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The Administration of *Caulerpa racemosa* Extract in Feed of Whiteleg Shrimp (*Litopenaeus vannamei*) After Infected by *Vibrio parahaemolyticus*

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Abstract

The purpose of this study was to determine the administration of *Caulerpa racemosa* extract in feed on the clinical sign and intestinal histopathological profile of whiteleg shrimp (*Litopenaeus vannamei*) after being infected by *Vibrio parahaemolyticus.* The study was conducted using a completely randomized design with six treatments and three replications. Shrimp were divided into six groups, two control groups without the administration of *C. racemosa* extract, the other four groups with the administration of *C. racemosa* extract with doses; 30 mg/kg (P1), 60 mg/kg (P2), 120 mg/kg (P3) and 240 mg/kg (P4) through the feed. On the 15th day, the control group was divided into two groups: negative control (K-) was injected with PBS and then positive control (K+) and four other treatments (P1, P2, P3, and P4) were infected with *V. parahaemolyticus*. The results showed that intestinal damage caused by *V. parahaemolyticus* infection was inflammation and necrosis. The administration of *C. racemosa* extract had a significant effect on the histopathological profile of the shrimp intestine. The lowest percentage of damage was found in the P4 treatment, which was 19.6% with an indication of light damage. Clinical signs appeared at 48 hours post-infection pale hepatopancreas, empty intestines, reddened uropods and gnats. Further, shrimp in the P4 showed a healthy condition without any clinical signs. It can be concluded that the administration of *C. racemosa* extract at a dose of 240 mg/kg is the best dose in reducing the damage level to the intestine and affects the health condition of *L. vannamei* infected with *V. parahaemolyticus*.

INTRODUCTION

Whiteleg shrimp is one of the brackish water fishery commodities with high economic value. World whiteleg shrimp production in 2018 was already above 34.9 million tons (FAO, 2020). The Ministry of Maritime Affairs and Fisheries targets the total production of whiteleg shrimp to reach 800 thousand tons

(MMAF, 2018). In recent years, the whiteleg shrimp culture industry has been attacked by many bacterial diseases caused by Vibrio, causing huge losses (Ngo *et al*., 2020). Cases of Acute Hepatopancreatic Necrosis Disease (AHPND) caused by *Vibrio parahaemolyticus* have infected whiteleg shrimp in China, Vietnam, Malaysia, and Thailand resulting in a mortality rate of 100% (Navaneeth *et al*., 2019).

Handling the disease is to create an optimal culture environment, quarantine whiteleg shrimp that are attacked by the disease, vaccination, and antibiotics (Gustiana *et al*., 2015). However, the administration of antibiotics harms the viability of whiteleg shrimp such as resistance and environmental pollution around the culture pond. Therefore, it is necessary to use natural plants for the prevention and treatment of a disease so as not to cause negative impacts on shrimp and the environment. Immunostimulants are substances derived from natural or synthetic materials that can increase the immune response, by giving -glucans and lipopolysaccharides (LPS) (Purbomatono and Husin, 2014). Natural ingredients to be used as immunostimulants can be obtained from bioactive compounds from animal and plant extracts. Immunostimulatory administration can be given orally, through injection, and immersion (Karunasagar *et al*., 2014).

Seaweed is a multicellular alga that has potential as a source of phytochemicals that have bioactive peptide activity with biological functions such as immunostimulant (Di *et al*., 2017), antioxidant, antimicrobial, and anticancer (Omar *et al*., 2018). The active substance in *Caulerpa racemosa* type seaweed that plays a role in increasing the immune system is sulfated polysaccharides. Sulfate polysaccharides have biological activities such as antibacterial, antiviral, and antioxidant (Xie *et al*., 2016), Activities in the immune system include an immune regulator with immunomodulatory functions and maintaining homeostatic balance through the proliferation of macrophages, lymphocytes, NK cells (Natural Killer cells) and the complement system. This substance is not only able to assist in the release of cytokines but also plays a role in improving the immune system. Sulfated polysaccharides can enhance the immune system by activating macrophage activity (Huang *et al*., 2019) The addition of *Gracilaria verrucosa* extract as an immunostimulant was able to increase the shrimp immune system which was characterized by an increase in total haemocytes and phagocytic activity (Sarjito *et al*., 2020).

Severe damage to organ structures will cause stress in shrimp, which will increase the risk of high sensitivity to viral and bacterial infections (Dharmawan *et al*., 2020), thus increasing the risk of death of shrimp. The importance of histopathological examination in the diagnosis of infectious diseases, apart from knowing the possible causes of infection, can also be classified based on the time and distribution of the disease. This study aimed to determine the administration of *Caulerpa racemosa* extract in feed of whiteleg shrimp after being infected by *Vibrio parahaemolyticus*.

METHODOLOGY

Place and Time

This research was carried out from February to April 2021. Extraction of *C. racemosa* was carried out at the Chemistry Laboratory and shrimp rearing was carried out at the Anatomy and Culture Laboratory, Faculty of Fisheries and Marine, Universitas Airlangga Surabaya. Histology preparations were made at the Pathology Laboratory, Jemur Sari Islamic Hospital, Surabaya.

Research Materials

The equipment used included a hotplate stirrer (THERMO Scientific CIMAREC SP88857105, USA), refrigerator centrifuge (Rotanta 460 Hettich, Tuttlingen, Germany), rotary evaporator, autoclave (Hirayama HVE50, Japan), spectrophotometer (AMTAST

AMV11), and an aquarium with a size of 40 x 30 x 30 cm. While the materials used were whiteleg shrimp (*Litopenaeus vannamei*) 5-7 cm in size and weighed 5 g/shrimp, *Caulerpa racemosa*, *Vibrio parahaemolyticus* bacteria with a density of 10^6 cells/ml, TSA media (MERCK 1054580500, Germany), TCBS media (MERCK 1102630500, Germany), PBS (Medcago AB, Uppsala, Sweden), 95% alcohol, hematoxylin, eosin, giemsa, CMC (*Carboxymethyl Cellulose*), Davidson solution.

Research Design

The research design used is the experimental method. This study used a completely randomized design (CRD) using 6 treatments with 3 replications. Shrimp were divided into five groups, one control group without the administration of *C. racemosa* extract, the other four groups were added *C. racemosa* extract at different doses; 30 mg/kg (P1), 60 mg/kg (P2), 120 mg/kg (P3) and 240 mg/kg (P4) were given through feed. After 14 days, the control group shrimp were divided into two groups of negative control (K-) injected with PBS then positive control (K+) and four other treatments (P1, P2, P3, and P4) infected with *V. parahaemolyticus*.

Work Procedure

The working procedure begins with the extraction of *C. racemosa* powder (20 g) extracted in 400 ml of water with the hot water method. This method is briefly carried out by maceration with hot water at 85°C for 2 hours (Hao *et al.,* 2019). Then the maceration results are filtered and then separated from the solvent using a rotary evaporator. Furthermore, the extract is mixed into the feed by adding 1% CMC and then sprayed onto the shrimp feed.

Shrimp measuring 5-7 cm with a weight of 5 g as many as 180 shrimp. Shrimp are fed three times a day with a feeding rate of 3%. On the 15th day of rearing, the shrimp were infected with *V. parahaemolyticus* intramuscularly in the third abdominal segment, for the control treatment, PBS was injected. Intestinal organ collecting was carried out at the end of maintenance, which was ten days after infection. The intestines were preserved with Davidson solution for further preparation for histology review (Li *et al.*, 2019).

Histological preparations include tissue fixation, tissue selection (trimming), tissue dehydration, tissue block making, tissue slicing, and tissue staining. The last step is observations made under a light microscope with a magnification of 400 ×.

Data Analysis

The data obtained from the intestinal histology scoring were analyzed using the nonparametric method of the Kruskal Wallis Test to determine the differences between the groups and the treatment groups. Followed again with the Mann Whitney Test method if the results showed significantly different ($P < 0.05$).

RESULTS AND DISCUSSION

Intestinal Histopathology Profile

Observation of the histopathological profile of shrimp intestine after the administration of *C. racemosa* extract and infected with *V. parahaemolyticus* could be seen in Figure 1.

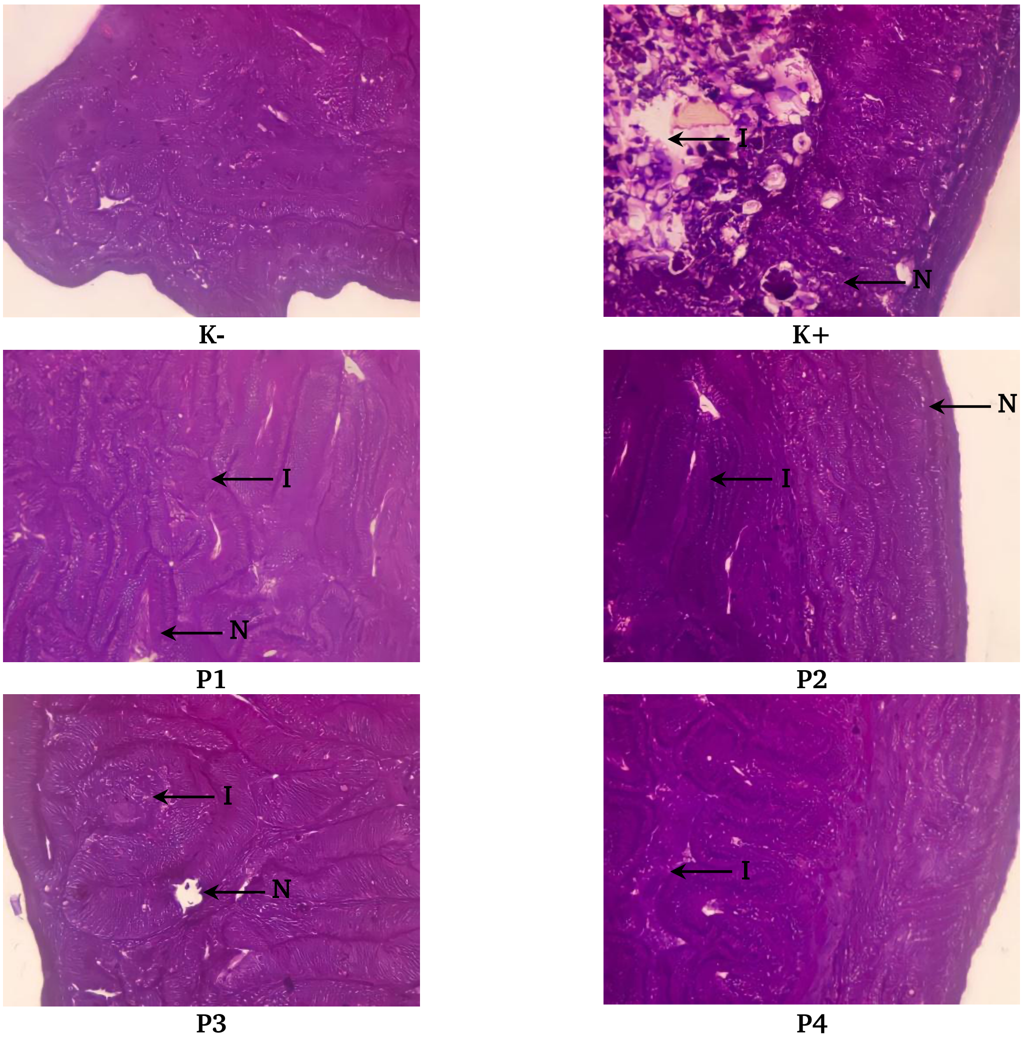

Figure 1. Shrimp intestinal histopathology profile. Description : (K-) negative control, (K+) positive control, (P1) treatment 30 mg/kg, (P2) treatment 60 mg/kg, (P3) treatment 120 mg/kg, (P4) treatment 240 mg/kg, (I) inflammation, (N) necrosis; Magnification 400× (Bar 40 μm).

The intestine of healthy shrimp shown in Figure K- (control) was normal. Histologically normal shrimp intestines show the intestinal mucosa, intestinal submucosa and the presence of elongated intestinal villi. In the intestine, villi are mucous extensions that function to expand the surface of the intestine so that nutrient absorption is more efficient (Chen *et al*., 2020).

Based on the observation of the intestinal profile of shrimp in the positive control (Figure 1), it was shown that the intestine was inflamed and necrotic. Each treatment shows a different level of severity. Organ damage first occurs in the digestive system, especially the intestines, then in the hepatopancreas and spreads to the muscle cells (Chen *et al*., 2020). Histological changes in the K+ treatment was in the form of inflammation along the intestinal mucosal layer, and necrosis at several points in the muscle tissue. Inflammation is a defense mechanism that occurs as a tissue response to damage, either locally or systemically inside the body of shrimp (Maftuch *et al.,* 2017).

Treatments P1, P2, and P3 also showed inflammation and necrosis of the intestinal mucosa. Necrosis is acute cellular damage and it can be focal or massive which results in the tissue not being completely formed again due to shrinkage of the nucleus (Victor *et al*., 2013). *Vibrio* infection can cause severe inflammation in the submucosal area and ulcerated hemorrhagic changes in the mucosa and submucosa (Kurniawan and Susianingsih, 2014). Whereas in the P4 treatment, there was no necrosis, only inflammation was seen at several points on the intestinal mucosa. This indicates that the intestinal organs in the treatment with the administration of *C. racemosa* extract at a dose of 240 mg/kg can reduce the occurrence of intestinal tissue damage.

The level of damage that occurs in each treatment gets a different percentage. The average level of damage that occurs is presented in (Table 1). The results of statistical analysis showed that there was a significant difference ($p<0.05$) on P4 to the treatment of K+, P1, and P2 but not significantly different ($p>0.05$) against P3.

The highest percentage of damage was treated with the administration of *C. racemosa* extract at a dose of 30 mg/kg (P1). While the lowest percentage of damage was treated with a dose of 240 mg/kg (P4) extract of *C. racemosa* (P4), which was 19.6% with a description of light damage. This indicated that the administration of *C. racemosa* extract through feed was able to reduce the damage caused by *V. parahaemolyticus* infection. This follows Suantika *et al*. (2018) who stated that the addition of polysaccharides from *Kappaphycus alvarezii* was able to reduce damage to the hepatopancreas of shrimp due to *V. harveyi* infection.

Table 1. Average level of damage to the intestines of shrimp.

Treatment	Average Level of Damage	Damage Percentages	Damage Rate
K-	1.00^{a}	11.3%	light
K+	2.67^{b}	53.0%	heavy
P1 (30 mg/kg)	2.00^{bc}	43.0%	medium
P2 (60 mg/kg)	2.00^{bc}	41.3%	medium
P3 (120 mg/kg)	1.67^{ab}	29.3%	medium
P4 (240 mg/kg)	1.00^{a}	19.6%	light

Note: Differences in superscripts in the same column show significantly different results ($p<0.05$).

Clinical Sign

Clinical sign caused by shrimp infected with *V. parahaemolyticus* are shown in Figure 2.

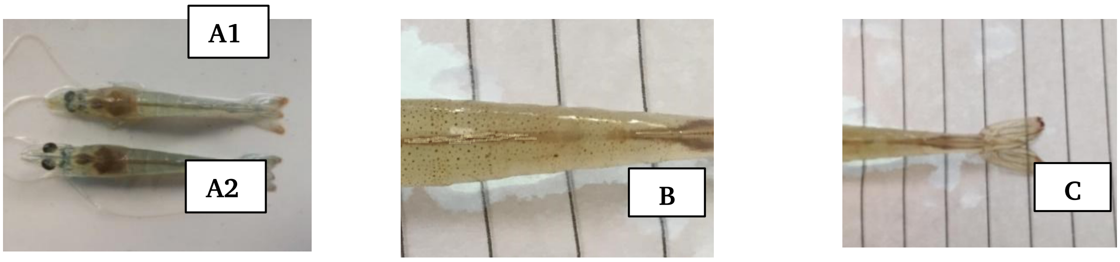

Figure 2. Clinical sign of shrimp infected by *V. parahaemolyticus*. Description : (A1) sick shrimp, (A2) normal shrimp, (B) empty intestine, (C) the uropod turned red and the gnats.

The results of the challenge test of shrimp with *V. parahaemolyticus* showed changes in behavior and morphology. After 24 hours of infection, the shrimp began to passively swim and tend to move toward the aerator as a source of oxygen. This shows that the shrimp are under stress so they need sufficient oxygen intake to recover from their condition. This follows the research of Parenrengi *et al*. (2013) which states that vannamei shrimp infected with Vibrio show a behavior change to become passive after 24 hours of being challenged.

Based on observations 48 hours after infection, shrimp showed clinical signs including pale hepatopancreas, empty intestines, reddened uropods, and gnats. This is because *V. parahaemolyticus* is a halophilic bacterium that attacks shrimp and causes lethargy, irregular swimming movements, empty intestines and pale hepatopancreas (Rudtanatip *et al*., 2017). In addition, the mortality of shrimp peaked on day 2 to day 6 after being infected with *V. parahaemolyticus*. This also happened in the study (Parenrengi *et al*., 2013), shrimp death occurred on day 2 to day 4 after Vibrio infection.

The dose of administration of *C. racemosa* extract affects the health of shrimp. It was shown by the P4 treatment with the highest dose of 240 mg/kg where the shrimp showed a healthy condition without any clinical symptoms after being infected with *V. parahaemolyticus*. This is in accordance with Pratiwi *et al.* (2021), that addition of *C. racemosa* extract with dose 6 μg/g by injection affects the health condition and increases the survival rates of shrimp after being infected with *V. parahaemolyticus*.

Water Quality

The data of water quality range value can be seen in Table 2.

Table 2. Water quality during research.

Parameter	Treatments					
	K-	K+	P1	P2	P3	P4
Temperature (°C)	28.4-28.7	28.5-28.9	28.6-28.9	28.5-28.8	28-28.4	28-28.3
Salinity (ppt)	15	15	15	15	15	15
pH	7-8.5	7-8.5	7-8.5	7-8.5	7-8.5	7-8.5
Dissolved oxygen (ppm)	6.3-6.5	6.0-6.2	6.0-6.2	6.0-6.3	6.4-6.6	6.2-6.5

The temperature during rearing ranged from 28 - 28.9 °C, the results follow the optimum temperature value of whiteleg shrimp which ranged from 28 – 33 °C (SNI, 2014). Salinity during maintenance is 15 ppt. These results are still within the tolerance limit for vannamei shrimp rearing, namely salinity 15 – 32 ppt (Hukom *et al.*, 2020). The average results of measuring pH parameters are 7 – 8.5 where these results are the optimum value for shrimp culture. The optimum pH value in shrimp culture is 7.5 – 8.5. The average results of dissolved oxygen parameters are in the optimum value for shrimp culture from 6.0 to 6.6 ppm. The optimum DO value in shrimp culture is > 4 ppm (SNI, 2014).

CONCLUSION

Alteration in the intestinal profile of whiteleg shrimp fed with the administration of *C. racemosa* extract and infected with *V. parahaemolyticus* in the form of inflammation and necrosis. Based on the average level of intestinal damage, treatment with the administration of *C. racemosa* extract as much as 240 mg/kg showed a slight damage profile. Furthermore, shrimp with the administration of *C. racemosa* extract with a dose of 240 mg/kg showed a healthy condition without any clinical symptoms after being infected with *V. parahaemolyticus*.

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