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COMMERCIAL HERBS ADMINISTRATION FOR PREVENTING *Vibrio parahemolyticus* INFECTION IN *Litopenaeus vannamei* SHRIMP

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ABSTRACT

Vibrio parahaemolyticus is one of the pathogens in crustaceans that can cause mass mortality in *Litopenaus vannamei* shrimp farming. This study aimed to evaluate the effect of administering the commercial herbs supplement Phycurma Aquatic (PA) through feeding at different doses to prevent *Vibrio parahaemolyticus* infection in *L.vannamei* shrimp. This study used a completely randomized design (CRD) consisting of five treatments and three replications, which are negative control group, positive control group, and PA at doses of 2.5, 5.0, and 7.5 mL kg^{-1} feed. Shrimps were reared for 30 days and fed five times a day. On the 31th day, shrimps were challenged with V*. parahaemolyticus* at a dose of 10^5 CFU mL^{-1}, except for the negative control group. The results showed that the administration of 5.0 mL kg^{-1} of PA in the feed gave the best growth performance ($P<0.05$) compared to other treatments. The administration of 5.0 mL kg^{-1} PA in feed also enhanced shrimp health status and significantly increased the total hemocyte count, phagocytic activity, respiratory burst, and phenoloxidase. Furthermore, the administration of PA also increased antioxidant activity, reduced malondialdehyde levels, decreased *V. parahaemolyticus* population in the intestine, and reduced hepatopancreas tissue damage. Moreover, the survival rate of vannamei shrimp before and after the challenge test in the treatment group with a dose of 5.0 mL kg^{-1} of PA was significantly higher ($p<0.05$) compared to other treatments.

KEYWORDS: *Commercial herbs; health status; phycurma aquatic; V. parahaemolyticus; L. vannamei*

INTRODUCTION

Litopenaeus vannamei which is usually called vanammei shrimp in the community is a major aquaculture commodity in Indonesia that holds high economic value with positive production trends. Vannamei shrimp farming ranks first among crustacean production, reaching 4.97 million tons in 2018 and rose to 5.81 million tons in 2020 (FAO, 2022). The value of Vannamei shrimp production represents 51.7% of the total crustacean production globally. Indonesia stands at the fourth rank, contributing 892 thousand tons to the crustacean production (FAO, 2022). This large number of productions was achieved through intensive farming with high density. However, intensive farming poses a higher risk of disease outbreaks with the potential in resulting significant losses, that lead to declines in production due to mass death (Hossain *et al*., 2022).

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Vibriosis disease in vannamei shrimp farming is one of the problems encountered by farmers that caused by *V. parahaemolyticus* infections (Abdel-latif *et al*., 2022). *V. parahaemolyticus* is a pathogenic bacterium in crustaceans with a wide host range, affecting all shrimp species and other crustaceans such as prawns, crabs, and lobsters (Chen *et al*., 2021). Infections of *V. parahaemolyticus* in vannamei shrimp can result in necrosis, growth inhibition, anorexia, and death (Valente & Wan, 2021). *V. parahaemolyticus* is also an agent of Acute Hepatopancreatic Necrosis Disease (AHPND), which has *Pir*A and *Pir*B toxins. AHPND is a type of disease that affects vannamei shrimp, showing signs of infection such as shrimp appearing lethargic and the hepatopancreas exhibiting atrophy, necrosis, and a pale appearance (Boonyawiwat *et al*., 2018). This disease can cause shrimp mortality up to 100% within 30-35 days post larvae stocking, resulting in significant economic losses in Asia amounting to 4 billion USD between 2009-2018. It has been reported to cause substantial financial losses (Shinn *et al*., 2018) and around 44 billion USD worldwide (Tang & Bondad-Reantaso, 2019).

One of the commercial herbs ingredients that is not fully studied and suspected to be effective in preventing shrimp diseases is Phycurma Aquatic. Phycurma Aquatic is an herbs supplement product that contains extracts of *Curcuma domestica 25 g*, *Curcuma xanthorrhiza 15 g*, and *Phyllantus niruri 10 g*. *C. domestica* or turmeric and *C. xanthorrhiza* or Javanese ginger contain bioactive compounds with various pharmacological effects such as anti-inflammatory, antioxidant, antibacterial, antiviral, and antifungal properties (Shan & Iskandar, 2018). The primary compounds in *C. domestica* are essential oils and curcuminoids (Kusbiantoro & Purwaningrum, 2018). *P. niruri* or meniran has the potential to treat diseases caused by fungal, viral, and bacterial infections, and can enhance the immune system due to its flavonoid, alkaloid, tannin, and vitamin C content (Nisar *et al*., 2018). It also can reduce blood glucose levels and boost the immunity of vannamei shrimp (Wachid *et al*., 2022). Several studies have applied the use of curcumin in fish. In the research by (Purbomartono *et al*., 2021), the use of *C. domestica* in fish was found to enhance the immune system, and in the study by (Wei *et al*., 2024), it resulted in good health status in fish. Additionally, the research by (Sarin *et al*., 2014) showed that the use of *P. niruri* could also improve the immune response in shrimp. The commercial herbs product Phycurma Aquatic, which combines compounds from *C. domestica*, *C. xanthorrhiza*, and *P. niruri*, is indicated as an immunostimulant that can enhance the immune system in shrimp. This study aimed to evaluate the effects of the commercial herbs product Phycurma Aquatic administration through diet at different doses to prevent *V. parahaemolyticus* bacterial infections in vannamei shrimp.

MATERIALS AND METHODS

Preparation of Maintenance Media and Test Animals

This research was conducted from January to September 2023 at the Laboratory of Aquatic Organism Health, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University. The experiment was conducted using a completely randomized design (CRD) used in this research with five treatments and three replications, including negative control group, positive control group, and Phycurma Aquatic at doses of 2.5, 5.0, and 7.5 mL kg^{-1} of feed. Vannamei shrimp (*L. vannamei*) used as a tested species with an average size of 1.5±0.01 g. Aquarium with size of 60×30×30 cm^3 (20 L volume of water) used as rearing media filled with seawater (salinity at 28 ppt) without resirculation system and equipped with heaters and aeration. Water quality conditions during the rearing period were maintained by siphoning and water exchange of 30% every three days. and adding treated sea water with a salinity of 28 ppt, if there is a decrease in salinity, seawater can be added until the salinity reaches the desired level. However, if the salinity value is high, the salinity can be reduced by adding freshwater until the desired salinity value is obtained. Water quality parameters during vannamei shrimp rearing are temperature (28.0-29.2°C) measured with a thermometer, pH (7.52-7.72) measured with a pH meter, DO (6.2-7.4 mg L^{-1}) measured with a DO meter, salinity (28-29 g L^{-1}) measured with a refractometer, with measurements every day at 07.00, 12.00, and 17.00, and TAN (0.094-0.339 mg L^{-1}) is read using a spectrophotometer at a wavelength of 630 nm and measured at the beginning and end of the maintenance. These results are managed by following national standard SNI 8008:2014 BSN (2014).

Preparation of Test Feed and Test Bacteria

Test feed was prepared by dissolving 3 g of Agribind binder in distilled water, followed by adding Phycurma Aquatic at doses of 2.5, 5.0, and 7.5 mL kg^{-1} of feed according to the treatment doses. The mixture was thoroughly stirred, then added to the feed and dried at room temperature before use. The shrimps stocked with a density of 20 individuals/aquarium. Shrimp were fed five times every day at 06.00, 10.00, 14.00, 18.00, and 22.00, with a 8-6% feeding rate. The challenge test was conducted after 30 days of administering the treatment feed and continued for 7 days to assess the commercial herbs effect in preventing *V. parahaemolyticus* infections in vannamei shrimp. The bacteria used for the challenge test were *V. parahaemolyticus* strains marked with rifampicin resistance (Rf^R) obtained from the Laboratory of Aquatic Organism Health, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University. The challenge test carried out using an injection method referring to the LD50 results (the concentration of *V. parahaemolyticus* that causes 50% shrimp mortality) using the method by Reed & Muench (1938).

Observation Parameters

Parameters observed in this study are growth performance, health status, bacterial abundance in the intestine, antioxidant activity, and histopathology. Growth performance parameters are survival rate (SR) (%) = (final shrimp number/initial shrimp number) × 100 (Yunarty & Renitasari, 2022), specific growth rate (SGR) (%) = [(ln final shrimp weight (g) - ln initial shrimp weight (g))/duration of the experiment (days)]

× 100 (Fuandila *et al*., 2019), and feed conversion ratio (FCR) = feed intake/(final weight (g) - initial weight (g)) (Yunarty & Renitasari, 2022), (Yang *et al*., 2015).

Health status parameters are total hemocyte count (THC). This involves taking 0.1 mL of shrimp hemolymph from the ventral sinus using a 1 mL syringe pre-filled with anticoagulant in a 1:2 ratio. Next, the hemolymph is dropped onto a hemocytometer and covered with a cover glass. The total hemocytes are observed and counted under a microscope at 100× magnification (Huynh *et al*., 2018), phagocytic activity (PA) measurement is done by mixing 100 µL of shrimp hemolymph sample with 25 µL of *Staphylococcus aureus* suspension (10^7 CFU mL^{-1}) and then incubating for 20 minutes. Next, a smear is prepared, dried, fixed with methanol for five minutes, and dried again. Then it is stained with Giemsa stain for 20 minutes and observed under a microscope at 400x magnification (Anderson & Siwicki, 1995), respiratory burst (RB) is carried out by incubating a mixture of 100 µL hemolymph and anticoagulant for 30 minutes at room temperature. The hemolymph is then centrifuged at 3000 rpm for 20 minutes, and the resulting supernatant is discarded. The pellet is treated with 100 µL of *nitro-blue tetrazolium* (NBT solution 0.3%) and incubated for 2 hours at room temperature. The NBT suspension is then centrifuged at 3000 rpm for 10 minutes, and the supernatant is discarded. Next, 100 µL of absolute methanol is added, and the mixture is centrifuged again for 10 minutes at 3000 rpm.

The resulting pellet is rinsed with 70% methanol. Then, 120 µL of KOH and 140 µL of *dimethyl sulfoxide* (DMSO) are added, and the sample is placed into a microplate well. The respiratory burst value is measured using a microplate reader at a wavelength of 630 nm. KOH or DMSO is used as a blank for standard values. The respiratory burst is expressed as the reduction of NBT per 10 µL of hemolymph (Hampton *et al*., 2020), and phenoloxidase activity (PO) is measured using the formation of dopachrome produced by L-DOPA. A mixture of 0.2 mL hemolymph and 0.8 mL anticoagulant is centrifuged at 1500 rpm for 10 minutes. The supernatant is discarded, and the pellet is gently resuspended by adding 1 mL of *cacodylate-citrate buffer* solution (0.01 M *sodium cacodylate*, 0.45 M *sodium chloride*, 0.10 M *trisodium citrate*, pH 7) and centrifuged again at 1500 rpm for 10 minutes. The supernatant is discarded once more, and the pellet is added to 200 µL of *cacodylate-citrate buffer* (0.01 M *sodium cacodylate*, 0.45 M *sodium chloride*, 0.10 M *trisodium citrate*, pH 7). The resulting suspension (100 µL) is incubated with 50 µL of *trypsin* (1 mg/mL in *cacodylate buffer*) as an activator for 10 minutes at a temperature of 25-26 °C, then L-DOPA (3 mg mL^{-1} in *cacodylate buffer*) is added and left for 5 minutes, followed by the addition of 800 µL *cacodylate buffer*. The optical density (OD) is measured using a spectrophotometer at a wavelength of 492 nm. The standard solution contains 100 µL of hemocyte suspension, 50 µL of *cacodylate buffer* (as a substitute for *trypsin*), and 50 µL of L-DOPA. The optical density of PO activity is expressed as dopachrome formation in 50 µL hemolymph (Ekasari *et al*., 2014).

Parameters measured for bacterial abundance in the intestine are total *Vibrio* sp. (TVC) and *V. parahaemolyticus* Rf^R (VPC) counted using the pour plate method. The intestines of shrimp (weighing 0.1 g) taken from one shrimp in each aquarium were homogenized in 0.9 mL of sterile *phosphate-buffered saline* (PBS). *Thiosulfate citrate bile salt sucrose* (TCBS) without *rifampicin* was used to count presumptive Vibrio (Liu *et al*., 2010), while TCBS with *rifampicin* (50 µg mL^{-1}) was used to count *V. parahaemolyticus* for the challenge test. The samples were serially diluted and 50 µL were spread on each of the media. The plates were incubated for 24 hours at 37°C, and then the total bacterial abundance was counted (Madigan *et al*., 2012).

Histopathology parameters are conducted on the hepatopancreas organ. Test shrimp were collected from each treatment to determine the extent of tissue damage caused by *V. parahaemolyticus* infection and the effects of the treatments. Organs and tissues from the test shrimp were first fixed with Davidson's fixative solution for 24 hours. The organs were cut into pieces of 3-5 mm and 1 × 1 cm, then subjected to dehydration, clearing, impregnation, embedding, and paraffin blocking. The tissue blocks were sectioned with a microtome at 5 ìm and stained with *hematoxylin-eosin* (Munaeni *et al*., 2020) and classification of the degree of tissue damage is carried out by scoring the histology results referring to Wolf *et al*. (2015) that is $P < 20\%$ (score 0, normal), $20\% \leq P < 40\%$ (score 1, mild damage), $40\% \leq P < 60\%$ (score 2, moderate damage), $60\% \leq P < 80\%$ (score 3, severe damage), $P \geq 80\%$ (score 4, very severe damage).

Antioxidant activity parameters consist of superoxide dismutase activity (SOD), the test solution is prepared by adding 20 µL of SOD *Assay Buffer* and 50 µL of SOD *Enzyme Solution* into 100 mL of cold PBS and then stirred until homogeneous. Subsequently, SOD analysis is conducted with sample preparation involving 0.15 g of tissue placed into 10 mL of 10 M H_2SO_4. It is then centrifuged (3000 rpm) to separate

the pellet and supernatant. The supernatant is collected and transferred into a sterile tube. Next, 20 μL of the test solution is added and left for 20 minutes. The reaction is stopped by adding 50 μL of BHT. The mixed solution is then measured using a spectrophotometer at a wavelength of 550 nm (McCord & Fridovich, 1969) and malondialdehyde levels (MDA) with the preparation of the test solution consisting of: 50 μL MDA Lysis Buffer + 50 μL TBA + 100 mL 10% KCl + 1 mL WST solution. Tissue samples weighing 0.15 g are prepared and immersed in EDTA until submerged. They are then left until the color of the tissue sample fades. Next, EDTA is discarded, and 2-5 mL of chloroform methanol is added to the sample, followed by centrifugation (3000 rpm) for 15-20 minutes to separate the pellet and supernatant. The supernatant is collected and transferred into a sterile reaction tube. 200 μL of the test solution is added to the supernatant. Then it is shaken until the mixture turns reddish or reddish-yellow. Afterward, it is measured using a spectrophotometer at a wavelength of 550 nm (Ohkawa *et al*., 1979).

Data Analysis

The data obtained were tabulated using Microsoft Excel 2023 software. Data analysis of survival rate, specific growth rate, feed conversion ratio, total haemocyte count (THC), phagocytic activity (PA), respiratory burst (RB), phenoloxidase (PO), bacterial abundance in the intestine, superoxide dismutase (SOD) activity, and malondialdehyde (MDA) levels was conducted using analysis of variance (ANOVA) with SPSS version 27. If significant differences were found, further testing was performed using Tukey's test with a confidence interval of 95%. Histopathological data were analyzed using scoring.

RESULTS AND DISCUSSION

Growth Performance

Growth performance of vannamei shrimp treated with commercial herbs supplements through feeding for 30 days of culture shown in Table 1. The initial weight did not significantly differ across all treatments ($p > 0.05$). The administration of the commercial herbs supplement significance affecting the final weight ($P < 0.05$), specific growth rate (SGR), and feed conversion ratio (FCR) compared to the control group. The survival rate (SR) in the treatment with a dose of 5.0 ml of PA was significantly higher and significantly different ($P < 0.05$) than the PA 2.5, PA 7.5, and control treatments. Overall, the administration of PA 5.0 resulted in the best growth performance, including final weight, SGR, FCR, and SR.

Tabel 1. Growth performance of vannamei shrimp treated with commercial herbs supplements Phycurma Aquatic through feeding for 30 days of culture.

Treatments	W0 (g)	W30 (g)	SGR (%)	FCR	SR (%)
K-	1.50 ± 0.01^{a}	3.24 ± 0.06^{a}	1.86 ± 0.10^{a}	2.07 ± 0.03^{c}	86.67 ± 2.89^{a}
K+	1.50 ± 0.01^{a}	3.21 ± 0.04^{a}	1.81 ± 0.05^{a}	2.08 ± 0.06^{c}	86.67 ± 2.89^{a}
PA 2.5	1.50 ± 0.01^{a}	3.85 ± 0.05^{c}	2.86 ± 0.08^{c}	1.72 ± 0.06^{b}	95.00 ± 5.00^{bc}
PA 5.0	1.50 ± 0.01^{a}	4.32 ± 0.05^{d}	3.46 ± 0.05^{d}	1.48 ± 0.02^{a}	98.33 ± 2.89^{c}
PA 7.5	1.50 ± 0.01^{a}	3.68 ± 0.04^{b}	2.61 ± 0.05^{b}	1.80 ± 0.11^{b}	90.00 ± 0.00^{ab}

Note: Different letters behind the mean value ± standard deviation in the same row indicating a significant difference ($p<0.05$). W0: initial weight, W30: final weight, SGR: specific growth rate, FCR: feed conversion ratio, SR: survival rate. Treatments: K- (negative control), K+ (positive control), doses of Phycurma Aquatic (PA) 2.5, Phycurma Aquatic (PA) 5.0, Phycurma Aquatic (PA) 7.5.

The use of herbs in vannamei farming can be an initial step in disease prevention and can enhance growth performance. The administration of a commercial herbs supplement in this study demonstrated improved growth performance compared to the control (Table 1). The commercial herbs supplement used contains curcumin which is also found in ginger, turmeric, and meniran, that can stimulate shrimp appetite and promote growth. This result is supported by the findings of (Gholian *et al*., 2022), who stated that curcumin compounds in turmeric enhance nutrient absorption and metabolism, thus improving the digestive system. The research by (Mulyadi *et al*., 2022) showed that the use of curcumin doses can enhance shrimp growth. Similarly, the study by (Putri *et al*., 2017) also found that curcumin can improve the growth rate in fish. The results of this study show better growth performance in the treatment with 5.0 mL kg^{-1} of commercial herbs supplement compared to other treatments (Table 1), with the highest survival rate (98%) during the 30-day culture period. This indicates that using the appropriate dosage of the commercial herbs supplement provides an optimal effect, stimulating protein synthesis and growth in vannamei shrimp.

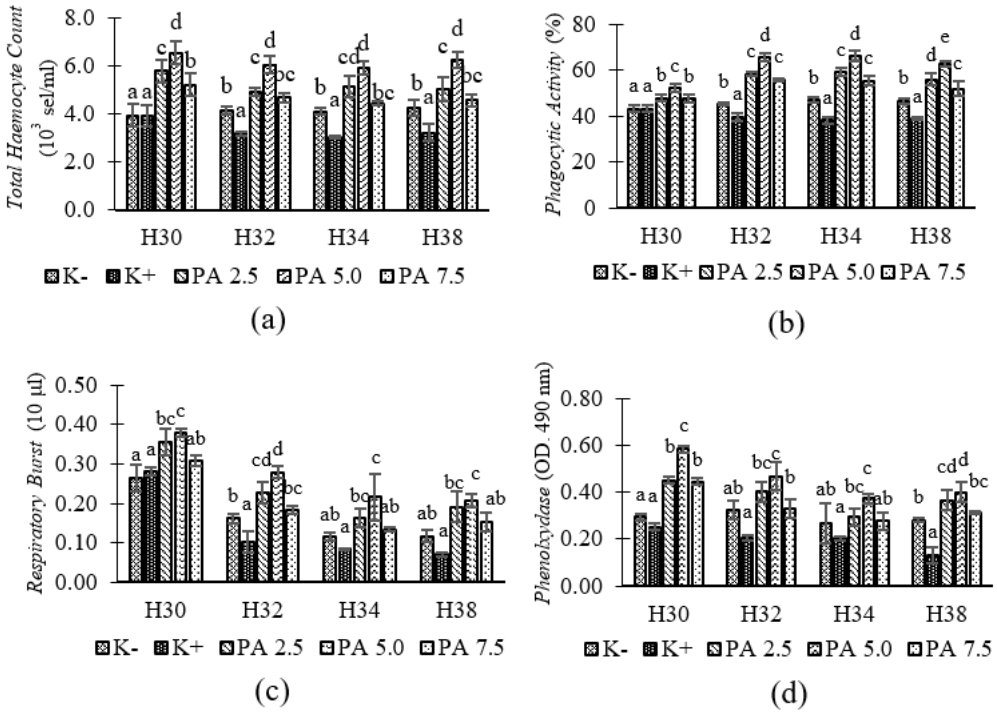

Figure 1. *Total haemocyte count* (a), *phagocytosis activity* (b), *respiratory burst* (c), and *phenoloxidase* (d) of vannamei shrimp at different doses of commercial herbs supplements through feeding. Different letters above the bars in the same observation period indicate significant differences ($P < 0.05$). Treatments: K- (negative control), K+ (positive control), doses of Phycurma Aquatic (PA) 2.5, Phycurma Aquatic (PA) 5.0, Phycurma Aquatic (PA) 7.5. Day 30 (before challenge test), 32nd day (1 day after challenge test), 34th (3 days after challenge test), and 38th day (7 days after challenge test).

Health status

Observations regarding health status indicate that the administration of commercial herbs supplements through feeding for 30 days resulted in significantly higher values of THC, PA, RB, and PO compared to the control (Figure 1). On the 32nd day (1 day after the challenge test), 34th day (3 days after the challenge test), and 38th day (7 days after the challenge test), the values of THC, PA, RB, and PO in vannamei shrimp treated with PA 2.5; 5.0; 7.5 showed significantly higher values ($P < 0.05$) compared to the positive control treatment. Overall, the administration of PA 5.0 led to better health status, including THC, PA, RB, and PO.

Health status of vannamei shrimp can be evaluated through hemocytes. Hemocytes act as a mechanism in response to cellular factors, such as phagocytosis, encapsulation, melanization, cell communication, and cytotoxicity (Eleftherianos *et al.*, 2021). The administration of a commercial herbs supplement in this study was able to enhance immune responses such as THC, PA, RB, and PO in vannamei shrimp before and after the challenge test compared to the control (Figure 1). Based on the result of this study, applying Phycurma Aquatic at a dose of 5.0 mL kg^{-1} of feed was the optimum dosage that could enhance the immune response in vannamei shrimp as can be shown from the high values of THC, PA, RB, and PO before and after the challenge test compared to other dosage treatments (Figure 1). Increasing the dosage of PA resulting in the decreasing immune response in the test shrimp, administration of Pycurma Aquatic at a dose of 7.5 mL kg^{-1} of feed may induce immunosuppression. This result matches with Rahmaningsih *et al.* (2021), who stated that using more than the optimal dosage of immunostimulants can lead to immunosuppression in shrimp. This is also in accordance with research by Munaeni *et al.*, 2021; Giri *et al.*, 2023), that use of more than the optimal dose of herbs can suppress the immune response in shrimp. The increase in THC and PA values (Figures 1a and 1b) is due to the presence of curcuminoids, essential oils, flavonoids, alkaloids, and tannins in the commercial herbs supplement, leading to enhanced regulation of the immune response in vannamei shrimp. This enhancement can improve their resistance to pathogens, which is a crucial defense system against invading pathogens in shrimp bodies (Andrianti & Baihani, 2022). Phagocytosis is a process of hemocyte cells devouring foreign particles to eliminate pathogens from the shrimp's body (Liu *et*

al., 2020). RB is part of the shrimp's immune system in responding to pathogen infections. In Chen *et al.* (2023), an increase in RB values occurred after the entry of disease agents after infection. Phenoloxidase activity is crucial in combating microbial infections (Santhosh *et al*., 2023). The mechanism of PO begins with phenol oxidation, leading to the formation of quinone that produces a dark brown pigment to inactivate and prevent the spread of pathogens. This process is called melanization (Boonchuen *et al*., 2021).

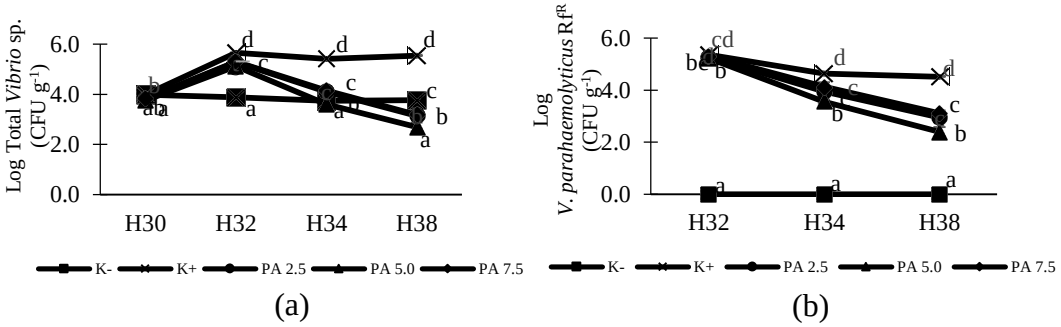

Figure 2. Total *Vibrio sp.* count (a) and *V. parahaemolyticus count* (b) in the intestine of vannamei shrimp at different doses of commercial herbs supplements through feeding. Different letters above the graph in the same observation period indicate significant differences ($P<0.05$). Treatments: K- (negative control), K+ (positive control), doses of Phycurma Aquatic (PA) 2.5, Phycurma Aquatic (PA) 5.0, Phycurma Aquatic (PA) 7.5. The 30th day (before challenge test), 32nd day (1 day after challenge test), 34th (3 days after challenge test), and 38th day (7 days after challenge test).

Bacterial abundance in the intestine

Administration of commercial herbs supplements for 30 days was able to suppress the population ($p<0.05$) of Total *Vibrio sp*. count (TVC) in the intestine of vannamei shrimp compared to the positive and negative control treatments before the challenge test (Figure 2a). The TVC value (Figure 2a) and *Vibrio parahaemolyticus* count Rf^R (VPC) (Figure 2b) increased on the 32nd day (1 day after the challenge test), then decreased on the 34th day (3 days after the challenge test) and 38th day (7 days after the challenge test), with PA 2.5, 5.0, and 7.5 treatments showing lower *Vibrio* populations, significantly different from the positive control. Overall, the PA 5.0 treatment provided lower TVC and VPC values ($P<0.05$) compared to other dose treatments before and after the challenge test.

Administration of a commercial herbs supplement in this study also effectively suppressed the growth of bacteria in the intestines of vannamei shrimp, including total *Vibrio* sp. and *V. parahaemolyticus* Rf^R (Figure 2a and 2b). The administration of Phycurma Aquatic at a dose of 5.0 mL kg^{-1} of feed resulted in lower values ($p<0.05$) compared to other dosage treatments. The commercial herbs supplement used in this study contains bioactive compounds with pharmacological effects, which have antibacterial properties. The research results of Munaeni *et al*. (2021) demonstrate that the use of herbs ingredients can suppress bacterial growth and enhance the immune response in shrimp infected with *V. parahaemolyticus*. In addition, the research by Rozik *et al*. (2022) found that the use of herbs in fish farming can be a preventive measure against the entry of diseases and can inhibit the growth of pathogenic bacteria and the research by Hamzah *et al*. (2021) also showed that the use of the herbs compound curcumin can inhibit the growth of *V. parahaemolyticus* in vannamei shrimp.

Histopathology of the hepatopancreas

Observation on histopathology in the hepatopancreas organ of vannamei shrimp on the 30th day (before the challenge test), 34th day (3 days after the challenge test), and 38th day (7 days after the challenge test) (Figure 3). The histopathological observations indicated a level of hepatopancreas damage in the PA 2.5, 5.0, and 7.5 treatments that were not as severe as in the positive control treatment. The hepatopancreas in the positive control treatment exhibited severe damage characterized by tubule necrosis, vacuolization, B-cell necrosis, and enlarged lumens due to infection.

The percentage of necrosis in the hepatopancreas organ was calculated using the method by Mustaqien *et al*. (2008), using 5 different microscopic fields, and was scored according to Wolf *et al*. (2015). The results of classification in tissue damage indicated that on the 30th day, all treatments were normal. On the 34th day (3 days after the challenge test), all commercial herbs treatment groups experienced moderate

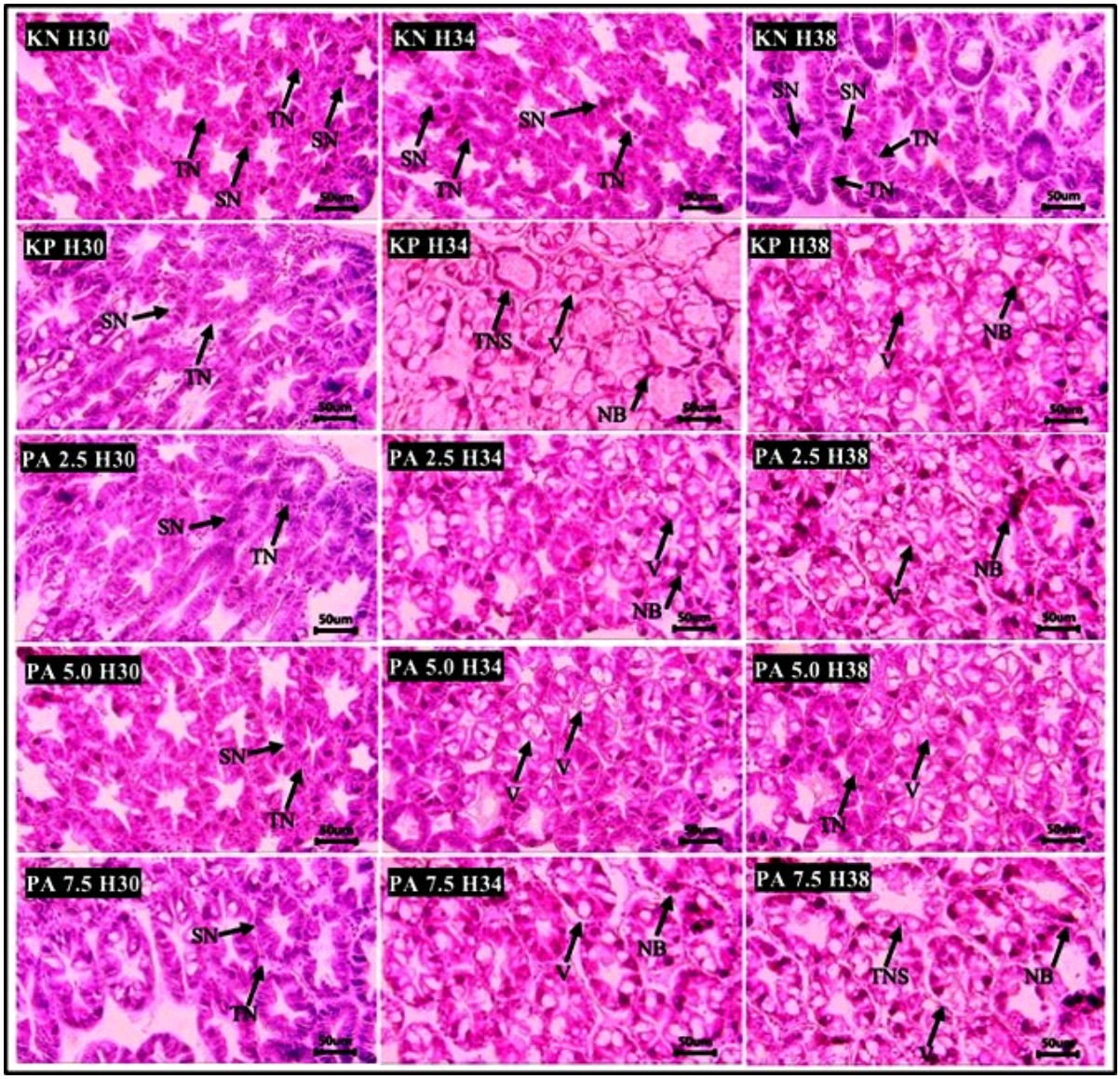

Figure 3. Histopathology of the hepatopancreas organ in vannamei shrimp at different doses of commercial herbs supplements through feeding. The 30th day (before the challenge test), 34th day (3 days after the challenge test), and 38th day (7 days after the challenge test). Treatments: KN (negative control), KP (positive control), doses of Phycurma Aquatic (PA) 2.5, Phycurma Aquatic (PA) 5.0, Phycurma Aquatic (PA) 7.5. Arrows in the observation periods indicate normal tubules (TN), B-cell necrosis (NB), vacuolation (V), normal cells (SN), and necrotic tubules (TNS).

damage, while the positive control suffered severe damage (Table 2). Furthermore, on the 38th day (7 days after the challenge test), the PA 2.5 and 7.5 treatments showed moderate hepatopancreas damage, the PA 5.0 treatment showed mild damage, whereas the positive control exhibited severe damage.

This can be shown from the reduction in lysis and vacuolization of tubules compared to the positive control (Figure 3). Histopathology is conducted to observe pathological changes at the microscopic level in shrimp tissues. The ability of pathogens to infect bacteria can affect tissues microscopically. According to Raja *et al*. (2017), shrimp that are infected with *V. parahaemolyticus* can cause damage to the hepatopancreas organ. Additionally, Suryana *et al*. (2023) stated that tissues can experience acute damage due to pathogen attacks, such as viruses or bacteria. *V. parahaemolyticus* attacks the hepatopancreas with a high level of acute damage. The hepatopancreas tissue in shrimp infected by *V. parahaemolyticus* exhibits a high level of necrosis or cell damage. According to Muharrama *et al*. (2021), necrosis in the hepatopancreatic tubules can cause B cell damage, rendering them non-functional and causing vacuolation. According to Kumar *et al*. (2021), the presence of vacuoles in tissue indicates that the tissue has experienced the most severe damage because it is an indication of cell necrosis. Tubule damage can also

Table 2. Scoring values for hepatopancreas organ damage in vannamei shrimp before and after the challenge test

Day 30			
Treatment	Necrosis	Score	Level of damage
K-	10.54%	0	Normal
K +	11.69%	0	Normal
PA 2.5	11.47%	0	Normal
PA 5.0	10.01%	0	Normal
PA 7.5	10.35%	0	Normal
Day 34			
Treatment	Necrosis	Score	Level of damage
K-	10.54%	0	Normal
K +	78.62%	3	Severe damage
PA 2.5	58.55%	2	Moderate damage
PA 5.0	51.16%	2	Moderate damage
PA 7.5	58.22%	2	Moderate damage
Day 38			
Treatment	Necrosis	Score	Level of damage
K-	10.54%	0	Normal
K +	67.13%	3	Severe damage
PA 2.5	46.97%	2	Moderate damage
PA 5.0	37.43%	1	Mild damage
PA 7.5	50.38%	2	Moderate damage

Note: P< 20% (score 0, normal), 20% d" P < 40% (score 1, mild damage), 40% d" P < 60% (score 2, moderate damage), 60% d" P < 80% (score 3, severe damage), P e" 80% (score 4, very severe damage). Treatments: K- (negative control), K+ (positive control), doses of Phycurma Aquatic (PA) 2.5, Phycurma Aquatic (PA) 5.0, Phycurma Aquatic (PA) 7.5.

lead to lumen dilation due to the tubules opening up (Tran *et al*., 2014). Nazaruddin *et al*. (2014) stated that, histologically, a normal hepatopancreas shows intact tubules with unchanged tubule lumens. In this study, the administration of a commercial herbs supplement was able to enhance the health status to fight *V. parahaemolyticus* infection, minimizing the damage to the shrimp's hepatopancreas. This is consistent with the research by Pratama *et al*. (2014), which found that the use of herbs ingredients can reduce organ damage in shrimp. This is further supported by the research by (He *et al*., 2022; Andayani *et al*., 2019), which found that the use of herbs ingredients can enhance the immune response and reduce organ damage in fish.

Antioxidant activity

Administration of commercial herbs supplements for 30 days significantly increased the *Superoxide dismutase* (SOD) levels in vannamei shrimp ($P<0.05$) compared to the positive and negative control treatments (Figure 4a). The PA 5.0 treatment provided significantly higher antioxidant values ($P<0.05$) compared to other doses before the challenge test. The antioxidant values continued to increase on the 34th day and 38th day (3 and 7 days after the challenge test) compared to the control. On the contrary, the *Malondialdehyde* (MDA) values after 30 days of commercial herbs supplementation were significantly lower ($P<0.05$) than the positive and negative controls (Figure 4b). The *Malondialdehyde* (MDA) values decreased on the 34th day and 38th day (3 and 7 days after the challenge test) compared to the control.

Administration of a commercial herbs supplement to vannamei shrimp resulted in increased antioxidant activity compared to the control (Figure 4a). This can be caused by commercial herbs supplements that contain extracts of *C. domestica*, *C. xanthorrhiza*, and *P. niruri*, which have bioactive compounds such as curcuminoids and flavonoids that serve as antioxidants. Based on the study by Bhoopathy *et al*. (2021), herbs ingredients containing flavonoids and curcumin enhance antioxidant activity. In addition, the research by (Ashry *et al*., 2021; Tawwab *et al*., 2022) also found that the curcumin content can enhance the antioxidant status in fish. The administration of PA at 5.0 mL kg^{-1} of feed was found to provide significantly higher values ($P<0.05$) compared to other dosage treatments. One cause of oxidative stress is the involvement of pathogenic bacteria that infect and induce the production of free radicals, leading to lipid peroxidation, protein damage, and DNA damage (Hosakote & Rayavara, 2020). The body's antioxidant status is observed through increased lipid peroxidation, indicated by the production of MDA values and SOD activity. When shrimp experience in-

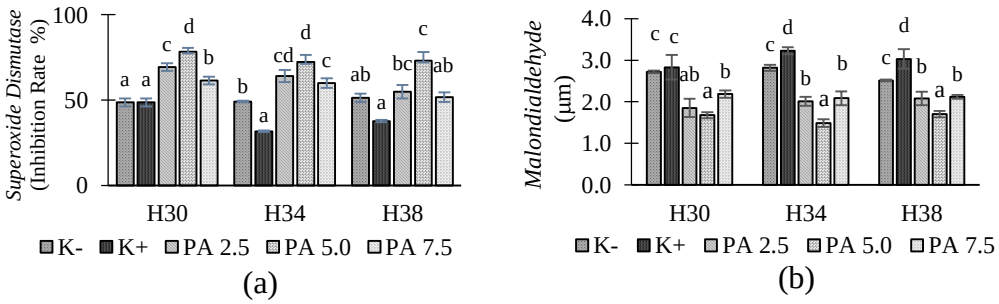

Figure 4. *Superoxide dismutase* (a) and *malondialdehyde* (b) values of vannamei shrimp treated with a commercial herbs supplement at different doses through feeding. Vertical lines above each data bar indicate standard deviation, and different letters above bars indicate significant differences ($P<0.05$). Treatments: K- (negative control), K+ (positive control), doses of Phycurma Aquatic (PA) 2.5, Phycurma Aquatic (PA) 5.0, Phycurma Aquatic (PA) 7.5. The 30th day (before challenge test), the 34th day (3 days after challenge test), and the 38th day (7 days after challenge test).

fection or get stress, hemocytes will produce Reactive Oxygen Species (ROS), and their concentration will be balanced by antioxidant enzymes, including SOD (Balta *et al*., 2022). Furthermore, the administration of a commercial herbs supplement plays an important role in reducing the production of lipid peroxidation or MDA in the shrimp's body, which can damage cells (Figure 4b). Increasing the dosage of the commercial herbs supplement can lead to a decrease in antioxidant activity in the test shrimp, administering a dose of Phycurma Aquatic at 7.5 mL kg^{-1} of feed triggers the formation of pro-oxidant compounds. This is in line with the research found by (Wolnicka & Wisniewska, 2023), which states that excessive use of curcumin, an herbs ingredient, can be toxic and act as a pro-oxidant. Additionally, research by Sotler *et al*. (2019) states that flavonoid compounds also have pro-oxidant properties, and one factor influencing antioxidant activity is the dosage.

Survival rate after challenge test

The observation result on the survival rate of vannamei shrimp after a 7-day challenge with *V. parahaemolyticus* bacteria shown in Figure 5. Vannamei shrimp treated with a commercial herbs supplement at doses of 2.5, 5.0, and 7.5 mL kg^{-1} of feed exhibit higher survival rates of (65%), (75%), and (57%), and significantly different ($P<0.05$) compared to the positive control treatment (43%). The best results are treatment with a dose of 5.0 mL kg^{-1} of feed, and this was significantly different ($P<0.05$) from both the positive control and the PA 7.5 treatments.

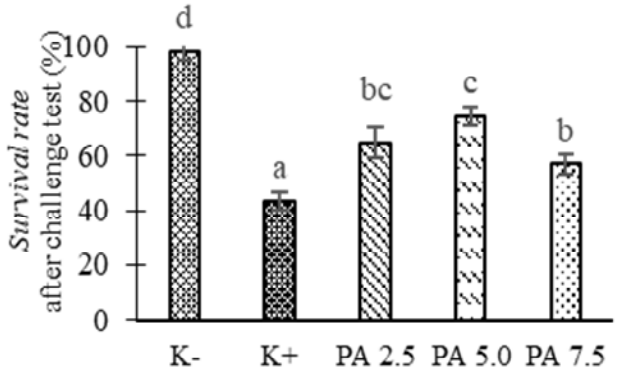

Figure 5. The survival rate of vannamei shrimp treated with a commercial herbs supplement at different doses through feeding after a 7-day challenge test with *V. parahaemolyticus.* Vertical lines above each data bar indicate standard deviation, and different letters above bars indicate significant differences ($P<0.05$). Treatments: K- (negative control), K+ (positive control), doses of Phycurma Aquatic (PA) 2.5, Phycurma Aquatic (PA) 5.0, Phycurma Aquatic (PA) 7.5. 38th day (7 days after challenge test).

The high survival rate in the administration of a commercial herbs supplement after the challenge test (Figure 5) indicates the presence of an effect that is capable of enhancing immune response in vannamei shrimp. Ding *et al*. (2020) stated that the use of herbs supplements in vannamei shrimp can improve survival rates, immune responses, and resistance to pathogen infections. Another study by Khieokhajonkhet *et al*. (2023) found that using herbs containing turmeric can also enhance the immune response and survival rate in carp farming and research by Natasya *et al*. (2022) also found that the use of herbs ingredients can improve the survival rate of vannamei shrimp and resistance to pathogen infections. The results of this study indicate that administering the commercial herbs PA at a dose of 5.0 mL kg^{-1} of feed is the optimal dose, capable of enhancing the immune response, reducing the population of *V. parahaemolyticus* in the intestines, decreasing hepatopancreatic damage, increasing antioxidant activity, lowering MDA levels, and achieving high survival rates in vannamei shrimp before and after the challenge test. This shows that the commercial herbs PA at a dose of 5.0 mL kg^{-1} of feed is better compared to other dosages of the commercial herbs PA. However, treatments with PA at 2.5 and 7.5 mL kg^{-1} of feed are still better than the positive control.

CONCLUSION

Supplementation of commercial herbs through feeding can enhance the growth performance, and immune response, and increase the resistance of vannamei shrimp to *V. parahaemolyticus* infection, with the best results observed at a dose of 5.0 mL kg^{-1} of feed.

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