Survival of *Penaeus monodon* shrimp fed diets supplemented with extract *Porphyridium aerugineum* and *Porphyridium sp.* after challenges by *Vibrio harveyi*

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**Abstract.** The prevention of vibriosis using microalgae as anti-quorum sensing was started in 2016. Five types of positive microalgae that have the ability to inhibit the Quorum sensing signal was found in 2016, namely: *Melosira sp*, *Porphyridium sp*, *Vulgaris sp*, *Phaeodactylum sp*, and *Nannochloropsis sp*. In order to obtain an environmentally-friendly alternative to prevent shrimp disease, a study was conducted in 2019 concerning the addition of *Porphyridium sp* and *Porphyridium aeruginosus* extract into feed as immunostimulants in shrimp. About 35% of microalgae extracts were added to shrimp feed. Tiger shrimp (*P. monodon*) fry with a weight of 0.5-2g was used in this study, and experimental design of 20 shrimps/treatment/repetition was applied. After 30 days of maintenance, an artificial infection was carried out using the pathogenic *Vibrio harveyi* by injection. Parameters observed included Total Hemocyte Count (THC), Pro-PO value and percentage of shrimp survival. The results showed that the addition of *Porphyridium aerugineum* extract in shrimp feed was successful in increasing the number of hemocytes and Pro-PO activity besides increasing the survival percentage of experimental shrimp. The results of this study are expected to help shrimp farmers in dealing with disease attacks in ponds in an environmentally friendly manner.

**1. Introduction**

Various efforts have been applied to treat disease attacks in shrimp farming, yet shrimp mortality continues to occur. The use of chemical medicine and antibiotics to control shrimp disease is banned by the Ministry of Marine Affairs and Fisheries for contaminating the environment and causing disease resistance. Therefore, the Research Institute for Brackishwater Aquaculture and Fisheries Extension (RIBAFE) must conduct a study to find an environmentally-friendly alternative to prevent shrimp disease.

One of the studies that have been carried out to prevent and control disease in aquaculture is the use of probiotics derived from bacteria in pond soil and marine water. Sources of bactericides and probiotic bacteria that have been investigated were marine water and sediment [1], corals [2], hatchery [3,4], mangrove leaves [5], and shrimp ponds [6]. [7] mentioned that the use of probiotic bacteria could suppress the mortality of tiger shrimp post larvae through the control of *Vibrio sp.* in the water. Other efforts included the use of natural substances to interrupt communication signals among pathogenic *Vibrio* bacteria. Some researchers concluded that cells of pathogenic bacteria communicate with each other before attacking an organism through a mechanism called quorum sensing [8–10].
One strategy that has been intensely examined regarding bacterial disease control is the intervention of bacterial quorum sensing mechanism [11,12].

One of the natural substances known to contain anti-Quorum Sensing is macroalgae and microalgae. Bacteria and microalgae co-exist in the aquatic ecosystem. Even though they are very different in terms of the evolutionary process, the study showed that such interactions occurred between these organisms [13]. One type of interaction by mutual communication is achieved through the Quorum Sensing (QS) system – communication among bacterial cells through the production, release, and detection of small signal molecules [14–16]. Therefore, QS is a potential therapeutic target, where inhibition of communication among bacterial cells could be a promising strategy to suppress bacterial virulence, hence controlling infection [17]. This way, quorum sensing intervention by microalgae may offer such an interesting opportunity for a disease prevention method in the shrimp culture industry.

The study conducted by RIBAFE in 2016 – 2017 has succeeded in investigating five species of microalgae that have the potential to inhibit QS signal, namely: Melosira sp, Porphyridium sp, Vulgaris sp, Phaeodactylum sp, and Nannochloropsis sp. In addition to its anti-QS potential, the result of the study also showed that microalgae Melosira sp, Porphyridium sp, and Phaeodactylum sp contain anti-bacterial properties.

This study was aimed to examine the effect of diets supplemented with microalgae extract as an immunostimulant in shrimp. Today, the application of immunostimulant has gained more attention to be developed as a disease control method in shrimp culture. Many pieces of evidence showed that immunostimulant added in feed could increased fish and shrimp resistance to disease infection through the increasing non-specific immune response [18]. Shrimps do not have a specific immune response and completely rely on the non-specific immune response. Despite being considered to be not satisfactory, the non-specific immune response may quickly and effectively recognize and destroy foreign materials, including pathogens [19].

2. Materials and Methods
This research was designed using Completely Randomized Design (CRD) with 4 treatments, namely: A) Shrimp diet + microalgae Porphyridium sp extract; B) Shrimp diet + microalgae Porphyridium aerugenum extract; C) Shrimp diet + microalgae Nannochloropsis sp extract; and D) Control (shrimp diet without the addition of microalgae extract). Each treatment was replicated 3 times. Test was conducted in 12 plastic tanks with a volume of 50 L. Each tank was filled with 30 L of water (salinity 28 ppt) that have been sterilized and continuously aerated. A total of 20 shrimp at size 0.5-2.0 g/shrimp were put in each tank and fed according to feed treatment 3 times/day for 30 days. Microalgae extract (35% of feed weight produced) was added into the feed formula. Dried feed was further put into a plastic bag and stored in the fridge before using it. Siphoning of uneaten feed and shrimp feces as well as water exchange up to 10% volume, were conducted daily before feeding to maintain water quality.

Parameter observed included Total Hemocyte Count (THC), Value of Prophenol Oxidase (Pro-PO), and Survival Rate of shrimp post-infection. Before artificial infection, sampling of hemolymph was done every 5-30 days to observe the immune parameter in the blood. Later, artificial infection through the injection method using pathogenic Vibrio harveyi was conducted on day-30 at a bacterial density of 10⁶ CFU/mL. Furthermore, the blood profile of shrimp was re-observed on day-1, day-4, and day-7 post-infection. A sampling of water quality was done on day-15 and day-30 of the maintenance period before infection. Observation of Survival Rate was done daily until day-7 post-infection. The data of survival rate obtained was further analyzed statistically, while other data were analyzed descriptively in the form of figure and table.

2.1. Collection and Calculation of THC
About 100µl hemolymph was mixed with 300µl anticoagulant. A mix of hemolymph-anticoagulant was homogenized and brought to the Laboratory for THC calculation. A mix of hemolymph and
anticoagulant was taken using a pipette and dropped to a hemocytometer. Moreover, THC was measured using a hemocytometer under a light microscope with a magnification of 40x.

The value of THC is calculated using the following formula:

\[
THC = \frac{A}{B} \times 25 \times P \times 10^4 \text{cell/mL} \tag{1}
\]

Where:
- THC = Total Hemocyte Count (cell/mL)
- A = number of cell counted
- B = number of small box in hemocytometer = 5 boxes
- 25 = number of small box in chamber of 1 x 1 mm
- P = dilution factor = 4

2.2. Observation of Pro-Phenoloxidase (Pro-PO) Activity

The activity of Pro-PO was measured according to the dopachrome formation produced by L-DOPA. The calculation of Pro-PO activity was done based on the procedure mentioned by Liu & Chen. First, 1 ml of a mixture of hemolymph-anticoagulant was centrifuged (700 g for 20 minutes at temperature 4°C). The supernatant was removed, and the pellet was slowly re-suspended in cacodylate-citrate buffer solution (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.10 M trisodium citrate, pH 7) and re-centrifuged. Pellet was further collected and suspended in 200 µl cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.01 M calcium chloride, 0.26 M magnesium chloride, pH 7).

Aliquot of 100 µl were incubated with 50 µl trypsin (1 mg.ml-1 Cacodylate buffer) as an activator for 10 minutes at temperature 25-26°C. Later, about 50 µl L-DOPA (3 mg/ml cacodylate buffer) was added, after 5 minutes, 800 µl cacodylate buffer, was also added to observe the value of Optical Density (OD) using Spectrophotometer 490 nm. A standard solution which contained 100µl hemocyte suspension, 50 µl cacodylate buffer (substitute for trypsin), and 50 µl L-DOPA was used to measure the background of PO activity in all treatment solution. Optical density (OD) obtained from PO activity of all treatment conditions was expressed as dopachrome formation in 50 µl hemolymph.

3. Result and Discussion

3.1. Observation of Total Hemocyte Count (THC)

Observation of THC before infection was conducted every 5 days until day-30. THC was seen to fluctuate from day-10 to day-30. On sampling day-0, it was observed that the highest value of THC was obtained by treatment PH, followed by treatment PM, N, and Control (Fig. 1). On sampling day-5, there was decreasing THC value in treatment PH and Control, while the increase in THC value was found in treatment PM on sampling day-5. However, on sampling day-10, there was a decreasing value of THC for all treatments. Similarly, THC decreased in all treatments, except for treatment PM that was found to be relatively stable on sampling day-15.
Figure 1. Hemolymph sampling before and after infection

The lowest THC during 30 days of maintenance before infection was observed on day-15. A significant increase in THC for all treatments occurred on day-20. Difference between treatments given feed added with microalgae extract and Control was not detected until observation day-30.

On day-30, the artificial infection was conducted through the injection method using pathogenic Vibrio at density $10^6$ (CFU/mL). After infection, hemolymph sampling was done on day-1, 4, and 7 post-infection. The highest value of THC until day-7 post-infection was found in treatment PH ($320 \times 10^4$ cell/mL), followed by treatment PM ($213.33 \times 10^4$ cell/mL). Furthermore, THC in treatment N and Control was no longer able to be observed since all shrimp were dead before 24 hours post-infection.

3.2. Calculation of ProPhenoloxidase (ProPO) value

Based on the result that showed in Figure 2, the highest value of Pro-PO activity at the end of the experiment was observed in treatment given diets supplemented with microalgae Porphyridium aerugenum extract (PH) (0.038). No significant difference was found in Pro-PO value between treatments given feed added with microalgae extract and Control. The highest Pro-PO activity occurred on the day-15 of observation. After the day-15, Pro-PO experienced a decrease. On sampling day-25 and day-30, the highest Pro-PO activity was observed in treatment given feed supplemented microalgae Porphyridium sp extract. After artificial infection using pathogenic Vibrio, rapid mortality occurred (less than 24 hours) in Control treatment. The lowest Pro-PO activity was observed on day-1 post-infection. Later, Pro-PO activity increased in treatment PH, PM, and N, yet Pro-PO activity in Control could not be observed since shrimps were all dead before 24 hours post-infection.
High activity of Pro-PO is related with high THC (Figure. 1) since shrimp hemolymph has a function in production and PO release to hemolymph in the form of inactive pro-enzyme or called Pro-PO. In a normal situation, a higher number of hemocytes leads to higher Pro-PO production. In crustacean, Pro-PO has a function in foreign material recognition system and melanization [20,21].

Immunostimulant is highly important to be used in disease control since it offers an alternative to the wide use of antibiotics in fish and crustacean farming [22,23] and has no side effect [23]. Immunostimulant is a substance that stimulates or increases the immune system by directly interacting with cells that activate the immune system [24]. The mechanism of action of immunostimulant in stimulating the body's immune system is done by increasing phagocytic cell activity [23]. Hence, immunostimulants could increase fish or shrimp resistance to pathogens simultaneously by stimulating non-specific immune responses [24]. Immunostimulant could be in the form of bacteria and bacteria products, yeast, carbohydrate complex, nutrition factor, animal extract, plant extract, and synthetic medicine [22,24,25].
Figure 3. Shrimp Survival Rate After Infection

Based on the data of shrimp survival (Fig. 3), it was found that the highest percentage of survival rate until day-7 post-infection was obtained by treatment PH, which is the addition of diets supplemented with microalgae *Porphyridium aerugenum* extract (33.33 – 38.1%). The result was followed by treatment PM and N, namely shrimp feed supplemented with the extract of microalgae *Porphyridium* sp and *Nannochloropsis* sp (19.04.1%). In Control treatment, no experimental shrimp was found alive during 24 hours post-infection (0%). To conclude, the addition of microalgae *Porphyridium aerugenum* extract was better to increase the survival rate of experimental shrimp compared to microalgae *Porphyridium* sp and *Nannochloropsis* sp also Control.

4. Conclusion
The addition of microalgae *Porphyridium aerugenum* extract at 35% of feed weight resulted in the highest shrimp survival compared to other treatments and Control treatment. A significant difference in THC and Pro-PO value was not found between diet supplemented with microalgae extract and Control treatment before artificial infection. However, after artificial infection, the highest value of THC and Pro-PO activity was found in the treatment fed diet supplemented with microalgae *Porphyridium aerugenum* extract.

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