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The Role of *Zoothamnium penaei* Crude Protein as an Immunostimulant to Improve Non-Specific Immunity and Survival Rate of Pacific White Shrimp *(Litopenaeus vannamei)* Against White Spot Syndrome Virus

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ABSTRACT

The immunogenic crude protein of *Zoothamnium penaei* (CPZp) has the potential to be developed as an immunostimulant agent in preventing white spot syndrome virus (WSSV) infection. This study aimed to evaluate the effectiveness of CPZp administration on the hematology, gene expression related immunity, and survival rates of the Pacific white shrimp (*Litopenaeus vannamei*) infected with WSSV. This study used a pre- and post-test laboratory experimental study design with two treatments P1 (3 ppm CPZp + WSSV infection) and P2 (without CPZp + WSSV infection). The observation was carried out for 7 days, while the CPZp booster was administered on the 4th day. The observed hematological parameters consisted of total hemocyte counts (THC), differential hemocyte counts (DHC), phenoloxidase (Po) enzyme, and gene expression related-immunity. WSSV was injected into shrimp on the first day (24 hours) after CPZp administration followed by quantitave analysis using qRT-PCR. Overall, the treatment of 3ppm CPZp increased the hematological parameters of the Pacific white shrimp. In addition, CPZp administration significantly increased the Po enzyme, as well as the expression of genes related to immunity, specifically *proPO* and *c-type lectin*, when compared to the control group. The survival rate of shrimp with 3ppm CPZp against WSSV (P1) was significantly higher when compared to control (P2) with 90 and 10%, respectively. Our results indicated an increase in resistance to WSSV infection as a result of the CPZp treatment.

INTRODUCTION

In Indonesia, the Pacific white shrimp, *Litopenaeus vannamei*, is one of the main commodities in the aquaculture industrialization. According to an FAO report **(FAO**

2017), Indonesian export activity of Pacific white shrimp in 2016 ranked fourth in the world, trailing only India, Vietnam, and Ecuador, with a total export volume of 220,000 tons. The Pacific white shrimp farmers are developing an intensive system on a small scale to increase production yields **(Yudiati *et al.*, 2016)**. However, there are still issues related to a decrease in environmental quality caused by stress from viral diseases such as the white spot syndrome virus (WSSV) **(Flegel, 2012; Syed Musthaq & Kwang, 2014)**. The declining Pacific white shrimp production due to WSSV attack can occur massively and relatively fast **(Flegel, 2012)**. The white shrimp mortality can reach 100% within 3-7 days due to WSSV infection **(Huang *et al.*, 2011)**. The WSSV-infected Pacific white shrimp showed a decrease in its immune system **(Wang & Zhu, 2017; Setyawan, 2019)**. Pacific white shrimp, as invertebrates, rely on their innate immune system to fight infection **(Jiravanichpaisal *et al.*, 2007)**.

Therefore, an effort to prevent pathogenic infections by increasing resistance in the Pacific white shrimp is a major concern. To improve immune response and disease control in aquatic organisms, immunostimulant administration is recommended **(Urbinati *et al.*, 2020)**. A study by **Meshram *et al.* (2015)** found a significant increase in phenoloxidase enzyme (Po) activity, nitroblue tetrazolim, phagocytic activity, total hemocytes counts (THC), and survival rates in *Macrobrachium rosenbergii* fed with 1.0g/ kg β-glucan after *Aeromonas hydrophila* infection. Furthermore, *Penaeus monodon* treated with mannan oligosaccharide-based immunostimulant and diets containing β-glucans showed higher growth rate, as well as a significant increase in its resistance to WSSV biochemistrically and immunologically **(Andrino *et al.*, 2014)**.

One of the solutions found in our previous study was an immunostimulant derived from the *Zoothamnium penaei* immunogenic membrane protein, which was shown to increase the Pacific white shrimp survival by 94% in a traditional shrimp pond culture system. According to the findings of our previous study, the use of *Zoothamnium penaei* immunogenic membrane protein had the potential to be developed as an immunostimulant that could increase the activity of shrimp defense cells in response to zoothamniosis attacks. A previous study by **Mahasri (2007)** also confirmed that the application of Zoothamnium immunogenic membrane proteins with molecular weights of 38 kDa, 48 kDa, and 67 kDa was capable of increasing the immune response (THC and DHC) of the tiger prawns (*P. monodon*). **Gustrifandi (2013)** reported a similar study in which immunostimulants derived from the *Zoothamnium penaei* immunogenic membrane protein reduced the prevalence of zoothamniosis in the Pacific white shrimp in the traditional pond and increased immune response (THC and DHC) by 41.55×10^6 cells/mL and 24.03% for 90 days. **Wiradana *et al.* (2019)** reported that using the crude protein of *Zoothamnium penaei*, they were able to increase the survival rate of the Pacific white shrimp by 86.6% on a laboratory scale at a concentration of 3ppm.

The examination of the immune response of shrimp to immunostimulants is currently focused not only on the hematological level but also on the expression level of

genes associated with shrimp immunity. C-type lectin is one of the most significant examples of pattern recognition proteins in invertebrates, and it plays a crucial role in protecting shrimp from a variety of viral and bacterial diseases **(Kumar *et al.*, 2022)**. When zymosan (carbohydrates from yeast cell walls), bacterial lipopolysaccharide (LPS), trypsin, calcium ions, urea, or temperature stimuli trigger melanization, subsequent immunity genes such as the *proPO* gene play a role in activating the formation of Phenoloxidase (PO). In addition, *proPO* activates shrimp defense mechanisms through encapsulation, nodule formation, phagocytosis, and activator of hemocytes **(Hirono *et al.*, 2011)**.

Based on the above description, it is still necessary to conduct research on the use of *Zoothamnium penaei* crude protein (CPZp) to control WSSV infection of the Pacific white shrimp. This study aimed to analyze the non-specific immune response, gene related immunity expression, and survival rate of the Pacific white shrimp *(L. vannamei)* due to the administration of CPZp against the WSSV that could be developed as an immunostimulant agent.

MATERIALS AND METHODS

Ethical approval

This research obtained a permit from the Ministry of Marine Affairs and Fisheries (KKP), Directorate General of Aquaculture, Brackish Water Aquaculture Center (BBPBAP), Jepara, Central Java, with Letter Number: 2088/BBPBAP/HM.320/VIII/2019.

Research design

This research was included in a randomized pre- and post-test laboratory experimental study. Seven days of adaptation were performed on 250 specific pathogen-free (SPF) Pacific white shrimp averaging 7-8 grams in weight and 60 days of age. Furthermore, 40 Pacific white shrimp were divided into two groups (20 shrimp each), the CPZp group (treated with CPZp immunostimulant 3 ppm/day + WSSV infection) and the control group (treated with WSSV infection only), for seven days.

Preparations isolates of WSSV

The virus samples were obtained from the WSSV-infected Pacific white shrimp at the Aquatic Animal Health Management Laboratory of the Brackish Water Cultivation Fishery Center (BBPBAP), Jepara, Central Java. The WSSV inoculum was prepared according to the method described in a previous study by **Hameed *et al.* (1998)** and **Hameed *et al.* (1998)**. In order to prepare the inoculum, 1.0 gram of WSSV-infected shrimp body was weighed after being washed 5 times with pH 7.3 phosphate-buffered saline (PBS), then crushed using a mortar, and dissolved into 9mL PBS. The solution was centrifuged at 1,107 ×g for 30 minutes and 7,871 ×g for 20 minutes each. The produced

supernatant was subsequently filtered using a 0.45µm millipore filter equipped with a filter holder and syringe. The obtained filtrate was stored at -20 ºC as WSSV inoculum.

Revirulence of WSSV

The viral revirulence was carried out to regenerate virus filtrate stored in the freezer. The stored viral filtrate was regenerated by re-infecting the healthy Pacific white shrimp. Ten healthy Pacific white shrimp were prepared in-stock containers (20L). The viral filtrate containing virus isolate was then collected in a microtube and injected to the shrimp by 0.1 mL/individual between the 3rd and 4th part of the Pacific white shrimp abdomen. The shrimp were then returned to the container and were left until infected. Shrimp with WSSV clinical symptoms were immediately collected and stored in –20ºC freezer. To ensure the presence of WSSV infection, a screening with Polymerase Chain Reaction (PCR) was carried out **Yudiati *et al.*, (2019)**.

Preparation of CPZp

The immunostimulant material to be tested was CPZp, which was produced and had been tested in the laboratory by **Mahasri (2007)**. This CPZp material contained 38 kDa, 48 kDa, and 67 kDa immunogenic proteins.

Treatment and challenge test with WSSV

The protective ability testing of the CPZp immunostimulant was carried out *in vivo* using 40 SPF/healthy Pacific white shrimp after PCR screening. Before being divided into treatment groups, white shrimp were acclimatized for ± 7 days at room temperature (26-28ºC). During the acclimatization period, the shrimp were given commercial feed at 3%/w/day four times a day. In addition, tapping was carried out before feeding the shrimp during the acclimatization, maintenance, and challenge test stages. After the acclimatization period ended, the white shrimp used were then divided into two groups with each group contained of 20 with the assumption of 1.0 shrimp/liter. As pre-test data, measurements of THC, DHC, and hemolymph PO levels were performed on the 7th day in each group.

The WSSV challenge test was carried out on the 2nd or 48 hours after pre-test. The WSSV challenge test was carried out through the dilution of the supernatant from the '10^{-3}' WSSV filtrate and filtered using a 0.45µm syringe filter. WSSV injection was carried out on the shrimp abdomen between the 3rd and 4th segments. It was then injected to 0.1mL/ individual with 10^{-3} dose corresponding to the LD_{50} value **(Wiradana *et al.*, 2019)**. The administration of immunostimulants was carried out after the infection and continued until the 7th day. The levels of THC, DHC, PO enzymatic activity, *c-type lectin* and *proPO* gene expression, and shrimp survival were measured again 7 days later as post-test data.

During the experimental period, the shrimp were fed commercial feed up to 3% of their body weight four times per day, at 6:00 a.m., 11:00 a.m., 5:00 p.m., and 10:00 p.m **(Yudiati *et al.*, 2016)**. The water quality detection and clinical symptom observations were carried out during the post-challenge maintenance period as supporting data.

Meanwhile, the clinical symptoms observation in the Pacific white shrimp was performed following the procedure provided by **Lightner (2011)** to determine the symptoms of abnormalities, especially in the treatment group, that was tested against WSSV.

Evaluation of hematological parameters

Total hemocyte counts (THC)

A mixture of 2µL hemolymph with anticoagulant was diluted with 8mL of 0.8% NaCl in distilled water. Furthermore, the hemolymph was placed in a hemocytometer to measure the total number of hemocytes using a light microscope (Axioskop, Zeiss, Germany) with 400 times magnification and counted by hand counter. The total of the five observations in the chamber Hemacytometer was multiplied by $1x10^6$ cells/mL **(Robalino *et al.*, 2004)**.

Differential hemocyte counts (DHC)

The hemocyte differential counts was aimed to determine the number, types, and percentage of hemocyte cells. Shrimp hemolymph was dropped onto a slide and a blood smear was prepared with Giemsa stain. The number of granular and hyaline hemocytes was counted up to 100 cells and the percentage was searched for each treatment ***(Robalino et al.*, 2004)**.

Phenoloxidase activity (PO)

The PO activity was measured spectrophotometrically by recording the formation of dopachrome produced by L-dihydroxyphenylalanine (L-DOPA) using the method by **Liu (2004)** with several modifications. In short, 100µL hemolymph was mixed with 100µL phosphate-buffered saline, then centrifuged at 277 ×g for 10 minutes at 4ºC. The supernatant was discarded and the pellets were slowly resuspended by adding 1.0mL cacodylate-citrate buffer solution and recentrifuged at 277 ×g for 10 minutes at 4ºC. The supernatant formed was removed and 200µL cacodylate-citrate buffer was added. The 100µL cell suspension was incubated with 50µL trypsin as an activator for 10 minutes at 25- 26ºC. Furthermore, 50µL L-DOPA was added, left for 5 minutes and 800µL cacodylate buffer was added. Optical density (OD) was measured using a spectrophotometer (R-Biopharm Well, Reader, Germany) at 49nm. OD value of PO activity expressed as dopachrome formation in 100µL hemolymph.

Gene related-immunity

The purpose of the gene expression test related to shrimp immunity was to determine the effects of administering immunostimulant materials and WSSV infection on the expression of genes associated with shrimp immunity. This expression was measured at the conclusion of the study period (7th day). The gene expression analysis followed the procedure outlined by **Yudiati *et al.* (2016)**, involving the following steps: 1. Isolation of hemolymph RNA using TRIzol reagent (Thermo Fisher Scientific, USA); 2. Measurement of RNA concentration and purity using a Nanodrop spectrophotometer (NanoDrop 1000, Thermo Scientific, USA) at a wavelength of 260/280nm; 3. Synthesis of cDNA (Reverse transcription) by converting the previously measured RNA into cDNA

using the Reverse transcription method. With nuclease-free water, the RNA concentration was equalized to 100ng/ l. Then, as much as 20l (10m) of a mixture of R primers from each target gene (*c-type lectin* and *proPO*) and an internal control gene (β-*actin*) was prepared. The primary sequences of genes associated with immunity are shown in Table (1) below.

Table 1. The list of primers related to shrimp immunity used in this study

Gene	Primer	Sequence	Acc. number	References
c-type lectin	*lectin* V-F	TTTGTAAACAACAGGCAG TTCCAC	EF583939.1	**(Subaidah *et al.*, 2012)**
	lectin V-R	CTGTCTTTCATCAGAATGCTACCT C		
proPO	*propo* -F	TTCAACGGTAGACCCGTGATTCTT C	AY723296.1	**(Yudiati *et al.*, 2016, 2019)**
	propo -R	TCTTGCCGGGTTTAAGGTGAACAGT		
β-actin	*βac*-F	CCTCCACCATGAAGATCAAGATCAT	AF300705.2	**(Subaidah *et al.*, 2012; Setyawan 2019)**
	βac-R	CACTCCTGTGAA CAATTGATGGTC		

The following step was to determine gene expression using Real Time quantitative PCR, which refers to the standard real time PCR protocol obtained from a previous study by **Nolan *et al.* (2006)**. Briefly, all the qPCR materials along with the cDNA template were mixed in a 96 well microplate specifically designed for qPCR. The sample was then put into the qPCR 7500 Fast Real-Time PCR System (Applied Biosystem, USA) machine and then adjusted to the PCR conditions. The melting curve was adjusted automatically by the PCR machine according to the probe used (SYBR Green) to prevent false positives. The DNA melting temperature of each gene was determined from the melting curve which showed a single peak. Then, the Cycle Threshold (CT) of each gene was recorded and then processed according to the equation of **Livak and Schmittgen (2001)**.

Survival rate

The protective ability was determined from the survival of Pacific white shrimp observed for 7 days after infection with WSSV or at the end of observation ***(*Robalino *et al.*, 2004)*** using the following formula:

$$Survival\ rate\ (\%) = \frac{Nt}{No} x100$$

Note: Nt = number of shrimp at the end of observation (individual)
No = number of shrimp early observation (individual)

The following step was to determine gene expression using real time quantitative PCR, which refers to the standard real time PCR protocol obtained from a previous study by **Nolan *et al.* (2006)**. Briefly, all the qPCR materials along with the cDNA template were mixed in a 96 well microplate specifically designed for qPCR. The sample was then put into the qPCR 7500 Fast Real-Time PCR System (Applied Biosystem, USA) machine and then adjusted to the PCR conditions. The melting curve was adjusted automatically by the PCR machine according to the probe used (SYBR Green) to prevent false positives. The DNA melting temperature of each gene was determined from the melting curve which showed a single peak. Then, the cycle threshold of each gene was recorded and then processed according to the equation of **Livak and Schmittgen (2001)**.

Data analysis

SPSS 23.0 (IBM Corporation, USA) was used to analyze the collected data. The normality of the data was tested using the Kolmogorov-Smirnov test. The results were evaluated using paired *t*-test (pre- and post-test) and independent test between groups with a confidence interval of $P< 0.05$ to determine the significant difference between each treatment. The gene expression measurement data were analyzed using the method of **Livak and Schmittgen (2001)**, namely by determining the fold change value ($2^{-\Delta\Delta CT}$), so that the relative changes in treatment gene expression were known in comparison to control shrimp and internal control genes. These results may indicate up-regulation or down-regulation of the target gene. The equation used is:

$$\text{Fold change} = 2^{-\Delta\Delta CT}$$

The results of observations were displayed in the form of pictures and tables. The graphic display was processed using GraphPad Prism 8.0 (GraphPad Software, Inc., San Diego, CA).

RESULTS

Total hemocyte counts (THC)

The THC values of the control and treatment groups were not significantly different (Fig. 1) before infection with WSSV. However, an increase observed occur in the treatment group after CPZp immersion on day 7 of 3.72 cells/mL, or significantly different compared to the control group of 0.44 cells/mL after WSSV infection. These results indicated that CPZp was able to maintain the amount of THC after WSSV infection. Taken together, these results indicated that the administration of CPZp after WSSV infection has protective properties by maintaining the amount of THC under normal conditions.

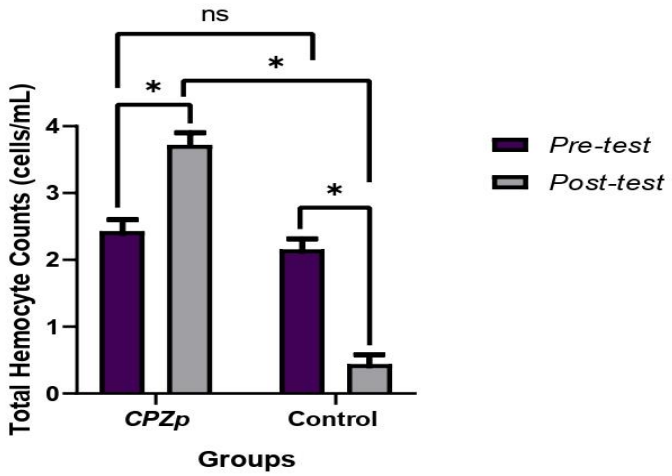

Fig. 1. Total hemocyte counts (THC) levels of Pacific white shrimp infected with white spot syndrome virus (WSSV). Based on paired *t*-test (pre-and post-test) and an independent test between groups, the * (asterisk sign) indicates a significant difference *(P<0.05)*, whereas NS indicates no significant difference *(P>0.05)*.

Granular and hyaline hemocytes

In the pre-test conditions, the mean granular and hyaline hemocyte of Pacific white shrimp did not show a significant difference ($P > 0.05$) between the two groups in this study. However, significant changes ($P < 0.05$) were observed in the mean granular and hyaline hemocytes between the CPZp-treated group and the WSSV-infected control group. Our study confirmed that the increase in granular and hyaline cells in the CPZp and WSSV-infected groups occurred as a result of receptor indications on hemocytes recognizing stimulants and responding quickly through the increased numbers of hyaline and granular cells (Fig. 2).

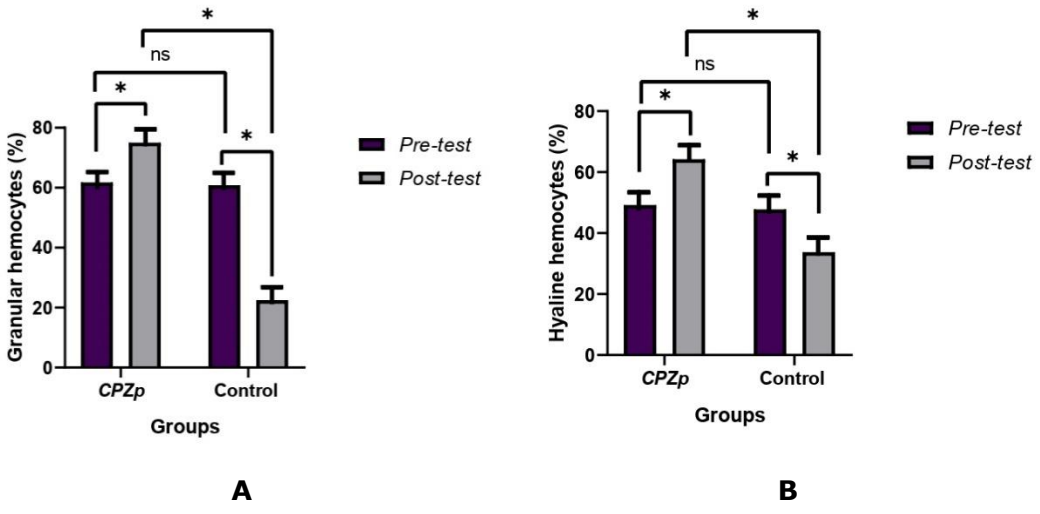

Fig. 2. A Granular and **B.** hyaline hemocytes of Pacific white shrimp infected with white spot syndrome virus (WSSV). Based on paired *t*-test (pre-and post-test) and an

independent test between groups, the * (asterisk sign) indicates a significant difference *(P<0.05)*, whereas NS indicates no significant difference *(P>0.05)*.

Phenoloxidase activity (PO)

The investigation found that Pacific white shrimp, before being infected with WSSV, had a mean value of PO activity that was not significantly different in the tested two groups. The PO activities in the CPZp-treated group and control groups before WSSV infection were 0.314 U/min/mg and 0.278 U/min/mg, respectively. The CPZp-treated group substantially showed an increased PO activity to 0.48 U/min/mg, which was significantly higher than the control group at 0.11 U/min/mg post-WSSV infection (post-test) (Fig. 3). These results confirm that the administration of CPZp acted as a curative agent against WSSV infection by increasing the activity of the PO enzyme, which in turn had an effect on the non-specific immune response of the Pacific white shrimp.

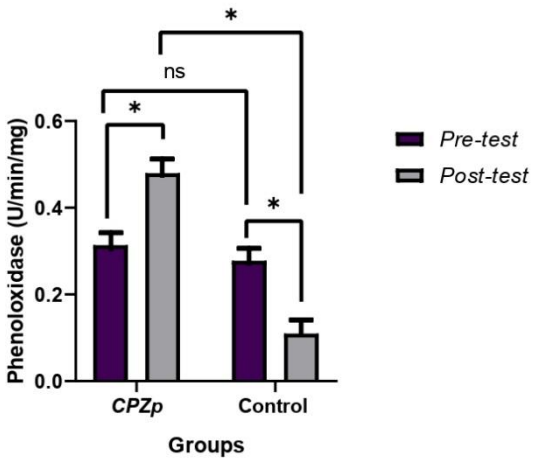

Fig. 3. The measurement of phenoloxidase (PO) enzyme activity of Pacific white shrimp infected with white spot syndrome virus (WSSV). Based on paired *t*-test (pre-and post-test) and an independent test between groups, the *(asterisk sign) indicates a significant difference ($P< 0.05$), whereas NS indicates no significant difference($P>0.05$).

Immune-related gene expression

The administration of CPZp significantly increased the mRNA expression of the *c-type lectin* and *propo* genes investigated in this study. The mRNA expressions of both genes were significantly upregulated at day 7-post WSSV infection and CPZp administration (Figure 4). The mRNA expression of the *c-type lectin* gene was significantly higher in the CPZp-treated group than that in the control group ($P<0.05$) at 7^{th} day observation. Likewise, the mRNA expression of the *proPO* gene was significantly higher in the CPZp-treated group compared to the control group after WSSV infection ($P< 0.05$).

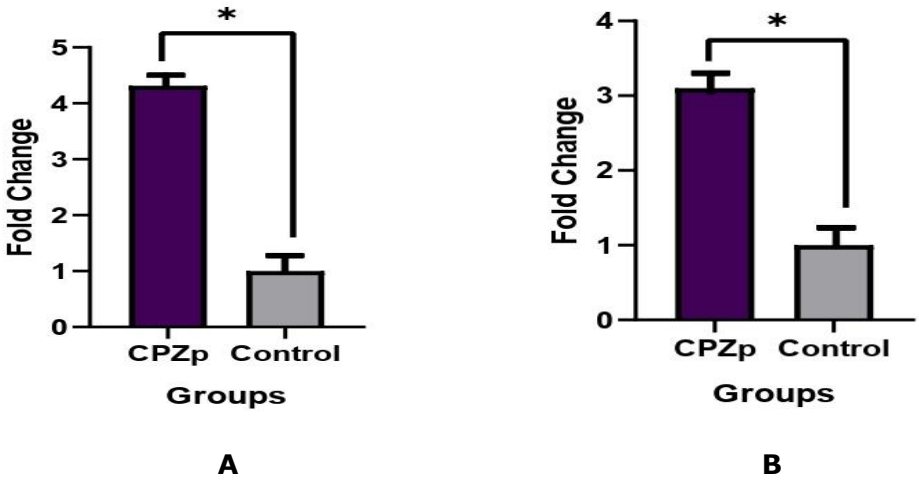

Fig. 4. A. *c-type lectin* and **B.** *proPO* gene expression at mRNA levels of Pacific white shrimp post-infected with white spot syndrome virus (WSSV). The * (asterisk sign) indicates a significant difference (P<0.05).

Survival rate

There was a difference in the survival rate of Pacific white shrimp between the two treatments. The overall result of survival was achieved by Pacific white shrimp inoculated with CPZp and infected with WSSV (P1). Survival was 10% in the P2 therapy group that was exclusively infected with WSSV. The Pacific white shrimp survival data were collected on the seventh day (D-7) after WSSV challenge and delivery of CPZp (Fig. 5).

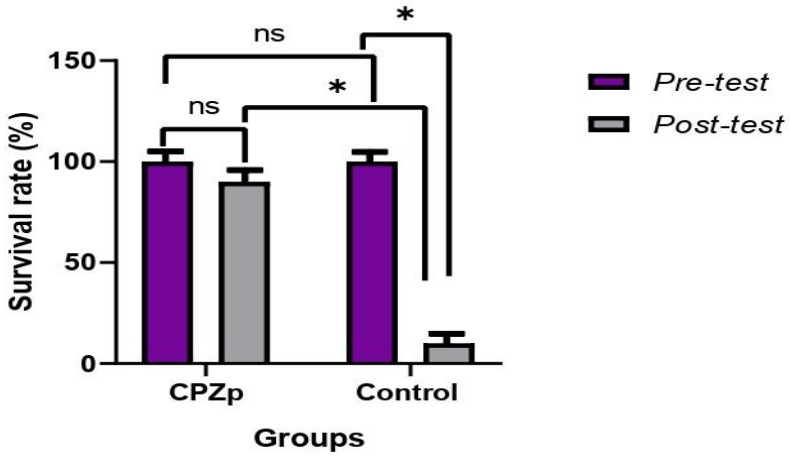

Fig. 5. Effect of CPZp on survival rate (%) of Pacific white shrimp infected by White Spot Syndrome Virus (WSSV). The * (asterisk sign) indicates a significant difference (P<0.05).

DISCUSSION

Disease attacks in the Pacific white shrimp culture system continue to be a concern for farmers since they can result in high mortality rates and significant economic losses. This study establishes the role of CPZp as an immunostimulant by increasing and maintaining a higher THC value in WSSV-infected Pacific white shrimp compared to the control group. Our results are supported by a previous study of **Setyawan (2019)** which stated that the THC amount of normal Pacific white shrimp ranges from 1.2×10^6 - 2.0×10^6 cells/mL. Similar results were also reported by **Prastiti (2017)**, who stated that the decreased THC value of Pacific white shrimp after the challenge test could indicate immune defense functioning. The decreased number of hemocytes is an effect of the shrimp defense system caused by infiltration and hemocyte accumulation in inflamed tissues due to the occurring infection and the death of hemocyte cells as the final result of apoptosis **(Cui *et al.*, 2020; Bouallegui, 2021)**. Treatment with an immunostimulant is also the first step that can be taken to restore the immune system which decreased due to the infection detected in the Pacific white shrimp. This can be observed from the increase in THC after booster administration on the 7^{th} day of observation. We recommended using immunostimulants during the larval phase or early development of shrimp, which have not yet formed an ideal immune system physiologically. Another reason for routine immunostimulant administration is that shrimp hemocytes have a very short lifespan (130 hours or about 5 days) **(Lin *et al.*, 2012)**, therefore the booster is very effective in restoring the immune system of post-infection shrimp.

The DHC in granular and hyaline Pacific white shrimp also increased due to the CPZp immunostimulant. The Pacific white shrimp hemocytes have three types of cells, namely granular, semi-granular, and granular cells. According to **Johansson and Soderhall (1989)**, the three types of hemocyte cells have a particular role in increasing immunity against foreign body infection, including pathogenic invasion, namely by carrying out phagocytosis, coagulation, and PO activities. **Jasmanindar *et al.* (2018)** reported the increase in hyaline cells at 24 hours after immunostimulant administration from *Gracillaria verrucosa* extract. Likewise, **Kitikiew *et al.* (2013)** reported that fucoidan in feed at doses of 0.5, 1.0, and 2.0g/ kg of feed can increase the number of hyaline cells after 14 and 21 days.

Decreased granular hemocytes following WSSV challenge can be attributed to granular cells protecting the shrimp body from WSSV attack by producing PO, which play a role in the non-specific immune system **(Yeh *et al.*, 2009)**. These results indicated that the CPZp immunostimulant material is able to stimulate the formation of granular hemocytes **(Sritunyalucksana *et al.*, 2005)** and inhibit WSSV invasion/replication in shrimp bodies **(Citarasu *et al.*, 2006; Flores-Miranda *et al.*, 2011; Zhao *et al.*, 2011)**. The granular hemocytes will be stimulated and degranulated spontaneously if there are active compounds or ingredients such as peptidoglycan **(Vargas-Albores & Yepiz-**

Plascencia, 2000). Active compounds may also include β-glucan **(Bai *et al.*, 2014; Li *et al.*, 2019; Sánchez-Salgado *et al.*, 2019)** and LPS **(Xu *et al.*, 2015)**. The decrease in the number of granular hemocytes on day 4 was also described by **Amparyup *et al.* (2009)** and **Kitikiew *et al.* (2013)**, who stated that several immunostimulant substances administered to shrimp actually showed the ability to activate the ProPO system, which can be initiated by lysis of granular and semi-granular cells against invading pathogens such as WSSV. In this context, **Mahasri (2007)** explained that the increase in granular hemocytes is due to Pacific white shrimp having no memory cells like other aquatic vertebrates, so they are unable to detect pathogenic materials. It can be argued that immunostimulant can induce a shrimp defense mechanism by increasing DHC numbers.

PO enzyme is an active product due to the activation of ProPO enzyme or proteolytical to PO through the serine proteinase cascade system to form quinine-quinone and other anti-microbial compounds **(Cerenius & Soderhall, 2004)**. The PO enzyme is able to catalyze the conversion of tyrosine to dihydroxyphenylalanine (DOPA), which is then converted to DOPA-quinone as a precursor to melanin **(Shao *et al.*, 2019)**. Melanin is a brownish-black pigment that inhibits fungal and bacterial activities **(Nosanchuk & Casadevall, 2006)**. THC and PO levels synergize, with granular hemocytes producing and releasing PO into the hemolymph in the form of an inactive pro-enzyme called PO **(Rahardjo *et al.*, 2022)**. Under normal conditions, the increase in the number of hemocytes is accompanied by an increase in PO **(Fagutao *et al.*, 2009; Hirono *et al.*, 2011)**.

C-type lectin plays an important role in innate immunity to be able to recognize and eliminate pathogens efficiently in crustaceans. The expression of *c-type lectin* has been studied and identified both in tissue and in response to a pathogenic stimulation in Pacific white shrimp **(Wei *et al.*, 2012)**. C-type lectins are known to belong to the pattern recognition receptor family, which can interact with pathogen-associated molecular patterns of invading microorganisms through a carbohydrate recognition domain approach. This interaction can cause a variety of immune responses through direct or indirect mechanisms **(Runsaeng *et al.*, 2018)**. In this context, the mRNA expression level of the *c-type lectin* gene was known to be upregulated after CPZp treatment even though WSSV infection occurred at the same time, indicating that CPZp was able to work through a molecular mechanism to inhibit virus attachment to the shrimp receptor.

These granular cells are the main repository for the ProPO system and are responsible for the storage and release of antimicrobial peptides (AMP) and various proteinase inhibitors **(Sricharoen *et al.*, 2005; Sutthangkul *et al.*, 2015)**. The results of this study show that the ProPO system, which is expressed and detected through the mRNA expression of immunity-related genes, plays a critical role in shrimp immune defense against WSSV pathogens, particularly in the control group. Furthermore, *proPO* gene expression was activated in groups that received both CPZp and WSSV infection, indicating that CPZp inhibition of WSSV infection occurred primarily through increased

activity of *proPO* gene mRNA expression in shrimp bodies. This is the first report demonstrating the effect of CPZp on modulating the immune response of WSSV-infected Pacific white shrimp through altering the mRNA expression of immunity-associated genes. The immunostimulant properties of CPZp can be useful for the health of cultured shrimp, although further research is still needed to prove this. The mRNA expression of the *proPO* gene in this study was also upregulated on the 7th day post WSSV infection. ProPO can be interpreted as a crucial encapsulation mechanism utilized by numerous invertebrates, such as shrimp, for granular cell maturation **(Leu *et al.*, 2013)**. These granular cells are the main repository for the ProPO system and are responsible for the storage and release of Antimicrobial Peptides (AMP) and various proteinase inhibitor.

The Pacific white shrimp survival data were collected on the seventh day (D-7) after WSSV challenge and delivery of CPZp. These findings support CPZp's protective effects as an immunostimulant in neutralizing WSSV infection in the Pacific white shrimp. Another discovery was that giving nucleotides and RNA from yeast extract at a level of 0.5 - 1 gram/kg of feed increased the survival of the Pacific white shrimp when they were challenged with *Vibrio harveyi* **(Novriadi *et al.*, 2021)**. Increased immune response has a significant role in shrimp survival during illness. Other putative survival strategies might be connected to water quality **(Kamaruddin *et al.*, 2021)** and shrimp stress levels **(Wiradana *et al.*, 2022)**.

CONCLUSION

Overall, the results showed that the ProPO system, as expressed by the mRNA expression of the gene, plays a crucial role in the immune defense of shrimp against WSSV pathogens, especially in the control group. On the other hand, the activation of *proPO* gene was enhanced in groups that received CPZp and WSSV infection, indicating that inhibition of WSSV infection by CPZp mostly occurred through the upregulation of mRNA expression of *proPO* and *c-type lectin* genes in the shrimp bodies. This is the first report demonstrating the effect of CPZp on modulating the immune response of WSSV-infected Pacific white shrimp through mRNA expression of immunity-associated genes. The immunostimulant properties of CPZp can be useful for the health of cultured shrimp, although further research is still needed to prove and unravel this.

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