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Research Article

The Effect of Immersion Duration of *Zoothamnium penaei* Crude Protein Extracts to Stimulate Immune System in *Litopenaeus vannamei* Against *Enterocytozoon hepatopenaei* (EHP)

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Abstract

Enterocytozoon hepatopenaei (EHP) is a spore-forming microsporidia intracellular parasite, which slough off the cells of the hepatopancreases of the shrimp. This study was intended to determine the effect of immersion duration and maintenance time to stimulate the immune system of shrimps by using a crude protein from *Zoothamnium penaei* to protect against EHP infections. The study used a completely randomized design where shrimps were divided into four groups each having five replicates. Groups P1, P2, P3 were immersed in immunostimulant for 10, 15, and 20 minutes respectively, except negative control. The phenoloxidase, phagocytosis activities, histopathology study were determined. Also, the molecular diagnosis of EHP was measured to confirm EHP infection in the shrimp samples. The phenoloxidase activity significantly increased after immersion for 20 minutes (P3) from 0.64-, 0.72-, and 0.78-units min^{-1} mil^{-1} of protein on the first week, second week and third week, respectively. The phagocytosis activities of the sample immersed for 20 minutes were significantly increased to 68% on the second week. The sample also showed improved hepatopancreases epithelial cells with only 26% necrotic cell, less EHP spore and vacuolation. There was an interaction between immersion duration and maintenance time of the *L. vannamei* immersed in crude protein from *Zoothamnium penaei*. Twenty minutes immersion were significantly found to stimulate the immune system of the shrimp against EHP. The present work revealed that, the application of crude protein from *Z. penaei* is effective against EHP in shrimp culture.

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1. Introduction

Enterocytozoon hepatopenaei (EHP) is a microsporidia parasite that infects the hepatopancreas of the shrimp, a disease called microsporidiosis. The clinical signs of microsporidiosis are hardly noticeable, although reports have shown that microsporidiosis is associated with slow growth. A previous study by Caro *et al.* (2020) has also provided evidence that EHP is associated with white feces syndrome (WFS). It is crucial to find a prophylaxis methodology to improve the shrimp immune system without causing harm to consumers and the environment in general.

Immunostimulant is an immune prophylaxis technique that enhances the immune system, primarily the innate immunity protecting the shrimp from different pathogenic diseases since crustaceans do not have adaptive immunity (Apines-Amar and Amar, 2015). Immunostimulants can either be chemical drugs that involve synthetic chemicals (Tassakka *et al*., 2020), whole bacteria (Flores-Miranda *et al*., 2011), immunogenic polysaccharides (Mohan *et al*., 2019), animal or plant extracts (Bairwa *et al*., 2012), probiotics and pre-biotic (Ganguly *et al*., 2010), nutritional feeds factors, immunogenic membrane, and cytokines (Barman *et al*., 2013). The secretion of innate immune of crustaceans after the application of immunostimulant involves prophenoloxidase activation, phenoloxidase, melanisation, anti-microbial peptide activation, and release of reactive oxygen species. On the other hand, cellular immunity involves encapsulation, nodulation and improves phagocytosis (Sritunyalucksana *et al*., 1999). Due to the significant loss caused by EHP to the farmers, measures should be taken to prevent the spread of EHP (Tang *et al*., 2017; Nkuba *et al*., 2021).

In immersion vaccination the immunostimulant is taken up by the exoskeleton of the shrimps, gills, and others that pass in the oral tract. When inside the body, the immune system starts to process it, responding to the elicitation of resistant compounds. Sajeevan *et al.* (2009) and Woraprayote *et al.* (2020) reported that overdosage leads to immunosuppression while the under dosage cannot stimulate the shrimp immune system.

A crude protein from *Zoothamnium penaei* with a size of 38, 48 and 67Kda was found to improve the innate immune system of white leg shrimp *Litopenaeus vannamei* by raising the hemocytes count and differential hemocytes count (Hidayat, 2017). In addition, it was found a high survival rate in a shrimp treated with immunostimulant from *Zoothamnium penaei* from 17% to 68% (Mahasri *et al*., 2018). However, the previous research were not able to show the effects of crude extracts from *Zoothamnium penaei* and its interaction on immersion duration against EHP infection. This study used the same crude protein from *Zoothamnium penaei* to determine the adequate immersion duration time for improving the immune system of the shrimp sample and protecting against the EHP microsporidia infection.

2. Materials and Methods

2.1 Shrimp Sample for Experiment

White leg shrimp *Litopenaeus vannamei* obtained randomly from their grow-out pond from a shrimp farm located in Boncong, Tuban Regency, Indonesia. The shrimps were 40 days old with an average body weight of 6 ± 0.2 g and length 7±0.5 cm. The shrimps were first acclimatized in tanks for one week with an average temperature of 28°C to 30°C. After acclimatization, shrimps were grouped into four groups according to their treatment (P0, P1, P2, and P3) 20L each. Each group has five replicates, making a total of 20 aquaria. Each aquarium was stocked with 50 shrimps. The complete randomized design (CRD) was used in this experiment.

2.2 The Crude Protein from Zoothamnium penaei

The immunostimulant used in this study were kindly provided by (Mahasri *et al*., 2018). Briefly to obtain crude extracts 6×10^8 of the colonies of *Zoothamnium penaei* were suspended with 10mM PBS (1 mL of 1 M Tris-HCl pH 7.4 to 99 mL of nuclease-free water.) then 100µL protease inhibitor was followed. The suspension was centrifuged to separate the insoluble cellular debris and supernatants containing whole protein extracts. Subsequently, the supernatants were taken and further resolved with 0.5% Nonidet P40 in a protease inhibitor solution. Sonication was then performed while stirring the mixture at medium speed. The suspension was then centrifuged at 4,000 rpm min^{-1} 4°C for 30 minutes and then it was collected for measurement. Protein measurement was conducted by SDS page electrophoresis.

2.3 The Immersion Treatment to the Crude Protein

Shrimp samples were randomly divided into four groups (30 shrimps per group in four separate aquaria according to their respective immersion duration): 0 minutes (negative control), 10 minutes, 15 minutes, and 20 minutes. Each group were set up in quintuplicate (five-fold). Shrimp were immersed in the

solution containing immunostimulant (3mg/L) after every seven days (Harijanto, 2012).

2.4 DNA Extraction and PCR Amplification

DNA extraction and amplification were done as in Rajendran *et al.* (2016) with some modifications. The genomic material for amplification was extracted from the hepatopancreas of the shrimp sample. 30mg of hepatopancreas was first mixed with 0.1 mg of proteinase K, lysis buffer solution (Tetra-acetic (EDTA) diamine of 1 m (50 mMTris), NaCl (500 mM) and 1% SDS) and then were homogenized and digested for approximately 15 minutes at 95°C. After homogenization, the mixture was centrifuged at 12,000 rpm for 10 minutes at 5°C. The supernatant from centrifugation was collected, and pure ethanol was added and kept at -20°C for 60 minutes. Next, the mixture was centrifuged at 12,000 rpm for 15 minutes at 5°C. Then the pellets were obtained and washed, ready for PCR amplification.

In this study, EHP was diagnosed by targeting its small 18S SU rRNA gene. The one-step PCR was used to detect the 510bp genes of EHP. The primers used to detect EHP were forward and reverse (510R: 5'-GCGTACTATCCCCAGAGCCCGA-3'; 510F: 5'-GCCTGAGAGATGGCTCCCACGT-3') (Tang *et al.*, 2017). The amplification was done with alternating change in temperature, denaturation was held at 95°C for 20 seconds, followed by annealing at 55°C, 30 seconds, and extension was done at 72°C for five minutes.

2.5 Haemolymph Collection Procedure

To evaluate the immune parameters such as phagocytosis and phenoloxidase (PO) activities, the shrimp haemolymph was collected from near the walking legs by using a 1 mL syringe. Anti-coagulant was prepared from sodium citrate to prevent the clotting of the haemolymph. A 1 mL syringe was used to withdraw 0.4 mL of anti-coagulant.

2.6 Phenoloxidase Activity

Phenoloxidase was measured by using a spectrophotometer following the procedure of (Yudiati *et al.* (2016). The dopachrome formation was recorded using the L-DOPA or L-dihydroxyphenylalanine as a substrate and used to read the amount of phenoloxidase in the haemolymph. 100 µL haemolymph was mixed with 100 µL of phosphate buffer saline (PBS) for dilution and then centrifuged at 700 g, at 4°C for 20 minutes. Then separated plasma (the pellets) was resuspended to 100 µL of cacodylate citrate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate, pH 7.0) and then centrifuged at 700 g, for 20 minutes at 4°C while the supernatant was discarded. The pellets were then re-suspended in 200 cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.01 M calcium chloride, 0.26 M magnesium chloride, pH 7.0). Then the solution was separated into two aliquots 100 µL each. 100 µL aliquot was activated with 50 µL trypsin for 10 minutes at 26°C. After activation, 50 µL of L-DOPA was then added followed by 800 µL of cacodylate buffer. Then the phenoloxidase activity was measured by reading the optical density in the spectrophotometer (Hitachi U-2000) at 490 nm. The published procedure from Lowry *et al.* (1951) was used to determine the protein content of hemocytic lysate from the shrimp samples. The rise in optical density (OD) per minute per milligrams (unit min^{-1} mg^{-1} of protein) was calculated as phenoloxidase activity.

2.7 Phagocytosis Activities

The phagocytosis activities were determined based on the procedure by (Yudiati *et al.*, 2016) with some modification, briefly, 20 µL of the haemolymph was mixed with 20 µL of PBS in micro well-plate. In the mixture, 20 µL of 10^8 cells/ ml of killed *Vibrio alginolyticus* was added. Then from the mixture 7 µL will be smeared gently and fixed with 95% ethanol, then washed with water. Next, the staining with 10% Giemsa for 20 minutes followed by washing and air-drying. Then the cells were visualized in the light microscope.

The phagocytic activity was calculated as Phagocytic rate as follow:

$$\text{Phagocytic rate (PR) } 100 = \times 100 \qquad \ldots\text{Eq (1)}$$

2.8 Histopathology

The histopathology study was performed using the haematoxylin & Eosin (H&E) staining technique following Rajendran *et al.* (2016) method with modification. The sample was taken and kept on ice for 60 minutes before dissection. Then were injected with Davidson's fixative agent into the hepatopancreas. Then hepatopancreas was dissected out and kept in Davidson's fixative agent for two days. Pre-embed of the tissues were followed with dehydrating in a series of alcohol concentrated liquids with 70%, 90%, and 100%. Then after dehydration, the tissues were treated twice in clearing agent xylene for one hour. The embedding of the tissues was followed by carefully positioning the samples infiltrated by paraffin inside base mold. Series

of sectioning was done by cutting 3 µm sections from the paraffin sample. Then the tissues were stained following the manual protocol by using H&E. Briefly, the slide was dipped in hematoxylin then washed with running water. Then was dipped in acid alcohol for two seconds before being dipped into the 0.1% aqueous eosin solution. The slide was washed and covered with thin glass. Finally, the stained tissues were mounted in a microscope and observed.

2.9 Data Analysis

Data were analyzed by statistical package for the social science (SPSS) 26 for windows. The Kolmogorov-Smirnov and Leven test was used to test the distribution and homogeneity of the data. The data of phenoloxidase, phagocytosis activities and the percentage of necrotic cells from histopathology were analyzed.

3. Results and Discussion

3.1 The Effects of Crude Protein Immunostimulant on Phenoloxidase Activity

PO activity was significantly higher in the group of shrimps immersed in crude protein P1, P2 and P3 when compared to P0 ($p>0.05$). Generally, phenoloxidase activity increased in the immersed group with the highest on the third week (Table 1).

Table 1. The effect of crude protein from *Zoothamnium penaei* on phenoloxidase activity (PO) on the first week, second week and third week.

	Phenoloxidase (unit min^{-1} mg^{-1} of protein) ± SD			
	P0	**P1**	**P2**	**P3**
Week 1	0.42^{a}	0.63^{c}	0.59^{b}	0.64^{c}
Week 2	0.52^{a}	0.71^{b}	0.70^{b}	0.72^{b}
Week 3	0.45^{a}	0.74^{bc}	0.72^{b}	0.78^{c}

P0 negative control, P1; immersed for 10 minutes, P2; immersed for 15 minutes, P3; immersed for 20 minutes. The difference in letters on each value indicates a significant difference ($p<0.05$).

The detection of immunostimulants led to activation of serine protease cascade, which involves proPO, PO, and other ant-microbial compounds, it is, therefore, caused the increase in the activity of PO (Maftuch *et al*., 2013). The result showed the highest phenoloxidase activity of 0.78 unit on shrimp immersed for 20 minutes in the third week. The same trend of the change of immune parameters after immersion of shrimp in the immunostimulant was observed by (Citarasu *et al*., 2006). Sung *et al*. (1994) reported the increase in the phenoloxidase and completely clearance of *Vibrio vulnificus* 24h following challenge test after immersion of shrimps in immunostimulants.

The variation in PO activities might be due to factors such as moult patterns of the shrimp (Xu *et al*., 2020), which causes the change in the immune response of crustaceans in the pre-moult stage and post-moult stage. Another factor demonstrated by Hernroth *et al*. (2012) that variation in environmental temperature, carbon dioxide amount and water pH level cause significant immune suppression to the crustaceans.

3.2 The Correlation between the Maintenance Time, Immersion Time with Phenoloxidase Activity on Shrimp Immersed on Zoothamnium penaei

The results showed a significant relationship between the immersion time and maintenance time with phenoloxidase activity (Figure 1). These results suggest that phenoloxidase activity depends not only on the amount of crude protein and its contents but also on the duration of immersion and maintenance. The same relationship of the dose frequency, time for maintenance of sample and immunostimulatory effects were obtained (Ojerio *et al*., 2018).

In the current study, immersion treatment was performed on day one and day seven. The second treatment was done to elevate the immune system enough to protect against pathogens. It was also explained in the previous experiment that after the sixth day, the immune parameter such as proPO, haematological factors such as THC and Oxyhaemocyanin was decreased, which then improved after the second treatment (Citarasu *et al*., 2006). In another study (Sajeevan *et al*., 2009) which investigated the dose frequency for the maximum survival of the shrimp sample reported that the sample which receives the dose every seven days for 21 days achieve maximum survival rate and improves in immune parameters as compared to the sample which receives once daily, once every two days, once every five days and once every ten days. It is considered that immunostimulants could be used on the shrimps to elevate the immune system for around six to seventh days; therefore, continuous administration of immunostimulants to the shrimps is necessary to ensure high immune protection against pathogens.

Table 2. The effect of crude protein from *Zoothamnium penaei* on phagocytosis activities on the first week, second week, and the third.

	Phagocytosis (%)			
	P0	**P1**	**P2**	**P3**
Week 1	32[a]	41[b]	47[b]	51[d]
Week 2	31[a]	71[d]	50[b]	68[c]
Week 3	28[a]	60[d]	41[b]	53[c]

P0 negative control, P1; immersed for 10 minutes, P2; immersed for 15 minutes, P3; immersed for 20 minutes. The difference in letters on each value indicates a significant difference ($p<0.05$)

3.3 The Effects of Crude Protein Immunostimulant on Phagocytosis Activities

Phagocytosis activity was significantly higher in shrimp immersed in crude protein for 10, 15 and 20 minutes for the first week, compared to the non-immersed control group (Table 2). These results agree with (Pope *et al*., 2011) reported the increase in the phagocytic activities and higher survival of the shrimp from *Vibrio harveyi* after stimulating the immune system. In the second week, the phagocytosis activities of shrimp immersed for 10 and 20 minutes were significantly different from the control group and 15 minutes immersed shrimp. In the third week, there was a significant increase in the phagocytic activities of the shrimp immersed in crude protein for 10, 15 and 20 minutes, when compared with the control group P0. These results suggest that the stimulation of phagocytic activities observed in the immersed group is caused by the crude protein immunostimulant from *Zoothamnium penaei*. Generally, the results showed that the immersed group in P1 and P3 was highest in the percentage of phenoloxidase with no significant change in the control group. The increase in the phagocytosis activities after administration of immunostimulants is in harmony with the study of (Smith and Söderhäll, 1983; Citarasu *et al*., 2006; Fu *et al*., 2007; Zhao *et al*., 2011; Declarador *et al*., 2014; Peraza-Gómez *et al*., 2014).

3.4 The Correlation between the Maintenance, Immersion Time with Phagocytosis Activities of Immersed Shrimp in the Crude Protein of Zoothamnium penaei

Phagocytosis activities were influenced positively with the immersion time at either time duration except for the control group. In contrast, it was found that there was no significant correlation with maintenance time. Similar results have been found in (Yeh *et al*., 2006), suggesting that phagocytosis activities of the *L. vannamei* were increasing after immersion in hot-water extracts of *S. duplicatum,* which results in the increased resistance against the *Vibrio alginolyticus* after the challenge test. The ingestion and elimination of pathogens form the crucial process in the immunity of the shrimp, which protects the shrimp from diseases (Figure 2).

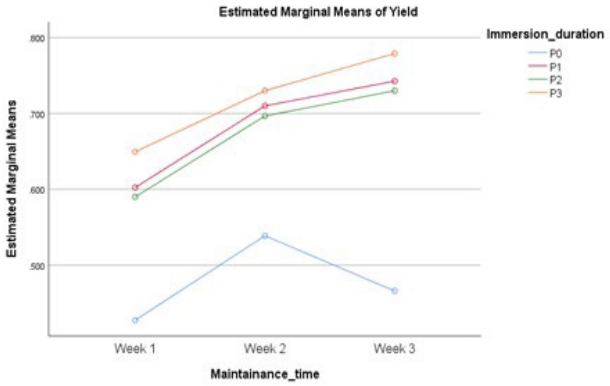

Figure 1. The interaction between the maintenance time and immersion time with PO (D); P0 negative control, P1; immersed for 10 min, P2; immersed for 15 min, P3; immersed for 20 minutes on phenoloxidase activity.

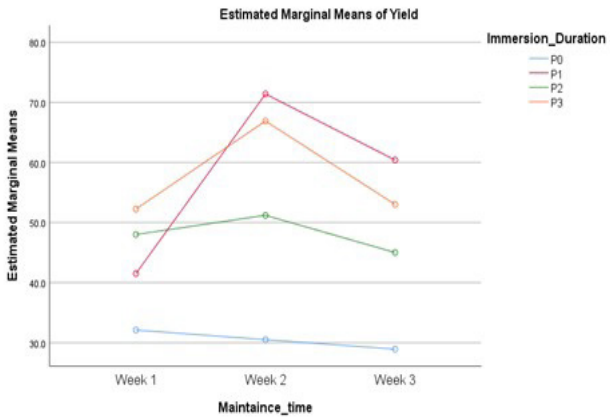

Figure 2. The interaction between the maintenance time and immersion time with PO (D); P0 negative control, P1; immersed for 10 min, P2; immersed for 15 min, P3; immersed for 20 minutes on phagocytosis activity.

3.5 Effect of Crude Protein from Zoothamnium penaei on Hepatopancreatic Cells

In the first week of maintenance, the control group P0 showed the highest percentage of necrosis (57%) while P3 were 49%. In the second week, P0 and P1 were 54%, the highest percentage of the necrotic cell, while the P2 was the lowest 33% (Table 3). In the

third week, P0 was significantly higher than P1, P2 and P3, which was 59, 39, and 28 respectively, while were P3 were 26%, which was the lowest percentage of necrotic cells than the rest. Generally, the rate of necrosis of hepatopancreatic cells was higher in the first week and lowest in the third week. The control group P0 was higher than the immersed group in the first, second and third week. These results are in harmony with the previous studies of (Huang *et al*., 2006; Babu *et al*., 2013; Declarador *et al*., 2014; Solidum *et al*., 2016).

Table 3. The effect of crude protein from *Zoothamnium penaei* on hepatopancreatic cells on the first week, second week and the third week

	Necrotic hepatopancreatic cells (%)			
	P0	**P1**	**P2**	**P3**
Week 1	57^{c}	56^{bc}	53^{ab}	49^{a}
Week 2	54^{c}	54^{c}	33^{a}	41^{b}
Week 3	56^{c}	39^{b}	28^{a}	26^{a}

The difference in letters on each value indicates a significant difference ($p<0.05$)

Table 4. PCR results before the immersion treatment

Source of the animal sample	Number of animals tested	Total positive	Total negative	Intensity of EHP (%)
From a normal grow-out pond	4	4	0	100

The photomicrographs of the groups that received the crude protein from *Zoothamnium penaei* were observed to show healthy hepatopancreas conditions. Cells such as epithelial cells, including E cell, reabsorption cell (R cell), fibrillate cell (F cell), blister cell (B cell), the rounded epithelial tubule were observed to be intact (Figure 4). In groups P0 and P1, the photomicrograph showed there were collapse, enlargement of the hepatopancreas tubules, degeneration of tubule lumen, sloughing off the hepatopancreas tubules, and degeneration and lacking epithelial cells, including the fibrillate cells (F cell), blister cells (B cell), reabsorption cells (R cell), and vacuolation in the hepatopancreas of the infected shrimp by EHP microsporidia spore (Figure 3). It showed the presence of sloughing cells, a lesion characteristic of EHP. The tubules containing numerous deep-dyed acidophilic small bodies which were fixed in the vacuoles within the cytoplasm of the hepatopancreatic cells were characterized as EHP spores. The spore was observed to measure 1.1 ± 0.2 by 0.6±0.2 µm. It was also observed the presence of numerous hemocytes cells near the infected hepatopancreatic cells.

The group immersed in the crude protein for 15 and 20 minutes in the third week were able to prevent the sloughing and necrosis of the hepatopancreases cells by 72 to 74 per cent, respectively, compared to the control group, which were 44 per cent. These results show that the crude extracts from the immunostimulant stimulated the hemocytes cells in the hepatopancreases, which prevent further sloughing and necrosis by the pathogens. The detection of EHP spores by histopathology was hardly distinguished with other similar shape and size particles such as basophilic inclusions, cytoplasmic inclusions, and others. This phenomenon might have been the source of errors when applying this method. The same observation was stated by (Tangprasittipap *et al*., 2013). Therefore, the incorporation of other high sensitivity methodology such as in situ hybridization (Santhoshkumar *et al*., 2017; Kumar *et al*., 2018) and PCR (Jaroenlak *et al*., 2016; Desrina *et al*., 2020) is highly advised.

Histopathology study showed the presence of EHP spores, lesions and sloughing off of the hepatopancreatic cells. Similar findings have been reported in (Tourtip *et al*., 2009; Rajendran *et al*., 2016). EHP cells can enter the cytoplasm of the hepatopancreases cells and use the cell nutrients to replicate. The matured spore enters the cytoplasm of the hepatopancreases by injecting its polar filament into the cell and ejecting its sporoplasm to replicate inside the cell (Aranguren *et al*., 2017). The replication results in an increase in the sporoplast and cause cell to burst and sloughing of the hepatopancreatic cells. The spore is released into the surrounding cell, and others are released out with feces through the gut. Other shrimp may become infected by ingesting spores previously released (Tourtip *et al*., 2009). Hepatopancreases is a digestive gland, which absorbs the digested food. The digestive enzymes are secreted in hepatopancreases; therefore, the rupture and sloughing of the hepatopancreatic cells result in problems in the food digestion of the shrimps. Due to digestion problems, the shrimp cannot eat and thus cause slow growth, the chief symptom of Molecular detection of EHP.

Initially, the prevalence study of EHP was performed to evaluate the intensity of EHP infection of the shrimp from their grow-out ponds. After immersion treatment to the crude protein in the third week, another molecular study to determine the intensity among the groups was performed. The results before the treatment show that all four samples were infected with EHP (Table 4). In contrast, the third-week results showed that

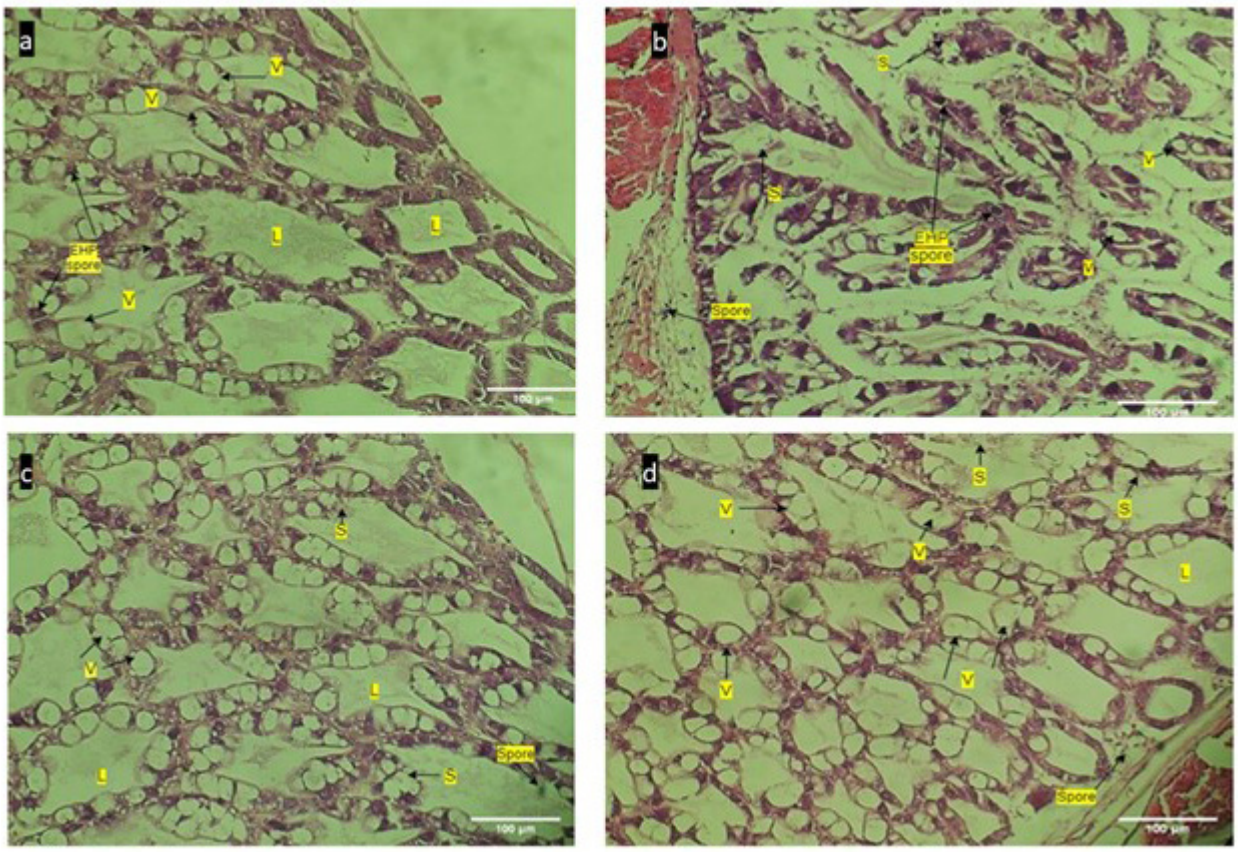

Figure 3. Photomicrograph of the hepatopancreases of the EHP-infected shrimp observed under ×200 magnification. Numerous presences of vacuolated cells (V), sloughed cells (S) around the Lumen (L) in diagram (b), (c), and (d), the presence of EHP spores in diagrams (a), (b), (c), and (d)

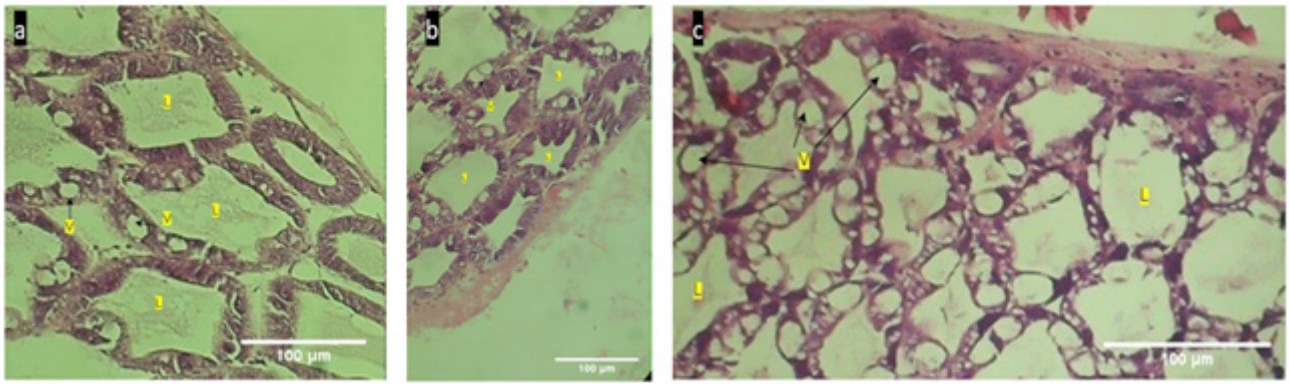

Figure 4. Photomicrograph of recovered hepatopancreases of the previously infected shrimp after immersion treatment in immunostimulant from *Zoothamnium penaei* observed under ×200 magnification. Presence of less vacuolization with improved cytoplasm of tubule epithelial cells (V). presence of structured lumen (L) with less or no EHP microsporidia spore(a), (b), and (c).

four samples from the control group were infected while P1 showed three positive EHP shrimp out of 4, P2, and P3 gave zero per cent EHP prevalence (Table 5).

The current study targeted the 18S rRNA gene of the *Enterocytozoon hepatopenaei* by using a PCR molecular detection method. It is well known that SSU rRNA genes are useful for species identification and determining the evolutionary relationship between species since the sequence of rRNA differ with the organism of different species and is the same organism of the same species (Marimuthu *et al*., 2021). Other studies that have done the molecular detection of EHP by targeting

Table 5. PCR results of week 3 after the treatment to the crude protein immunostimulant.

Treatment group	Number of animals tested (N)	Total positive (TP)	Total negative (TN)	Intensity of EHP (%) = TP / TN
P0	4	4	0	100
P1	4	3	1	75
P2	4	0	4	0
P3	4	0	4	0

the SS rRNA with nested PCR (Tangprasittipap *et al*., 2013), one-step PCR (Tourtip *et al*., 2009), visual loop-mediated isothermal amplification (LAMP) (Kumar *et al*., 2018), and real-time PCR (Mai *et al*., 2020). In contrast, a study by Kurcheti *et al.* (2019) reported one shortcoming of targeting 18S rRNA is the cross-reactivity of designed primers with other closely related species, which may give false-positive results. The presence of Species such as *Enterospora canceri*, *Nucleospora* sp., *Paranucleospora theridon* (Kurcheti *et al*., 2019) and *Enterocytozoon bieneusi* (Marimuthu *et al*., 2021) may cause false positive with the 18S rRNA detection method due to close relatedness with *Enterocytozoon hepatopenaei*. The prevalence of 75% EHP in the group of shrimps immersed in 10 minutes in the current study may be due to the false positive as explained by Kurcheti *et al*. (2019). Jaroenlak *et al.* (2016) suggested the way to solve the false positive problem by applying nested PCR to target the spore wall protein (SWP) of *Enterocytozoon hepatopenaei* due to its high sensitivity and specificity compared to SS rRNA. Therefore, future research may target the spore wall protein (SWP) of *Enterocytozoon penaei* to prevent false positive results.

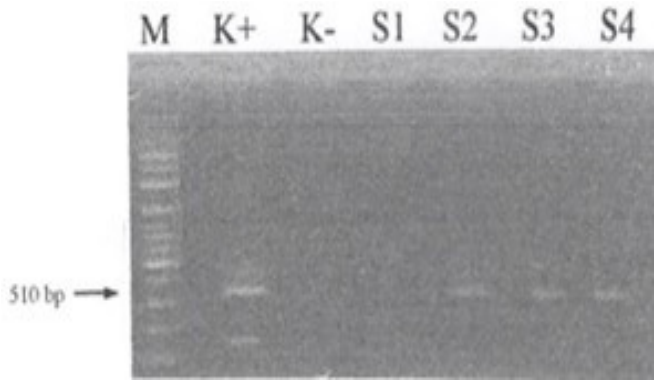

Figure 5. Agarose gel electrophoresis of the PCR products amplified from hepatopancreatic DNA of *Litopenaeus vannamei* (representatives). The primers were designed to target 18 S ss rRNA, and A 510 bp products size were obtained in the positive sample. M- molecular weight marker, K- and K+ are positive control and positive control respectively, S1-S4 are samples.

The current study showed that the immersion of shrimp in crude protein from *Zoothamnium penaei* of shrimp for 15 and 20 minutes is optimal for stimulating the innate immune system of white leg shrimp *Litopenaeus vannamei*. The results are generally in agreement with the previous study of Mahasri *et al.* (2018), Marwiyah *et al.* (2019), and Wiradana *et al.* (2019), who reported the effectivity of crude protein from *Zoothamnium penaei* on immunostimulant of the innate immune system of white leg shrimp *Litopenaeus vannamei.*

The PCR amplification showed a product size of 510bp for the positive samples (Figure 5). The DNA obtained from the hepatopancreases of the sample was subjected to PCR amplification by using primers designed to target 18S SSU rRNA of *Enterocytozoon hepatopenaei.* The results showed a product size of 510 bp in all four samples before the immersion treatment (Figure 5). Three sample shrimp from the group P0 were positive, while only one showed a negative EHP. The sample of four shrimps in group P2 and four in group P3 were negatives, and no positive samples were obtained.

4. Conclusion

The results obtained in this study showed that the immersion of shrimp in crude protein immunostimulant from *Zoothamnium penaei* for 20 minutes stimulate phenoloxidase, phagocytosis activities and protect against EHP infection. The three-week maintenance time with immersion after each 7 days of the *Litopenaeus vannamei* to the crude protein from *Zoothamnium penaei* improves the PO, phagocytosis activities and hepatopancreatic cells of the EHP infected shrimp. There is an interaction between immersion duration and maintenance time of the white leg shrimp *Litopenaeus vannamei* immersed in crude protein from *Zoothamnium penaei*, which led to an increase in phenoloxidase, phagocytosis activities, and reduction of the damaged hepatopancreatic cells while 20 minutes immersion effectively reduce the percentage of EHP intensity.

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Authors' Contributions

The authors contribution to the manuscript were as follows, Anord; collected the data and wrote the original manuscript. Gunanti; designed, supervised, reviewed, and edited the research and also managed project administration. Nunuk; reviewed, edited, and supervised the research. Mwendolwa; proofread, reviewed, and edited the final manuscript. All authors discussed and contributed to the final manuscript.

Conflict of Interest

The authors declare that they have no conflict of interest.

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