 1: 3 (m: v) or methanol equal dissolved with 3 replications, then proceed with the encapsulation process using the freeze dry method. This study uses the freeze-drying method according to, the ethanol extract of candis acid obtained previously was mixed with maltodextrin, with a coating formulation of 30% (w / v) against the solvent, and extract 20% (w / w) against the coating, with a speed of 1800 rpm for 10 minutes, after 10 minutes, after it is frozen in the freezer [15]. The encapsulation process with freeze dry for 2 x 24 hours. This study uses 6 treatments, namely positive control (A), negative control (B), treatment of immunostimulant from *S. polycystum* (C), immunostimulant of *P. australis* (D), immunostimulant of *E. cottonii* (E), immunostimulant of G. verrucossa extract (F). The treatment was carried out for 3 weeks, then continued with the WSSV challenge test and observed the morphology and behaviour of vanamei shrimp for 5 days.

Observation of morphology and behaviour of vanamei shrimp is classified by using a score according to the level of vanamei shrimp infection. After 5 days of observation, a Polymerase Chain Reaction (PCR) test was performed to detect the presence of WSSV DNA in the body of the shrimp. Observation of the total number of haemocyte cells was carried out in the second week, third week and after WSSV infection [16]. The number of haemocyte cells can be calculated using the formula according to as follows:

$$DHC = \frac{The\ number\ of\ every\ haemocytye\ cell}{Total\ haemocyte} x\ 100$$

Observation and calculation of haemocyte cell differentiation using the formula according to [17]. Which is as follows:

$$THC = \frac{The\ number\ of\ haemocyte\ counted}{Volume}\ x\ dilution\ x\ 10^6$$

Super dioxide Dismutase (SOD) and Respiratory Brust (RB) parameters were used using the immunohistology spectrophotometer reader method, as well as for water quality parameters measured, namely: temperature, acidity (pH), dissolved oxygen (DO), salinity and ammonia.

Preparation of experimental diet

One hundred and eight of healthy shrimps were randomly divided into three replicate tanks. They were placed in aquarium, and the water temperature set at 28°C for 24 h. The experimental shrimps, with an average weight of 5.4±0.7g, were obtained from a commercial farm. The shrimps were immediately transported to the lab and acclimated in 500 L filtered, aerated (oxygen pump) seawater tanks at least 4 days before experiments. During the acclimation stage, the water salinity and temperature in tanks were consistent with that of the culture ponds (salinity 5‰, pH 8.3±0.1 and temperature 28±1°C). Commercial shrimp feeds were given two times per day (5% of shrimp body weight per time).

Challenge with White Spot Syndrome Virus

Each infected shrimp supplying (only one dose) the WSSV in each aquarium (three replicates per treatment). For this, both tissues infected and non-infected at the correspondent proportions are homogenized and distributed to the aquariums. Shrimp pertaining to a double negative control group (three replicates) are fed with 10% shrimp biomass of WSSV-non infected shrimp muscle. Shrimp of treatments and double negative control group are not fed with the commercial diets for the next 24h and afterward, shrimp are fed with a commercial diet for nine days at 5% of its daily biomass. finally, they all administrated by each marine algae extract. Then observed after 24 h.

PCR Test

PCR test using a rapid PCR assay for detection of white spot syndrome virus (WSSV) based on the nested PCR procedure described by (Lo et al. 1996) and outlined as the recommended PCR diagnostic assay in the Manual of Diagnostic Tests for Aquatic Animals published by the Office of International Epizootics [18].

Haematological analysis

Haemolymph Cell Differentiation is analysed by using published method by, while Total Haemocytes Count (THC) is analysed by using published method by, and Differentiation Haemocytes Cell (DHC) test was using published method by [19-22].

Significance of Freeze Dry Seaweed Allowance to Overall Results of DHC Parameters

SOD Enzyme activity

Samples of shrimp muscle were thawed in an ice bath; 200 mg of each tissue were put in a cooled Eppendorf tube containing 1 mL of phosphate buffer (50 mM, pH 7.8). Each sample was homogenized with a motorized Kontes pellet pestle. The homogenate was centrifuged at 5724 g for 5 min at 4°C; the supernatant was recovered and heated for 5 min at 65°C. A new supernatant was obtained after a second centrifugation (crude extract) and stored at −20°C. SOD activity was determined by the

protocol of [23]. Briefly, 2 mL reaction mixture (0.1 mM EDTA, 13 µM methionine, 0.75 mM Nitro Blue Tetrazolium (NBT) and 20 µM riboflavin in 50 mM of phosphate buffer, pH 7.8) and from 0 to 100 µL of crude extract were placed under fluorescent light in a spectrophotometer (Beckman DU 600) for 1 min or until absorbance (A560) in control tubes (without crude extract) reached 0.2 to 0.25 optical densities. Results of enzymatic specific activity were obtained using a computer program and calculated in units per mg protein [24]. The ratio of specific SOD activity from treated shrimp or scallop samples to specific SOD activity of the controls was expressed as an index, the relative enzymatic activity (REA).

Respiratory Brust

Respiratory burst (RB) activity of haemocytes was quantified using the reduction of nitrobluetetrazolium (NBT) to formazan as a measure of superoxide anion production following the method of with slight modifications [25]. The optical density of formazan was measured at 630 nm using a 96-well microtiter plate and microplate reader Respiratory burst was expressed as NBT-reduction in 10 µL of haemolymph.

Statistical analyses

The composition and structural data were analysed using SAS 9.1.3 software (SAS Institute Inc., Cary, USA) and done using Analysis of variance (ANOVA). A comparison between the harvests periods was analysed with the measure of the least significance difference (LSD) at a significant level of 5%. Means with the same letter are not significantly different (at 0.05). The GC measurements were analysed with a factorial experiment. The effect of the harvest period was analysed as a 4 (harvest period) and 3 (extraction) by ANOVA for each monosaccharide. A comparison between harvest periods was analysed for each mono- saccharide with the measure of the least significance difference (LSD) at a significant level of 5%. Means with the same letter are not significantly different (at 0.05).

Results and Discussion

Physiological Analysis

According to *L. vannamei* infected with WSSV physically there are white spots on carapace with diameters ranging from 1.5-2.8 m. From the results of morphological analysis, in the positive control treatment group there were 3 shrimps that had characteristics of being infected with WSSV with a score of 1 (+) and the number of dead shrimps 9 shrimps from 20 shrimps. Shrimp in positive control that show clinical symptoms such as red tails but do not indicate infection with the WSSV virus, this can occur because of physiological stress experienced by the shrimp itself. The negative control treatment group contained 18 shrimps indicating a WSSV infection rate with a score of 1 (+) and 2 shrimps indicating a level of infection with a score of 2 (++), the number of shrimps dying was 13 shrimps from 20 shrimps. The immunostimulant treatment group of *Sargassum polycystum* (C) extract contained 11 shrimps indicating a WSSV infection level with a score of 1 (+) and no shrimp indicating a level of infection with a score of 2 (++) or 3 (+++), the number of shrimps that were 6 shrimp died out of 20 shrimp. The immunostimulant treatment group of Padina australis (D) extract consisted of 13 shrimps indicating WSSV infection level with a score of 1 (+) and no shrimp indicating infection level with a score of 2 (++) or 3 (+++), the number of shrimps that were 10 shrimp died out of 20 shrimp. The immunostimulant treatment group of *Eucheuma cottoni extract* (E) contained 6 shrimps indicating the level of WSSV infection with a score of 1 (+) and there was no shrimp indicating the level of infection with a score of 2 (++) or 3 (+++), the number of shrimps that were 5 dead shrimp out of 20 shrimp. The immunostimulant treatment group of *Gracillaria verucossa* (F) extract contained 14 shrimps indicating the level of WSSV infection with a score of 1 (+) and no shrimp indicating the level of infection with a score of 2 (++) or 3 (+++), the number of shrimps that were 10 shrimp died out of 20 shrimp.

Challenge with White Spot Syndrome Virus

PCR Test

The results obtained from testing shrimp vanamei samples using the PCR (*Polymerase Chain Reaction*) method can be seen in **Table 1**.

Based on PCR results from vanamei shrimp samples after the challenge test in the table, it is known that in the negative control group the results were positive for the presence of WSSV Virus DNA because in the treatment group they were not treated with immunostimulant marine algae freeze dried. This is in line with the morphological characteristics obtained in the negative control treatment group, in vanamei shrimp carapace, white spots with diameters ranging from 1.5-2.8 m, which are clinical symptoms of shrimp infected with WSSV virus [26].

Haematological analysis

Haemolymph Cell Differentiation

The results of the histogram average *L. vannamei* hyaline cells were applied to the addition of immunostimulant freeze dry seaweed in feed with before and after WSSV infection seen in **Figure 1**.

From the above data in this experimental research the average treatment of B and A is the same, treatment D is different and treatment E, F and C are the same. Thus, the treatment variable only significantly influences the difference in the average treatment of E, F and C. Seaweed treatment C (*S. polycystum*)

Table 1: PCR test result.

No	Shrimp Sample	PCR Result
1	Positive control	Negative
2	Negative control	Positive
3	Shrimp die in the immunostimulant treatment of marine algae freeze dried	Negative
4	Shrimp live on marine algae freeze dried immunostimulant treatment	Negative

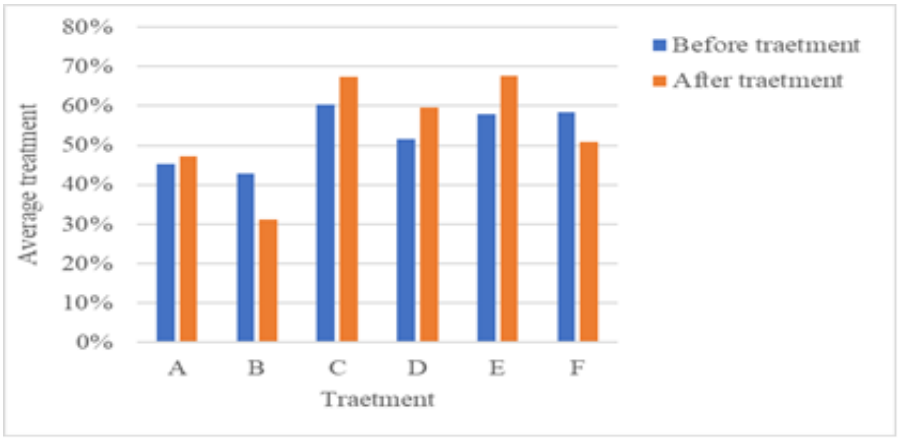

Figure 1 The average histogram yield of L. vannamei hyaline cells which was applied by addition of immunostimulant freeze dry seaweed to feed with before and after infected WSSV.

A: Positive Control
B: Negative Control
C: Treatment of S. polycystum
D: Treatment of P. australis
E: Treatment of E. cotonii
F: Treatment of G. verucossa

Most significantly in increasing hyaline cells After the WWSV challenge test proves that this experimental research the average treatment B is different, treatment A is different, the average treatment D and F are the same and treatment C and E are the same. Thus, the treatment variable only significantly influences the difference in average treatment C and E. Seaweed treatment E (*E. cotonii*) at most significant in increasing hyaline cells.

Based on two-way ANOVA analysis, each treatment was significantly different from the hyaline cells of shrimp exposed to WSSV with a confidence level of 95%. The homogeneity of the data areas prior to the experimental WSSV challenge test was that Treatment A and B were the same, Treatment A and E were the same, Treatment E, C and F were the same, and Treatment D was different. Thus, the treatment variable only had a significant effect on treatment D. Treatment of seaweed D (*P. australis*) Was most significant for increasing hyaline cell area. After the WSSV test proved that the average treatment B and F were the same, treatment F, A, D, C was the same and treatment E was different. Thus, the treatment variable only had a significant impact on the difference in the mean of treatment E. The treatment of algae E (*Eucheuma cotonii*) Was most significant for increasing the area of hyaline cells. Hyaline plays an important role in the shrimp defense system, especially in the phagocytosis process. Hyaline becomes the main cell in phagocytosis. Hyaline cells do not have cytoplasmic agranular cells. The size is smaller among the haemocyte cells. According to, the increase in hyaline cells due to LPS can induce the proliferation of haemocyte cells [27]. Cell proliferation occurs because the LPS polysaccharide in the extract can stimulate the immune response of aquatic animals. This is expected to accelerate the process of maturation of haemocytes in the hematopoietic tissue by stroke cells where new haemocytes are released into the hemolim. The release of new haemocytes increases the number of hyaline cells and accelerates the maturation process of the haemocytes in the connective tissue, so that the frequency of the granular cells in the hemolim is awakened during a pathogenic infection.

Added that if an attack occurs hyaline cell pathogens are cells that have a greater role than other cells. Hyaline cells become the main cells in the process of phagocytosis and semi-granular cells play a role in the freeze dry process which indicates the incorporation of several haemocyte cells to block foreign particles in the blood circulation [28]. Thus, the increase in hyaline cells in haemocytes is one of the parameters of improving the health status or body resistance of *L. vananmei*, which obviously cannot be separated from other cell types in haemocytes.

Total Haemocytes Count (THC)

The results obtained from testing the blood samples of vanamei shrimp on parameters that describe the non-specific immune system of shrimp are the total number of haemocyte cells and can be seen in **Figure 2**.

The number of haemocyte cells in vanamei shrimp after WSSV infection in the third week undergoes a pretty drastic change. The negative control treatment group of haemocyte cell counts decreased drastically to 0.51 x 107 cells / L. This can be caused by haemocyte cells in vanamei shrimp cannot fight the pathogens that enter the body, while the treatment group extract *Sargassum polycistum* (C) rose to 0.70 x 107 cells / L, the treatment group extract Padina australis (D) rose to 0.65 × 107 cell / L, the treatment group extract of *Euchemma cottoni* (E) rose to 1.13 × 107 cells / L, the treatment group extract of *Gracillaria verucossa* (F) rose to 0.97 × 107 cells / L. This can be due to shrimp that have been given immunostimulant seaweed extract can increase the level of shrimp immunity when pathogens enter the body by increasing the number of haemocyte cells.

According to, the ability of immunostimulants to increase shrimp immune response and increase protection against pathogenic

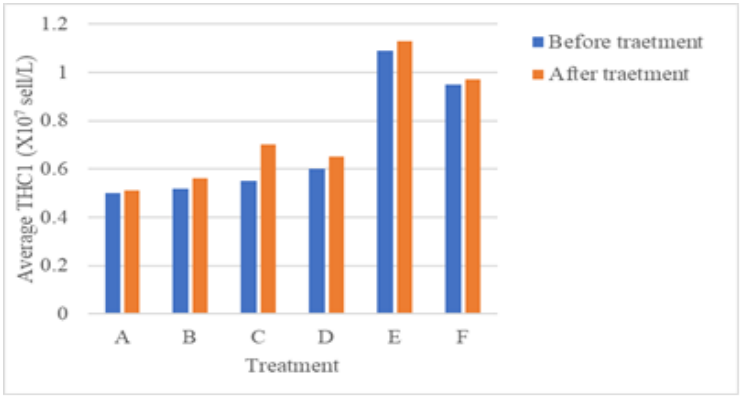

Figure 2 Total Haemocyte Count.

infections can be affected by application dosages [29]. Giving immunostimulants with doses below the minimum value for the occurrence of an immune response will not have an effect on increasing the number of haemocytes, whereas at too high a dose can also have no effect or behave as an inhibitor. According to, that an increase in the total number of hemocytes is assumed to be a form of cellular immune response in shrimp bodies, because hemocytes are the body's defense mechanism from shrimp [30]. A decrease in the number of hemocytes as a result of a specific disease infection [31].

The results of observing the number of hemocyte cells before infection differ in the effect of treatment lies in the treatment groups C, D, E, and F with groups B and A. The difference in the effect of treatment results of observation of the number of hemocyte cells after infection lies in the treatment groups A, B, and D. Treatment group A is different because it is a control and not given any treatment.

Based on two-way ANOVA analysis, each treatment was significantly different from THC shrimp exposed to WSSV with a confidence level of 95%. With the best treatment is E (immunostimulant extract *E. cottonii*) and F (immunostimulant extract *G. verrucossa*). Treatment group B, the average number of haemocyte cells decreased because they were not given immunostimulant from seaweed extract, so when there are pathogens that enter the body, haemocyte cells are not strong enough to fight and cause a decrease in the number of haemocyte cells. Treatment group D showed almost the same as treatment group B because it could be due to immunostimulant administration of seaweed extract *P. australis* (D) less than the maximum in terms of the number of haemocyte cells not as much as treatment C, E, and F.

DHC Granular cells

Histogram results of average L. vannamei granular cells were applied to the addition of immunostimulant freeze dry seaweed in feed with before and after WSSV infection seen in **Figure 3**.

From the above data in this experimental research the average treatment of C and E are the same, while the treatments of E, F and D are the same and B and A are the same. Thus, the treatment variables only significantly influence the difference in the average treatment B and A are the same. The most significant was in treatment A. It can be concluded that treatment D (*Padina australis*) Seaweed had the most significant increase in granule cells. After the WSSV challenge test proved this experimental research the average treatment C was different, treatments E and D were the same, treatments D and F were the same, treatment A was different and treatment E was different. Thus, the treatment variable only significantly influences the difference in the average treatment E being the same. It can be concluded that the most significant treatment of E (*Eucheuma cotonii*) Seaweed experienced an increase in granule cells.

Based on two-way ANOVA analysis, each treatment was significantly different from shrimp cell granules exposed to WSSV with a 95% confidence level. The homogeneity of the data range before the experimental research WSSV challenge test is that treatment A is different, treatments B, C and D are the same, treatments C, D and E are the same and the average treatment F is different. Thus, the treatment variables only significantly influence the difference in the average treatment F. It can be concluded that the seaweed treatment F (*Gracillaria verucossa*) Which most significantly increased the range of granule cells. After the WSSV challenge test proves that this experimental research - treatments A, B and C are the same, treatments C, D and F are the same, treatments D, F and A are the same and treatment B is different. Thus, the treatment variable only significantly influences the difference in the average treatment B. It can be concluded that the seaweed treatment F (*Gracillaria verucossa*) Which most significantly increases the range of granule cells. Granule cells are characterized by having a large number of granules and performing proPO and cytotoxic functions. Granular cells are cells with a ratio of lower cell nuclei from cytoplasm. These cells function in storing and releasing proPO and as a cytotoxic together with semi-granule cells (33).

Granular cells were observed to have decreased After being infected with WSSV, this shows that granular cells play a role in maintaining the body's resistance to shrimp from a petogen attack, WSSV. The function of granular cells is more in the process of producing phenol oxidase enzymes which have an important role in non-specific defense systems. Supamattaya explained that granules in haemocyte granular cells consist of propenol oxidase. In the activation of prophenoloxidase (proPO)

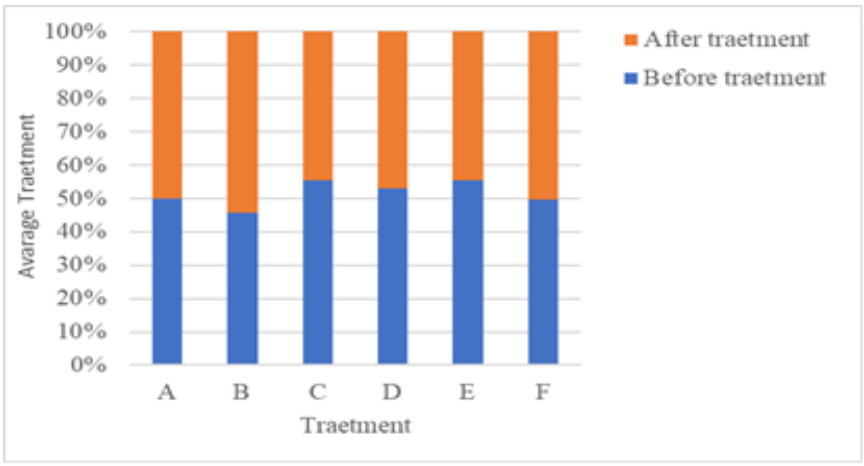

Figure 3 The average histogram yield of L. *vannamei* granular cells which was applied by addition of immunostimulant freeze dry seaweed on feed with before and after infection of WSSV.

will free an enzyme from granular cells. This system is also driven by the presence of microbial components such as β-glucan. The propenol oxidase process is responsible for the production and secretion of toxic metabolites such as quinone. The final product of this system is the appearance of blackish nodules which are usually located around the gills or exoskeleton. When a pathogen attacks, granular and semi-granular cells will undergo degranulation, cytotoxicity and lysis of the material so that the number of granular cells circulating in the hemolim will decrease.

DHC Semi Granule Cells

Histogram results of the average semi-granular cells of L. vannamei which were applied to the addition of immunostimulant freeze dry seaweed in feed with before and after WSSV infection are shown in **Figure 4**.

From the above data in this experimental research the average treatment of E, F and C are the same, while the treatments F, C and D are the same, C, D and B are the same and A is different. Thus, the treatment variable only significantly influences the difference in the average of treatment A most significantly. Seaweed treatment D (*P. australis*) Most significantly increased semi-granule cells. After the WWSV challenge test proves that this experimental research the average treatment of E, F and C are the same, treatment F, C and D are the same, treatment D and A are the same and treatment B is different. Thus, the treatment variable only significantly influences the difference in the average of the most significant treatment B. Seaweed treatment D (*Padina australis*) Most significantly increased semi-granule cells.

The homogeneity of the range before the WSSV experimental research challenge test, the average treatment of F, D, E and C are the same and treatments A and B are the same. Thus, the treatment variable only significantly influences the difference in the average of treatment A most significantly. Seaweed treatment C (*S. polycystum*) Most significantly increased the range of semi-granule cells. After the WSSV challenge test proves that this experimental research the average treatment of A, E, D, C and F are the same and the treatments of C. F and B are different. Thus, the treatment variable only significantly influences the difference in the average of the most significant treatment B. Seaweed treatment F (*G. verucossa*) Most significantly increased the range of semi-granule cells. These cells play a role in the freeze dry process which indicates the incorporation of several haemocyte cells to block foreign particles in the blood cells. Semi-granule cells are characterized by small amounts of granules. According to, semi-granular cells are the ripening of hialyne cells which when there is a pathogen attack then the first role is hyaline cells, so these cells do not develop into semi-granular cells and there is a decrease in the number of semi-granular cells contained in haemocytes.

The observation shows that semi-granular cells have decreased after infection except in the control (-) treatment, this means that semi-granular cells have only a small role in the process of shrimp survival, because in fact semi-granular cells are only ripening from hyaline cells that play a role in the freeze dry process. suspected pathogenic hyaline cells play a role more than semi-granular cells so that the number of semi-granular cells decreases. Semi-granular cells are the maturation of hyalin cells which when a pathogen attacks then the first role is hyalin cells, so these cells do not develop into semi-granular cells and a decrease in the number of semi-granular cells contained in haemocytes Semi-granular cells are characterized by the presence of granules in the cytoplasm. These cells are able to respond to polysaccharides from bacterial cell walls or β-glucan derived from fungi. These semi-granular cells can do the freeze dry process and have little role in the phagocytosis process. freeze dry is a defense reaction against particles in large numbers and unable to be phagocyted by haemocyte cells.

Significance of Freeze Dry Seaweed Allowance to Overall Results of DHC Parameters

Significant seaweed freeze dry treatment can increase hyaline cells, based on SPSS One-way ANOVA test as follows: based on percentage criteria before WSSV challenge test: C treatment (*S. polycystum*). After WSSV challenge test: treatment E (*E. cotonii*). Based on the range criteria before the WSSV challenge test: D

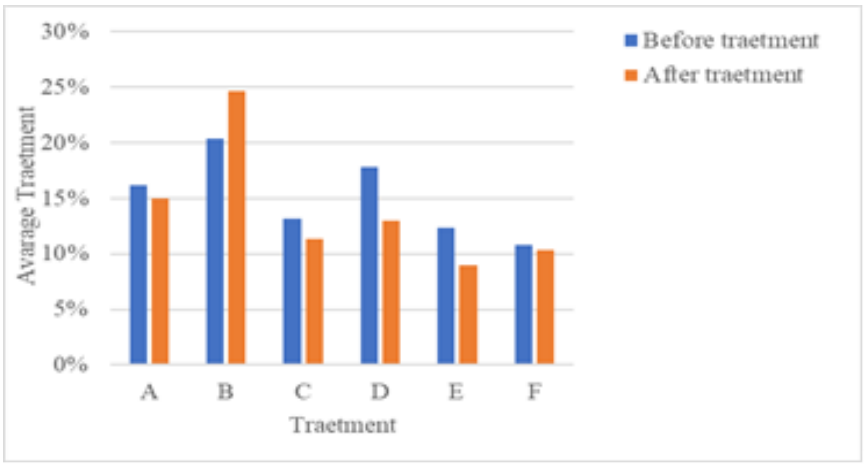

Figure 4 Average Histogram Semi Granule L. *vannamei* that applied freeze dry seaweed immunostimulant on feed (A) before and (B) After WSSV infection.

treatment (*P. australis*). After WSSV challenge test: treatment E (*E. cotonii*).

The seaweed freezes dry treatment that is able to increase granule cells, based on SPSS One-way ANOVA test as follows: percentage criteria before WSSV challenge test: D treatment (*Padina australis*). After WSSV challenge test: F treatment (*G. verucossa*). Based on the range criteria before the WSSV challenge test: F treatment (*G. verucossa*). After WSSV challenge test: F treatment (*G. verucossa*).

Seaweed treatment (*P. australis*) of all DHC parameters tested based on the criteria proved to be the most significant increase. This is due to antigens entering the body, then the antigens will be snared by macrophages in such a way that they can be known as foreign material. The foreign material will be sent to the antibody-forming system and antibody formation occurs. So that (*P. australis*) Has the potential to have active ingredients that can increase non-specific *L. vannamei* immune cells infected with WSSV.

SOD Enzyme Activity

Based on the results of research conducted, the SOD value before the WSSV challenge test was the lowest in treatment B (negative control) of 0.55 and the highest in treatment C (S. polycystum) which was 0.80. The lowest SOD value after the WSSV test in treatment B (negative control) is 0.40 and the highest in treatment E (E. cottonii) is 1.2. This is due to the negative control (B) not given immunostimulatory feed treatment so that there is no significant effect on the immune response in vannamei shrimp. Increasing SOD after infection showed that treatment (Euchema cottonii) was significantly different from other treatments, and seaweed (E. cottonii) showed the best type of immunostimulant against WSSV attack on vannamei shrimp. The average vannamei shrimp SOD value is presented in **Figure 5**.

SOD enzymes are produced in animals as antioxidants, but their numbers will decrease sharply when there is a lot of damage to cell metabolism [32]. When shrimp get infected or get stressor, haemocytes will produce reactive oxygen species (ROS) and their concentration will be balanced with antioxidant enzymes, one of which is SOD [33]. This increase in SOD aims to reduce the explosion of cellular superoxide during defense against viral infections and to protect shrimp cells from damage [34]. Superoxide is a free radical that is a molecule that has unpaired electrons. Therefore, it is very reactive and its reactivity can injure molecules in the body. SOD enzymes catalyse the transformation of superoxide into hydrogen peroxide and oxygen. Superoxide dismutase has become an alternative way to minimize tissue damage due to free radicals [35].

Based on two-way ANOVA analysis, each treatment was significantly different from SOD of shrimp exposed to WSSV with a confidence level of 95%. The best treatment is E (immunostimulant extract *Eucheuma cottonii*) and F (immunostimulant extract *Gracillaria verrucossa*). Antioxidants function to stabilize free radicals by completing the lack of electrons and inhibiting the chain reaction of free radical formation [36]. Free radicals are molecules or atoms which have one or more unpaired electrons in their outermost orbitals. Free radicals are unstable, very reactive and can grab electrons from other molecules in an effort to get their electron pairs. Molecules that lose electrons can be reactive [37]. The presence of natural antioxidants (such as phenolic compounds) and synthetics can inhibit lipid oxidation, prevent damage and changes in organic components. Antioxidant components can be found in marine algae.

Respiratory Brust (RB)

The results obtained from the testing of vanamei shrimp blood samples on parameters describing respiratory brust activity can be seen in **Figure 6**.

RB value in vanamei shrimp after WSSV infection in the third week also experienced a pretty drastic change. The negative control treatment group RB value decreased dramatically to 0.58, while the treatment group extract *Sargassum polycistum* (C) rose to 1.3; *Padina australis* (D) extract treatment group increased to 1.01; the *Euchemma cottoni* (E) extract treatment group increased to 1.50; *Gracillaria verucossa* (F) extract treatment group increased to 1.32.

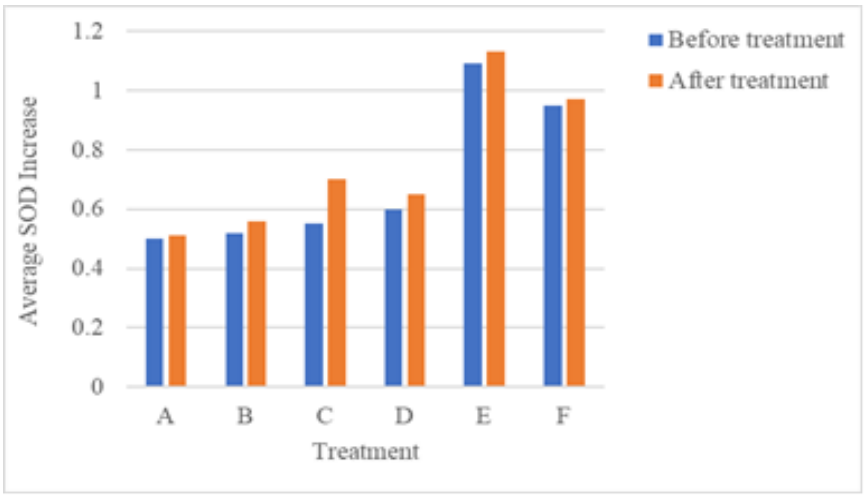

Figure 5 Graph Value of Superoxide Dismutase (SOD) Before and after Challenged Test WSSV treatment (A) control (+), (B) control (-), (C) *S. polycystum* 10 gr / Kg feed, (D) *P.australis* 10 gr / Kg feed, (E) *E. cottonii* 10 gr / Kg feed and (F) *G. verrucosa* 10 gr / Kg feed.

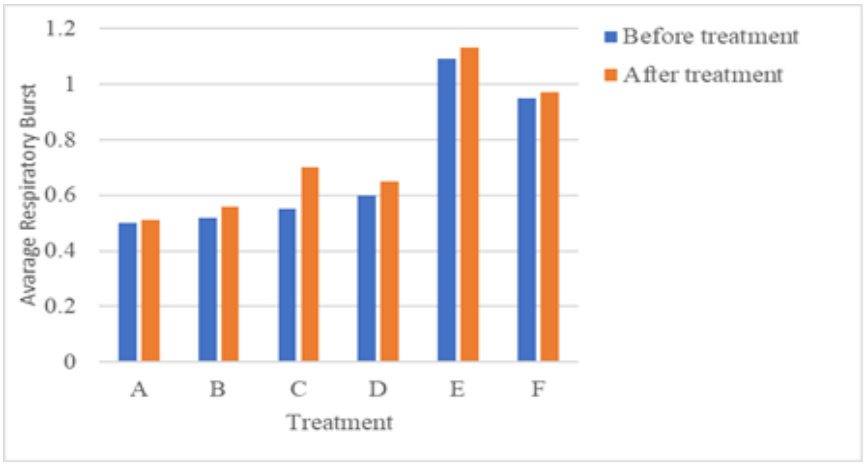

Figure 6 Graph of Respiratory Burst Activity Result.

According to states that to examine immunotoxicity, biomarkers are focused on innate immune functions that cannot be avoided because they have a strong response to foreign bodies [38]. One of the most frequently studied functions in ecotoxicology is RB activity. Respiratory burst (RB) is the basic form of the antibacterial system that exists in the fish's body. The increase in RB value can be correlated with an increase in phagocytic cell activity [39]. Respiratory burst can increase oxygen consumption so that it can result in the formation of superoxide anions and this process is accelerated by NADPH-oxidase, a multi-component enzyme that has been attached to the inner surface of the plasma membrane after activation to carry out phagophytic [40].

Based on two-way ANOVA analysis, each treatment was significantly different from RB of shrimp exposed to WSSV with a 95% confidence level. The best treatment is E (immunostimulant extract *Eucheuma cottonii*) and F (immunostimulant extract *Gracillaria verrucossa*). According to, in the phagocytic process a superoxide anion will be produced as a result of the RB process and will be confirmed by enzymes producing toxic hypochlorous acid (HOCl), nitricoxid (NO) and peroxinitrit (ONOO-) which will be used to destroy pathogens, thus if the phagocytic process increases, it will increase RB activity.

Conclusion

Freeze dried marine algae exract has significant effect to increasing the immune system of vannamei shrimp (*L. vannamei*) challenged with White Spot Syndrome Virus (WSSV). The best treatment is E (immunostimulant extract *Eucheuma cottonii*) and F (immunostimulant extract *Gracillaria verrucossa*) that causing positive affect on the parameter i.e., Total Haemocyte Count (THC), hyaline cells, granule cells and semi granule cells, Superoxide dismutase (SOD) activity, and Respiratory Brust (RB).

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