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ADDITION OF *Caulerpa racemosa* EXTRACT ON RPS AND CLINICAL SIGNS OF VANAME SHRIMP AFTER *Vibrio parahaemolyticus* INFECTION

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Abstrak

Penanggulangan penyakit AHPND pada udang dapat dilakukan dengan pemberian immunostimuant, salah satunya berasal dari ekstrak rumput laut yaitu Caulerpa racemosa. Tujuan dari penelitian ini adalah untuk mengetahui pengaruh pemberian ekstrak Caulerpa racemosa terhadap relative percent survival (RPS), gejala klinis dan perubahan tingkah laku udang vaname setelah diinfeksi bakteri Vibrio parahaemolyticus. Penelitian ini menggunakan Rancangan Acak Lengkap dengan lima perlakuan dan tiga ulangan dengan masing masing perlakuan yaitu K-, K+ dan 3 perlakuan dengan penambahan ekstrak Caulerpa racemosa yaitu P1 (3 µg/g), P2 (6 µg /g) dan P3 (9 µg /g). Proses infeksi dengan bakteri Vibrio parahaemolyticus dilakukan 24 jam setelah injeksi dengan ekstrak Caulerpa rasemosa. Hasil penelitian ini menunjukkan bahwa nilai Relative Percent Survival (RPS) tertinggi terdapat pada P3. Gelaja klinis udang yang terinfeksi Vibrio parahaemolyticus ditandai dengan usus kosong serta hepatopankreas yang memutih, selain itu, terdapat perubahan tingkah laku pada udang yang terinfeksii Vibrio parahaemolyticus yaitu penurunan nafsu makan, pergerakan udang pasif di dasar aquarium serta gerkaan berenang udang yang tidak menentu. Berdasarkan hasil ini dapat disimpulkan bahwa perlakuan terbaik terdapat pada P3 dengan dosis 9 µg /g . Penelitian ini dapat memberikan informasi mengenai dosis terbaik penggunaan ekstrak C. rasemosa untuk menanggulangi penyakit AHPND pada udang.

***Kata Kunci**: Udang vaname, C. racemosa, Imunostimulan, V. parahaemolyticus, AHPND*

Abstract

Prevention of AHPND in shrimp can be done by giving immunostimulants, one of which comes from seaweed extract, explicitly *Caulerpa racemosa*. This study aimed to determine the effect of the administration of *Caulerpa racemosa* extract on the relative percent survival (RPS), clinical signs, and behavioral changes of white shrimp after being infected with *Vibrio parahaemolyticus*. This study used a completely random design with five treatments and three replications, namely K-, K+, and three treatments with the addition of *Caulerpa racemosa* extract, names P1 (3 µg/g), P2 (6 µg /g), and P3 (9 µg/g). The infection process with *Vibrio parahaemolyticus* bacteria was carried out 24 hours after being injected with *Caulerpa racemosa* extract. The results of this study indicate that the highest Relative Percent Survival (RPS) value was P3. Clinical signs of shrimp infected with *Vibrio parahaemolyticus* were characterized by empty intestines and a whitened hepatopancreas. In addition, there were behavioral changes in shrimp infected with *Vibrio parahaemolyticus*, namely decreased appetite, passive shrimp movement at the bottom of the aquarium, and erratic swimming movement of shrimp. Based on these results, it could be said that the best treatment is in P3 with a dose of 9 µg/g. This study can provide the best quantity of *C. racemosa* extract to treat AHPND in shrimp.

***Keywords**: Vaname shrimp, C. racemosa, Immunostimulant, V. parahaemolyticus, AHPND*

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1. INTRODUCTION

Vannamei shrimp (*Litopenaeus vannamei*) is one of the largest cultivated commodities in Indonesia and also the largest export commodity in Indonesia (Sitompul *et al*., 2018). The demand for

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vaname shrimp in 2018 reached 150 thousand tons (KKP, 2018). The high demand for vaname shrimp has led to the intensification of aquaculture with the application of a super-intensive aquaculture system (Syah *et al*., 2017). The process of intensification of this cultivation cannot be separated from the causes of negative impact on the existence of a disease. Disease attacks can be caused by decreased water quality due to waste from unconsumed feed and increased feces (Kharisma and Manan, 2012; Hermawan *et al*., 2014). The disease that often attacks shrimp is acute hepatopancreatic necrosis syndrome or known as AHPND, caused by *Vibrio parahaemolyticus* bacteria called AHPND-causing strain of *Vibrio parahaemolyticus* (Vpahpnd) (Lee *et al*., 2015). AHPND disease in shrimp can cause death in shrimp within 24 hours and can cause death up to 100% in shrimp aged 20-45 days (Tblackadee *et al*., 2016; Choi *et al*., 2017; Schofield *et al*., 2020). *V. parahaemolyticus* bacteria that attack shrimp can release PirA and PirB toxins which are released into the hepatopancreas and cause damage to the hepatopancreas by exfoliating tubular epithelial cells (Tran *et al*., 2013; Muthukrishnan *et al*., 2019). AHPND disease has clinical signs, such as an empty intestine, a white and atrophic hepatopancreas, and growth retardation (Hong *et al*., 2016).

Based on the research of Soto-Rodriguez *et al*., (2018) stated that the phase of AHPND attack on shrimp is characterized by three stages of infection, such as the initial phase marked as partially or empty intestine, after which is followed by an acute phase with clinical signs, anorexia with the digestive tract which is open and the loss of pigmentation in the hepatopancreas which is characterized by the start of whitening of the color of the hepatopancreas, after which there is a terminal phase which is characterized by a white hepatopancreas with an utterly empty intestine. According to research by Aguilar-Rendon *et al*., (2020) stated that shrimp death due to AHPND could occur within 17 hours after showing the signs of clinical infection in the acute phase with a relatively high mortality rate of up to 50%, after 24 hours in the terminal phase with a lower mortality rate than during the acute phase.

The high incidence of disease in shrimp in recent years has led to the increased use of antibiotics (Zhao *et al*., 2018). However, the use of antibiotics in shrimp can potentially cause residues that can cause problems regarding food safety, and some pathogens have shown resistance to antibiotic products (Hai, 2015). Efforts to overcome the negative impact of using antibiotics, it can be made to prevent bacterial infections by using immunostimulants. The application of immunostimulants in shrimp can cause enhancement in both humoral and cellular immune responses (Wang *et al*., 2016). The humoral immune response includes releasing shrimp immune components into the hemolymph, such as prophenoloxidase (Propo) and the release of antimicrobial peptide (AMP). At the same time, the cellular immune response is mediated by hemocytes, including phagocytosis, nodule formation, and encapsulation (Kaizu *et al*., 2011). Several types of immunostimulant materials used in shrimp are polysaccharides, oligosaccharides, antibacterial peptides, probiotics, and added vitamins (Wang *et al*., 2016). In addition, there are sulfated polysaccharides that can be used as immunostimulants (Xie *et al*., 2016).

Sulfate polysaccharides can be found in the extracellular fluid of bacteria, yeasts, and fungi and various parts of plants, including seeds, leaves, and stems of herbal plants (Singh *et al*., 2012). In addition, sulfated polysaccharides can be obtained from several groups of seaweed, namely rhodophyta, phaeophyta, and chlorophyta (Costa *et al*., 2010). One seaweed containing sulfated

polysaccharides is *Caulerpa racemosa*, 34.19 ± 0.46 mg/g. In addition, *Caulerpa racemosa* extract has several active ingredients, such as phenols and flavonoids, with respective values of 1.93 ± 0.04 mg/g and 4.80 ± 0.10 mg/g (Hao *et al*., 2015). Sulfate polysaccharides have benefits as antitumor, antioxidant, antiviral, and can be immunomodulators. Using sulfate polysaccharides in shrimp can increase the immune response, characterized by increased phenoloxidase activity, survival value, RPS value, and increase in total hemocyte count (Pholdaeng and Pongsamart, 2010; Cantelli *et al*., 2019). RPS is a calculation to show the effectiveness of the use of certain compounds in anticipating mortality in organisms (Marbun *et al*., 2019). Based on this description, it is necessary to research the administration of *Caulerpa racemosa* extract on the relative value of survival percentage, clinical signs, and changes in the behavior of vaname shrimp after being infected with *Vibrio parahaemolyticus* bacteria.

2. MATERIAL AND METHOD

2.1 Research Time and Place

This research was conducted in March-June 2022. Vannamei shrimp rearing was carried out at the Aquaculture and Anatomy Laboratory, Faculty of Fisheries and Marine, Airlangga University, Surabaya. Extracts of *C. racemosa* were made at the Chemistry and Analysis Laboratory, Faculty of Fisheries and Marine, Universitas Airlangga Surabaya. The bacterial culture of *V. parahaemolyticus* was carried out at the Laboratory of Microbiology and Fish Diseases, Faculty of Fisheries and Marine, Airlangga University, Surabaya.

2.2 Research Method

This study used a completely random design (CRD) with five treatments (K-, K+, P1, P2, and P3) and three replications. Treatments P1, P2, and P3 were added with extracts of *C. racemosa* at a dose referred to by Tayag *et al*., (2010) with a dose of 3.6,9 g/g shrimp weight on the first day, respectively. After 24 hours, *V. parahaemolyticus* was injected into K+, P1, P2, and P3, while in the K- treatment, PBS was injected. After seven days post-infection, parameters were observed, including relative percent survival (RPS), clinical signs, and changes in shrimp behavior.

2.3 Aquarium Preparation and Maintenance Media

This study used an aquarium measuring 40x30x30 with a total of 15 units. Preparation for the aquarium by cleaning using soap until clean, then filling the water with sterilization using one dose of chlorine in 30 ppm as a disinfectant. The aquarium was soaked for 24 hours then the chlorine water was removed and rinsed with clean water. Subsequently, drying the aquarium was carried out for 24 hours. The sterilized seawater was used for the maintenance with a 30 ppm dose of chlorine.

2.4 Hot Water Extraction of *C. racemosa*

Caulerpa racemosa was air-dried first without being exposed to direct sunlight. After drying then, *C. racemosa* is mashed using a blender. After that, 10 grams of *C. racemosa* flour was mixed with 200 ml of distilled water in a ratio of 1:20. In The following process, *C. racemosa* flour was mixed with distilled water and put into a glass beaker to which hot water was added at a temperature of 85^{o}C. Next, the beaker glass is placed on a hotplate for 2 hours while maintaining a temperature of 85^{o}C. After that, the extract is centrifuged

at 3000 rpm for 15 minutes. Next, solvent evaporation was carried out to obtain the sulfate polysaccharide extract using a rotary evaporator at a temperature of 65 ^{o}C (Hao *et al*., 2019).

2.5 Shrimp Preparation

The test animal used in this study was *Litopenaeus vannamei*, with ten shrimp filling each aquarium. Acclimatization is done by placing a plastic bag containing shrimp into the tub and leaving it until the plastic bag is dewy or by adding water little by little into the plastic bag until the water temperature in the bag and rearing tank is the same and letting the shrimp come out by itself from the plastic bag (Faisyal *et al*. al., 2016). The prawns were previously adapted to a new environment for one week (Muarif and Rosmawati, 2011). During the adaptation process, the shrimp were fed commercial pellets with a feeding frequency of three times a day and a feeding rate (FR) of 3% of the biomass/day (Satyantini *et al*., 2016).

2.6 Maintenance of Shrimp

The maintenance of vannamei shrimp lasts for nine days with the frequency of feeding three times a day, namely at 09.00 WIB, 13.00 WIB, and 17.00 WIB, with a feeding rate of 3% (Satyantini *et al*., 2016). The feed is used in the form of commercial feed with a protein content of 36%. Water changes are carried out every day by siphoning and removing water as much as 20% of the total volume of the aquarium. The addition of new water is adjusted to the initial volume of water.

2.7 Challenge Test with *V. parahaemolyticus*

The challenge test was carried out on day one after administration of *C. racemosa* extract in rearing by injection of 0.1 mL/shrimp *V. parahaemolyticus* with a density of 10^6 CFU/ml each shrimp intramuscularly in the third abdominal segment (Mameloco *et al*., 2020). . The negative control treatment was injected with 0.1 mL of PBS/shrimp.

2.8 Hemolymph Sampling

Shrimp hemolimation was carried out three times during the study period. Namely, one day after administration of *C. racemosa* extract, one day after *V. parahaemolyticus* injection, and the 7th day after infection. Shrimp hemolymph was taken from the ventral sinus of the shrimp (Pourmozaffar *et al*., 2019).

2.9 Relative Percent Survival (RPS)

The value of Relative percent survival (RPS) in this study was calculated based on the formula according to Satyantini, (2013), namely:

$$= (1 - \frac{\text{Death of each treatment (\%)}}{\text{Death of control (\%)}})\text{X } 100\ \%$$

2.10 Data Analysis

Data analysis in this study used analysis of variance (ANOVA). If the analysis results show a significant or very significant difference, it will be continued with Duncan's Multiple Range Test (DMRT) to compare the treatment effect.

3. RESULTS

3.1 Relative Percent Survival (RPS)

Calculating RPS value was performed to determine the effectiveness of immunostimulants from the extract of *C. racemosa* in vaname shrimp after being infected with *V. parahaemolyticus* bacteria. The research process was carried out by observing the mortality of vaname shrimp every day until the maintenance was complete. The death of vaname shrimp

infected with *V. parahaemolyticus* bacteria was recorded 24 hours after infection with *V. parahaemolyticus* bacteria on K+ (without adding *C. racemosa* extract) until day six after infection with *V. parahaemolyticus* bacteria. The K- treatment (without the addition of *C. racemosa* extract and *V. parahaemolyticus* infection) had an RPS value of 40% which was significantly different ($p<0,05$) against K+ and P3 but not significantly different ($p>0,05$) with P1 and P2. Meanwhile, treatments P1 (addition of 3 g/g of *C. racemosa* extract) and P2 (addition of 6 g/g of *C. racemosa* extract) each had an RPS value of 60%. The P3 treatment (addition of 9 g/g of *C. racemosa* extract) had the highest RPS value of 80% (Table 1). The cumulative mortality value of shrimp with the addition of *C. racemosa* extract had a lower value than the control treatment, namely K- and K+. The highest cumulative mortality value of vaname shrimp was found in K+ with a value of 33.33%, which was significantly different from treatments P1, P2, and P3 ($p<0,05$), followed by K- with a value of 20.33%. In addition, the cumulative mortality in P1 and P2 had a value of 13.33% each, and the lowest cumulative mortality value was found in the P3 treatment with a value of 6.67% (Figure 1).

Table 1. Relative Percent Survival (RPS) of white shrimp after infection with *V. parahaemolyticus*.

Treatment	Cumulative Death (%)	RPS (%)
K+	33.33^{b}	0^{c}
K-	20.33^{ab}	40^{b}
P1	13.33^{a}	60^{ab}
P2	13.33^{a}	60^{ab}
P3	6.67^{a}	80^{a}

Description:(K-) Injection with sterile PBS; (K+) VP infection 10^6 CFU/ml per shrimp; (P1) Addition of *C. racemosa* 3 g/g shrimp weight + VP infection 10^6 CFU/ml per shrimp; (P2) Addition of *C. racemosa* 6 g/g shrimp weight + VP infection 10^6 CFU/ml per shrimp; (P3) Addition of *C. racemosa* 9 g/g shrimp weight + VP infection 10^6 CFU/ml per shrimp.

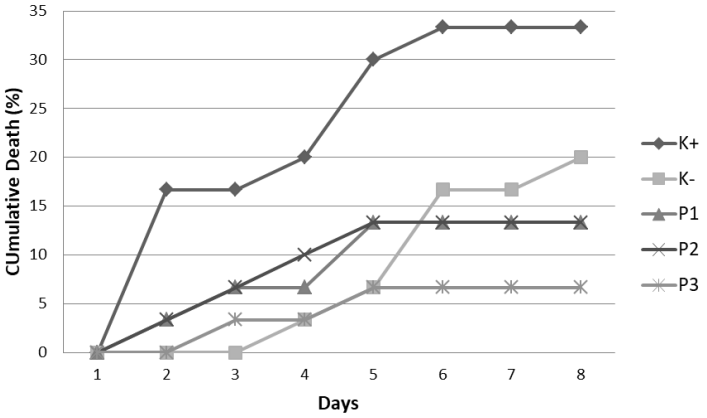

Figure 1. Cumulative mortality of vaname shrimp. (K-) Injection with sterile PBS; (K+) VP infection 10^6 CFU/ml per shrimp; (P1) Addition of *C. racemosa* 3 g/g shrimp weight + VP infection 10^6 CFU/ml per shrimp; (P2) Addition of *C. racemosa* 6 g/g shrimp weight + VP infection 10^6 CFU/ml per shrimp; (P3) Addition of *C. racemosa* 9 g/g shrimp weight + VP infection 10^6 CFU/ml per shrimp.

3.2 Clinical Signs

Vannamei shrimp in this study had different clinical signs for each treatment.

K- treatment (without adding *C. racemosa* extract and without *V. parahaemolyticus* infection) did not show any clinical signs of the attack of *V. parahaemolyticus* bacteria with black hepatopancreas and full intestines (Figure 2A). This was different from the K+ treatment (without adding *C. racemosa* extract), which showed clinical signs in shrimp infected with *V. parahaemolyticus* bacteria with the indicated symptoms of an empty intestine and a whitened hepatopancreas (Figure 2B). The treatment with the addition of *C. racemosa* extract, namely treatments P1 and P2 (2C, D), showed signs of loss of tissue pigmentation in the hepatopancreas and caused the hepatopancreas to blanch. The intestines to empty, but treatment P3 (2E) did not show clinical signs with full intestines and black hepatopancreas.

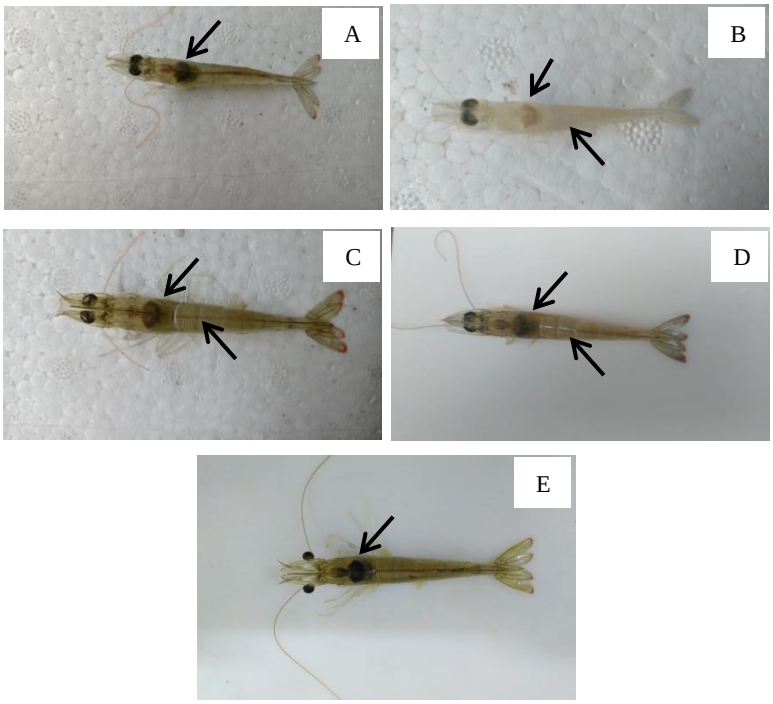

Figure 2. Clinical signs of shrimp in each treatment. (K-) Injection with sterile PBS; (K+) VP infection 10^6 CFU/ml per shrimp; (P1) Addition of *C. racemosa* 3 g/g shrimp weight + VP infection 10^6 CFU/ml per shrimp; (P2) Addition of *C. racemosa* 6 g/g shrimp weight + VP infection 10^6 CFU/ml per shrimp; (P3) Addition of *C. racemosa* 9 g/g shrimp weight + VP infection 10^6 CFU/ml per shrimp.

3.3 Behavior Change of Shrimp

Changes in the behavior of vaname shrimp after injection of V. parahaeomolyticus bacteria in K+, P1, P2, and P3 treatments decreased shrimp appetite and erratic swimming behavior. In addition, the shrimp settle at the bottom of the aquarium with a passive movement. After that, vaname shrimp infected with V. parahaeomolyticus showed behavioral changes in the form of loss of balance and shrimp death. Vannamei shrimp in K- treatment did not show any changes in behavior and could swim normally.

4. DISCUSSION

RPS (Relative percent survival) is a calculation to show the effectiveness of the use of certain compounds in anticipating

mortality in organisms (Marbun *et al*., 2019). The results of the research that has been carried out show that the RPS value in shrimp increased along with the addition of *C. racemosa* extract in shrimp. The highest RPS value was found in P3 with an RPS value of 80%, besides that, there were P1 and P2 treatments with an RPS value of 60% each. The lowest RPS value in this study was K- (control), with an RPS value of 40% (Table 1). According to Costa *et al*., (2011) stated that a good RPS value has a value of 60% of the total organism. Pholdaeng and Pongsamart (2010) said that adding polysaccharides to shrimp can have an RPS value of up to 60%. The increase in the RPS value in shrimp corresponds to the cumulative mortality value in shrimp, with the lowest cumulative mortality value found in P3 with a value of 6.67%, followed by P1 and P2 with a cumulative mortality value of 13.33%. The cumulative mortality value of shrimp with the addition of *C. racemosa* extract was lower than the control treatment, namely K- with a value of 20%. The highest cumulative mortality was found in K+, with a value of 33.33% (Table 1).

Based on the research that has been done, the administration of *C. racemosa* extract can increase the RPS value and reduce mortality in shrimp. This is due to the polysaccharide sulfate content in *C. racemosa*, which can function as an immunostimulant (Xie *et al*., 2016). The addition of sulfate polysaccharides can cause an increase in non-specific immune parameters of shrimp, namely Phenoloxidase activity, and can increase the survival value of shrimp (Cantelli *et al*., 2019). In addition, the addition of sulfate polysaccharides in shrimp can stimulate haemocyte cells to proliferate and cause an increase in the value of THC (Total haemocyte count) (Pholdaeng and Pongsamart, 2010). The increase in total haemocyte count (THC) in shrimp can be caused by sulfated polysaccharides that can stimulate macrophages to increase phagocytic activity that can destroy pathogenic cells, cytokine secretion, and enzyme activity (Huang *et al*., 2019).

Based on the research results, clinical signs of shrimp in K+ treatment show signs of AHPND disease, which is characterized by whitened hepatopancreas and empty intestines. In addition, clinical signs of AHPND disease were found in treatments P1 and P2, which were marked by a pale color change in the hepatopancreas and an empty intestine. This was different from the K+ and P3 treatments which did not show any clinical signs and were characterized by black hepatopancreas and full intestines. This statement is supported by Hong *et al*., (2016), who state that shrimp infected with AHPND disease have clinical signs, namely white hepatopancreas and slow growth in shrimp. In addition, according to Pang *et al*., (2019) stated that shrimp infected with AHPND disease showed signs of an empty stomach, atrophy of the hepatopancreas, and soft carapace. According to Soto-Rodriguez *et al*., (2018), there are 3 phases of AHPND infection in shrimp: the initial, the acute, and the terminal. The acute phase can reduce the survival rate by up to 50% at 17 hours post-infection, whereas after 24 hours post-infection, shrimp show clinical signs in the terminal phase with a lower decrease in survival value than in the acute phase (Aguilar-Rendon *et al*., 2020)

Changes in the behavior of vaname shrimp infected with *V. parahaemolyticus* in K+, P1, P2, and P3 treatments decreased appetite and erratic swimming movements. After irregular swimming movements, vaname shrimp infected with *V. parahaemolyticus* bacteria became passive in moving. They remained at the bottom of the aquarium, followed by a loss of balance in vaname shrimp, and continued with shrimp death. This change in the behavior of vaname shrimp was not found in the K- treatment. The K- treatment did not show differences in conduct which was indicated

by the normal swimming movements of vaname shrimp. This statement is supported by Morales-Covarrubias *et al*., (2016), who state that shrimp infected with *V. parahaemolyticus* bacteria show behavioral changes in the form of irregular swimming behavior, the shrimp stays at the bottom of the aquarium and ends with the death of shrimp.

5. CONCLUSION

The addition of *C. racemosa* in shrimp can affect the highest RPS value at P3 with a value of 80% at the end of the study period, and shrimp infected with *V. parahaemolyticus* showed changes in clinical signs, namely empty intestine, changes in the hepatopancreas to white color and changes in behavior in shrimp that tend to passively moves and loses balance in swimming. Based on this research, adding *Caulerpa racemosa* extract can prevent AHPND disease in shrimp.

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