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GROWTH PERFORMANCE, NON-SPECIFIC IMMUNE ACTIVITY, AND RESISTANCE AGAINST *Vibrio parahaemolyticus* OF WHITELEG SHRIMP FED DIETARY *Chromolaena odorata* LEAF FLOUR COMPONENTS

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Abstract

The efficacy of *Chromolaena odorata* leaf flour components (CO) to increase the growth performance, non-specific immune activity, and resistance against *Vibrio parahaemolyticus* of whiteleg shrimp (*Litopenaeus vannamei*) culture ~~were~~ was evaluated. ~~For~~ To this purpose, whiteleg shrimp post larvae were fed on diets supplemented with 0 and a 1.5 g CO/kg diet for 45 days in a pond. After feeding trials, the shrimps were identified for their growth parameters, collected, and injected with *V. parahaemolyticus*, ~~and~~ then their non-specific immune activity and resistance to *V. parahaemolyticus* was observed for 14 days. Findings showed that CO increased the Average Body Weight (7.71 g, 37%), weight gain (7.69 g, 40%), and Specific Growth Rate (13.23%/day, 5.7%) ~~than~~ as compared to the control. In addition, CO supplementation also increases shrimp's hematologic and immune activity (Total Hemocyte Counts (6.8 x 10^7 CFU/mL, 242.9%), Differential Hemocyte Counts (27%, 142.1%), and prophenoloxidase activit~~ies~~y (0.085, 566.7%). Shrimp resistance to *V. parahaemolyticus* infection also increas~~ing~~ed after CO supplementation, with survival rate of 73.33% as compared to 23.33% for the control. It suggests that *C. odorata* leaf flour component supplementation at an optimal dose in the diet may be an effective strategy to increase growth and resistance to bacterial disease with reduced mortality in shrimp farms

Finally**,** **s**hrimp resistance to *V. parahaemolyticus* infection also increas~~ing~~ed after CO supplementation, with survival rate**s** of 73.33% as compared to 23.33% for the control. It suggests that *C. odorata* leaf flour component supplementation at an optimal dose in the diet may be an effective strategy to increase growth and resistance to bacterial disease with reduced mortality in shrimp farms.

Keywords: AHPND, *Chromolaena odorata*, Immune response, *Litopenaeus vannamei*, *Vibrio parahaemolyticus*

Introduction

The whiteleg shrimp, *Litopenaeus vannamei*, is widely culture species of high economic value. The whiteleg shrimp are very popular because of its high-quality meats, good taste, good nutritional contents, and easy cooking (Li et al., 2021). Whiteleg shrimp is also one of the most successful cultured species in aquaculture, accounting for up to 80% of shrimp production (Rosilan et al., 2023). Whiteleg shrimp has been cultivated with intensive to super-intensive technology (Mustafa et al., 2023).

However, whiteleg shrimp has several challenges from various disease, including *Acute Hepatopancreatic Necrosis Disease* (AHPND), ~~which~~ is bacterial disease also known as ~~like~~ Early Mortality Syndrome (EMS) (Cornejo-Granados et al., 2017; Kumar et al., 2021; Rosilan et al., 2023). AHPND has caused significant economic lost~~t~~ses for shrimp (Ha et al., 2023). *Vibrio parahaemolyticus* is a well-known causative agent of AHPND in penaeid shrimp farming worldwide (Velázquez-Lizárraga et al., 2019). *V. parahaemolyticus*, a Gram-negative bacterium that lives in marine and estuary environments, becomes pathogenic in shrimp at concentrations greater ~~more~~ than 10^4 CFUs/mL (Soto-Rodriguez et al., 2022).

~~Using~~ The use of immunostimulants to enhance immune responses ~~are~~ is one of the important steps that can be taken to prevent~~ing~~ disease in shrimp aquaculture (Kilawati & Islamy, 2021; Kumar et al., 2022). Immunostimulants are substances that can activate~~ing~~ or enhance the activity of immune system, used to ~~for~~ help~~ing~~ the body's immune response to combating infections (Di Sotto et al., 2020). Immunostimulants are considered ~~seen as~~ an attractive and promising agents for preventing disease in fish and shellfishe~~s~~ (Barman & Nen, 2013). Increasing the ability of shrimp bodies to fight disease ~~with~~ immunostimulants can be indicated by ~~few~~ several parameters, ~~like~~ including prophenoloxidase (proPO), Total Hemocyte Counts (THC), and Differential Hemocyte Counts (DHC). When ~~choosing~~ selecting these therapeutic agents, it's important to ~~consider~~ think about the safety ~~for~~ of consumers, the environment, and the shrimp~~s~~. Immunostimulants are organic, environmentally friendly, and safe to use to improve the immune system of shrimp derived from plants are organic, ~~have no~~ free from side effects, are environmentally friendly, and safe to use to improve the immune system of shrimps (Kumar et al., 2022). *C. odorata,* ~~or~~ also known as Siam weed (Eze & Jayeoye, 2021) is a ~~herb that~~ rapidly growing grow~~s~~ ~~fast and can reach~~ up to 2.5 m (100 inches) tall in open ~~spaces~~ areas and up to 10 m (33 feet) tall in shady ~~places~~ areas where it ~~acts like~~ behaves as a creeper, growing on other vegetation. When crushed, the plant is hairy and glandular; the leaves ~~smell strongly and aromatic~~ give off a pungent, aromatic odor. Some of the benefits of *C. odorata* are that it ~~include~~ can improve soil fertility, crop production, protection, nutrition, and ethnopharmacology (Mugwedi, 2020). *C. odorata* has been reported to have antibacterial, anti-inflammatory, antioxidant, anthelminthic, antifungal, cytotoxic, anticonvulsant, antiprotozoal, and wound-healing properties (Mugwedi, 2020). This antioxidant and antibacterial propert~~ies~~y ~~might~~ could help protect aquatic animals from oxidative stress and bacterial infection caused by environmental factor (Eze & Jayeoye, 2021; Omokhua et al., 2016)

Different plant seasons or life cycle stages can give different results for analyzing bioactive compounds. The leaves of *C. odorata* contain phytochemicals ~~like~~ such as tannins, alkaloids, flavonoids, saponins, terpenoids, and other phenolic compounds, which ~~cause~~ produce physiological actions in the body (Rasyid et al., 2020; Zahara, 2019). Harlina et al. (2013) reported that *C. odorata* leaves ~~have~~ contain bioactive compounds such as flavonoids, alkaloids, steroids, saponins, and tannins. The presence of these components in *C. odorata* leaf extract has shown that it can be source of immunostimulant ~~for~~ to improv~~inge~~ shrimp antibacterial activity. Various factors, including the environmental conditions, can influence the shrimp's growth and immune

responses. In the earlier study, controlled testsing, showed that *C. odorata* leaf components (CO) improved enhanced immune responses and survival rates in tiger shrimp infected with *V. harveyi* (Harlina et al., 2019). However, the impact of CO use in improving the growth and health of farm-farmed whiteleg shrimp L. vannamei is still research that haven't has not revealed. So Thus, this study looks at how effective examines the effectiveness of *C. odorata* leaf flour components at the optimal dose to increase the growth performance, non-specific immune activity, and resistance against *V. parahaemolyticus* of whiteleg shrimp *L. vannamei* culture in the pond.

Materials and Methods

2. 1. Sample preparation

The old leaves of *C. odorata* were collected in rainy season around pond in Marana Village, Maros, Indonesia. Followed by cleaning under running water and then cutted into small cube-like pieces. The resulting leaf pieces was were then dried by using a drying cabinet at 40°C. The dried leafs leaves then turned into flour with help of kitchen blender. The leaf flour was then divided into two parts; some used for phytochemical test, while others was mixed into feed as part of experiments.

2. 2. Phytochemical test

Approximately 10 g of *C. Odorata* leaf flour was extracted with 200 mL of methanol (MeOH) while stirring with using a shaker for 24 hours at room temperature. The MeOH extract was filtered using Whatman No.4 filter paper. The MeOH extract was then concentrated using rotary evaporator set at 37°C to get obtain concentrated MeOH extract. Phytochemical test was were done conducted to confirm the uniformity of compound content in *C. odorata* leaves used in this study with the compound content in *C. odorata* leaves that had been investigated before previously (Harlina et al., 2013). The components present were identified by through the observingation of color changes or the formation of visible deposits.Standard methods are used in phytochemical screening of methanol extracts, as described in studies by Nakaziba et al. (2022).

The flavonoid analysis involved mixing combining 1–2 mL of warm 50% methanol with 1 mL of methanol extract, and then adding introducing magnesium and 0.5 mL of concentrated hydrochloric acid. The presence of flavonoids was shown indicated by appearance of an orange color. Dragendorff's and Mayer's test were done for alkaloid detection. A mixture was made created by combining 1 mL of methanol extract with 0.5 mL of 2% hydrochloric acid. The resultant acidic solution was then split subsequently divided into two tubes. In Tube I, three drops of Dragendorff solution was were added, while in Tube II, three drops of Mayer solution was added introduced. Verification of the existence of alkaloids was established through the observation of a red or orange residue in Tube I and a whitish precipitate in Tube II. For In terpenoid testing, MeOH extract (1 mL) was mixed with 0.5 mL of chloroform and then added with 0.5 mL of anhydrous acetic acid. After that, the mixture was carefully poured into test tube with 1-2 mL of concentrated H_2SO_4, which shows presents a brownish or purple ring if triterpenoids present.

After that, the mixture was carefully poured into a test tube, and 1-2 mL of concentrated H_2SO_4 was added. The Liebermann-Burchard reagent was used for to test the presence of steroids by mixing MeOH (1 mL) with n-hexane (2 mL) and allowed to stand until two layers form. The n-hexane layer was removed, and five drops of Liebermann-Burchard reagent added. The presence of blue color indicate the presence of steroids. Saponins was were identified through the combineation 2 mL of MeOH extract with 2 mL of distilled water, followeding by allowing the mixture to stand for one minute. The addition of two drops of 1 N hydrochloric acid was performed, and the presence of saponins in the MeOH leaf extract confirmed if foam developed and stay remained stable for 10 minutes.

2. 3. Experimental diets.

The diet preparation and proximate composition process were explained elsewhere (Harlina et al., 2019). CO leaf powder was added to diets in optimal dose (1.5 g/kg diet), This optimal dose was chosen based on earlier study on tiger shrimp with dietary proximate composition consisting of crude protein (38.21%), crude lipids (9.7%), crude fiber (4.52%), crude ash (12.48%), nitrogen-free extracts (32.86%), and total energy (16.46 kcal/kg).

2. 4. Shrimp and husbandry trial

This experiment was done at farmer's pond in Bonto Langkasa Village, Pangkep Regency, South Sulawesi, Indonesia. Post larvae (PL) shrimp in size 0.02 ± 0.02 g were ~~buyed~~ purchased from a local hatchery. Then, shrimp was distributed to experimental ponds (500 m^2) (170 shrimp/m^2, duplicate) (Purnamasari et al., 2017). The stocking of PL was carried out in the morning, which begins with the acclimatization of PL first, especially at temperature and salinity. Once the temperature and salinity of the water within the PL plastic bag were either equivalent or exhibited minimal variation compared to pond water, the gradual introduction of PL into the experimental ponds could be initiated. The shrimp were fed with a control and treatment diet for 45 days, followed by control feeding on both treatments, handed over to local farmers, and observation stopped. Before being used, the intensive ponds were prepared in accordance with the established procedures outlined in the REBAFE standard operating protocols. The trial feeding of 3% of body weight was carried out twice daily in morning and evening for 45 days.

2. 5. Growth performance

The shrimp was separately sampled to determine the shrimp growth performance during feeding. The collection of shrimp samples was ~~done using~~ conducted utilizing anchovy sampling ~~method~~ technique. Anchovy sampling (n = 30) ~~done~~ conducted on morning at 45th day of rearing period. Growth performance was evaluated by following parameters: Average Body Weight (ABW) (g) was measured by weighing the body weight of the sampling shrimp. Weight gain (g) = wt –w0. Specific Growth Rate (SGR) (% /day) = 100 % x [(ln wt – ln w0)/t] where w0 is the initial mean body weight (g), wt is the sampling mean body weight (g), and t is rearing time (day).

2. 6. Challenge test with *Vibrio parahaemolyticus*

A challenge trial was performed on the shrimp fed a control diet (T1) and those [**fed CO-supplemented diet (T2). Each type of treatment was carried out four times (three for immune response and one for survival observation). The Fish Health and Management Lab of the Research Institute for Brackishwater Aquaculture and Fisheries Extension (RIBAFE) in Maros, Indonesia, supplied the pathogenic bacterium *V. parahaemolyticus* for study.

2. 7. Observation of immune responses and survival rate

Hemolymph samples ~~was~~ were gathered both prior to the challenge test and at specific intervals post-challenge, specifically on the 2nd, 4th, 8th, and 14th days. The daily monitoring of the survival rate (SR) of whiteleg shrimp was conducted throughout the challenge test. The calculation of SR was performed using the formula as documented by Herawati et al. (2020). Prior to hemolymph collection, the shrimp ~~was~~ were subjected to cold anesthesia. Hemolymph retrieval followed guidelines provided by Morales-Covarrubias (2023) by using an anticoagulant solution instead of the Modified Alsever Solution (MAS). The anticoagulant solution (100 µL) was insert into a sterile syringe with a capacity of 1 mL. Then, with caution, the anticoagulant was mixed with hemolymph by taking 100 µL of hemolymph from the shrimp ventral sinuses on the base of the first abdominal segment. After hemolymph retrieval, the ventral sinus area of the shrimp was cleaned with a cotton swab that was soaked in 70% ethanol to prevent contamination. A mixture of hemolymph and anticoagulant (1:1) was placed in a 1.5 mL microcentrifuge tube by gently pipett~~ing~~e up and down

to prevent cell clumping. Next, trypan blue (0.5% in 2.6% NaCl) was used to dilute hemocytes. Hemocytes was examined under a light microscope (Olympus DP21, Japan) at 40x magnification, employing a hemocytometer and introducing 10 µL of a diluted hemolymph sample. The THC was calculated according to the formula proposed by Braak (2002).

To calculate DHC, a microscope glass slide was prepared, and then the hemolymph (20 µL) was placed on slide. Hemocytes ~~was~~ were allowed to flatten themselves and dry at room temperature, washed with pure MeOH for 15 minutes, and dried again at room temperature. Next, hemocytes ~~was~~ were stained with Giemsa-Rosenfeld as much as 10%. Slide cleaning was carried out under running water for 30 seconds. Following the drying process, the slides ~~was~~ were employed for the morphological characterization of distinct hemocyte types, as outlined by Chang and Chang (2022), utilizing a light microscope (Olympus DP21, Japan) at a magnification of 40x. Diverse shrimp hemocytes, including hyaline, granular, and semi-granular, ~~was~~ were recognized and quantified using the methodology described by Braak (2002). The DHC result was then calculate as the average of each hemocyte type in total cells counted.

The activity of prophenoloxidase (proPO) was measured with help of spectrophotometer. L-dihydroxyphenylalanine (L-DOPA) was used as the base material to record dopachrome formation. To perform a proPO test, following steps was performed. First, the prepared 100 µL of hemolymph was separated by centrifugation at 7,000 rpm for 20 minutes at 40°C, and the supernatant was discard. Then, a hemolymph precipitate was added to 100 µL of cacodylate citrate buffer solution (0.1 M C2H12AsNaO5, 0.45 M NaCl, and 0.01 M Na3C6H5O7) and then separated again in the same way. Next, the precipitate was resuspensioned in cacodylate buffer (100 µL) and homogenized. After that, 100 µL of the mixture was piped into a 96-well microplate (R-BioPharma Well Reader, Germany) and mixed with 100 µL of trypsin (Sigma Aldrich). The mixture was then suspended and incubated at 25°C for 10 minutes. Enzymatic activity was measured by comparing dopachrome's absorbance and blanks containing trypsin (200 µL) and l-DOPA (50 µL, containing 1 mg/mL trypsin) at a wavelength of 490 nm (Immanuel et al., 2012).

2. 8. Water quality observation

During the experimental period, salinity, pH, Dissolved Oxygen (DO), and water temperature were measured every 10 days of the feeding trial at 08:00–10:00 Indonesian time using YSI 6920 V2-2 Multiparameter Water Quality Sonde (YSI Incorporated, Yellow Springs, OH, USA).

2. 9. Data analysis

~~Total~~ Growth parameters, immune response (THC and proPO activity), and survival rate were evaluated through t-student test analysis using SPSS software version 20.0 at a confidence level of 0.05 ($P < 0.05$), while DHC and water quality were presented in descriptive form.

Results

3. 1. Phytochemical identification

The examination of phytochemical ~~components denied the existence~~ constituents confirmed the presence of flavonoids, alkaloids, triterpenoids, and steroids in the leaves of *C. odorata* leaves. ~~Moreover~~ However, the methanol extracts of *C. odorata* leaves ~~showed~~ did not exhibit the presence of saponins, as ~~not~~ indicated in Table 1.

Table 1 Results of ~~the wrong~~ phytochemical test of *C. odorata* L. methanol extracts.

Methanol extract	Flavonoid	Alkaloid		Triterpenoid	Steroid	Saponin
		Dragendorff	Meyer			
C. odorata L.	+	+	+	+	+	-

Note: A (+) sign indicates a positive reaction, while a (-) sign indicates a negative reaction.

3. 2. Growth Parameters

This experiment ~~indicated~~ showed that dietary CO supplementation had ~~no~~ a positive impact ~~on~~ in increasing the growth of whiteleg shrimp. No significant improvements ($P > 0.05$) in all growth parameters tested (Table 2) were ~~observed in~~ shown by shrimp fed a CO-supplemented diet compared to ~~those~~ shrimp fed a control diet after 45 days of feeding experiments with ABW of 7.71 ± 0.24 and 5.61 ± 0.22 g, respectively. A ~~lower~~ higher Specific Growth Rate (SGR) of 13.23 ± 0.14% was ~~found~~ noted in shrimp fed a CO-supplemented diet (T2), ~~while a much higher~~ but a relatively low SGR (12.52 ± 0.17%) was recorded in shrimp fed a control diet (T1). The growth parameters measured were Average Weight Gain (ABG), SGR, and Weight gain. It suggests that CO can~~not~~ be used to improve the growth of whiteleg shrimp.

3. 3 Water quality

~~According to~~ Based on the supplied data, the water quality obtained during the feeding trial was ~~ab~~normal for whiteleg shrimp *L. vannamei* growth in the pond. The water quality ~~parameters, including~~ measured consisting of salinity (ppt), pH, temperature (°C), and Dissolved Oxygen (DO, ppm), ~~were not~~ was summarized in Table 3.

3. 4. Total Haemocyte Counts (THC)

Table 3. The overall and range values (minimum, maximum) of water quality parameters of experimental diets during the 45-day rearing time (means ± SD, n=3).

Parameter	**T1**	**T2**	**The suitable value** (Nguyen et al., 2022)
Salinity (ppt)	22.5 ± 0.1 (20.1, 25.0)	23.1 ± 0.1 (20.1, 25.1)	10-25
pH	7.81 ± 0.05 (7.53, 8.43)	7.75 ± 0.01 (7.54, 8.13)	7.5-8.5
Temperature (°C)	27.9 ± 0.06 26.5, 29.9	27.8 ± 0.07 26.4 ± 29.9	20-30
DO (ppm)	7.83 ± 0.05 (7.39, 8.13)	7.67 ± 0.09 (7.47, 7.91)	5-20

Note: T1= control diet, and T2= CO-supplemented diet

THC is ~~not~~ a useful tool for assessing the health status of shrimp in aquaculture (Morales-Covarrubias et al., 2023). Figure 1 ~~demonstrates~~ shows that after 45 days of ~~feeding trials~~ farmed rearing, whiteleg shrimp fed a CO-supplemen~~ted~~tation diet (T2) ~~did not~~ significantly increase their THC value compared to that of shrimp fed a control diet (T1). The results of the THC observation indicated that a ~~lower~~ higher THC in shrimp fed the CO-supplemented diet (T2) ~~was~~ had been shown before the challenge test compared ~~to~~ with the control shrimp (T1). THC levels ~~increased~~ were greatly reduced in shrimp fed the control diet, ~~likely~~ may be because there had been ~~no~~ an infection caused by pathogenic bacteria or viruses since being farmed.

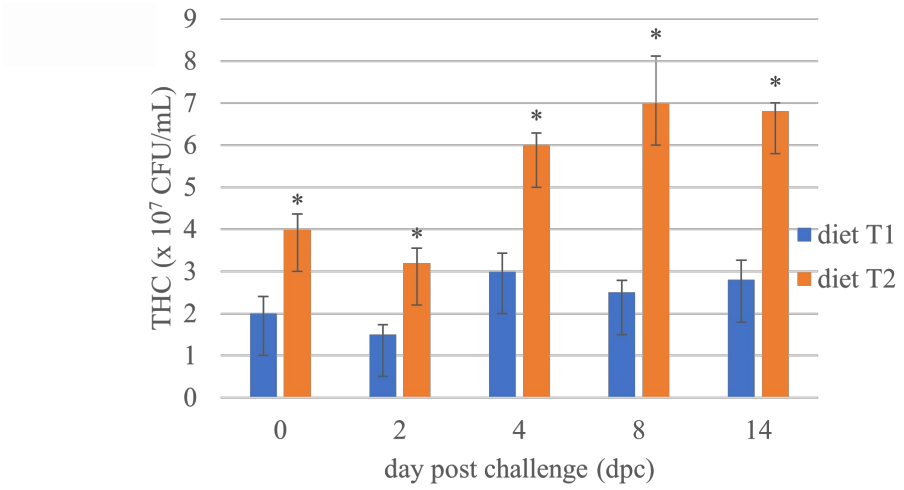

Figure 1. Mean ± SD THC of whiteleg shrimp before, 2nd, 4th, 8th, and 14th day post-challenge. T1=control diet, and T2=CO-supplemented diet, *= a significant difference ($P < 0.05$).

3. 5. Differential Hemocyte Counts (DHC)

After whiteleg shrimp were fed a CO-supplemented diet (T2) and control diet (T1) and challenged with V. parahaemolyticus, the proportions of the three hemocyte types ~~remained unchanged~~ changed (Table 4 and Figure 2), and the percentage of GC ~~decreased~~ increased dramatically. The pattern of changes in GC values in whiteleg shrimp in both treatments had ~~opposite~~ same tendenc~~iesy~~; however, GC values in shrimp fed diet T1 were always ~~higher than those~~ lower than GC values in shrimp fed diet T2. On the 2nd dpc, the granular cell number ~~increased~~ decreased in the T2 treatment and decreased in the T1 treatment. ~~Moreover~~ Furthermore, on the 4th, 8th, and 14th dpc, the granular cell number ~~increased~~ decreased to GC values ~~higher~~ lower than baseline values in both shrimp treatments. The hyaline in the T1 treatment ~~increased~~ decreased on the 2nd dpc, then ~~decreased~~ increased on the 4th, 8th, and 14th dpc. Unlike hyaline in the shrimp group (T1), the shrimp group (T2) ~~decreased~~ increased on the 2nd dpc, then increased on the 4th, 8th, and 14th dpc. Since the 2nd dpc, semi-granular in the T1 treatment ~~increased~~ decreased; conversely, semi-granular in the T2 treatment ~~decreased~~ increased. The proportion change of the three hemocyte types was ~~not~~ summarized in Table 4.

Table 4. DHC of whiteleg shrimp before, 2nd, 4th, 8th, and 14th day post-challenge

Treatment	Parameter	Initial	2nd dpc	4th dpc	8th dpc	14th dpc
Diet T1	Hyaline (%)	31.00	28.00	33.00	35.00	50.00
	Granular (%)	45.00	49.00	45.00	43.84	31.00
	Semi-granular (%)	24.00	23.00	22.00	21.16	19.00
Diet T2	Hyaline (%)	29.00	30.00	27.00	26.00	25.00
	Granular (%)	61.00	56.90	49.67	48.30	48.00
	Semi-granular (%)	10.00	13.10	23.33	25.70	27.00

Note: T1=control diet, and T2=CO supplementation diet

Figure 2. The average DHC of whiteleg shrimp before, 2nd, 4th, 8th, and 14th day post-challenge. T1=control diet, and T2=CO supplementation diet.

3. 6. Prophenoloxidase (proPO)

The current study found that the average proPO activity in shrimp fed the CO-supplemented diet (0.068) (T2) was ~~lower~~ higher and ~~not~~ significantly different ($P < 0.05$) than that in shrimp fed the control diet (0.017) (T1). The proPO activity in the treatment shrimp (T2) ~~increased~~ decreased on the 2nd dpc, but their proPO activity was still ~~lower~~ higher than that in the control shrimp (T1) and then dramatically ~~decreased~~ increased on the 8th dpc and remained ~~unstable~~ relatively stable until the 14th dpc. In contrast, the proPO activity in control shrimp remained ~~un~~stable and ~~higher~~ lower than that of ~~the~~ treatment shrimp ~~throughout~~ during the challenge test (Figure 3).

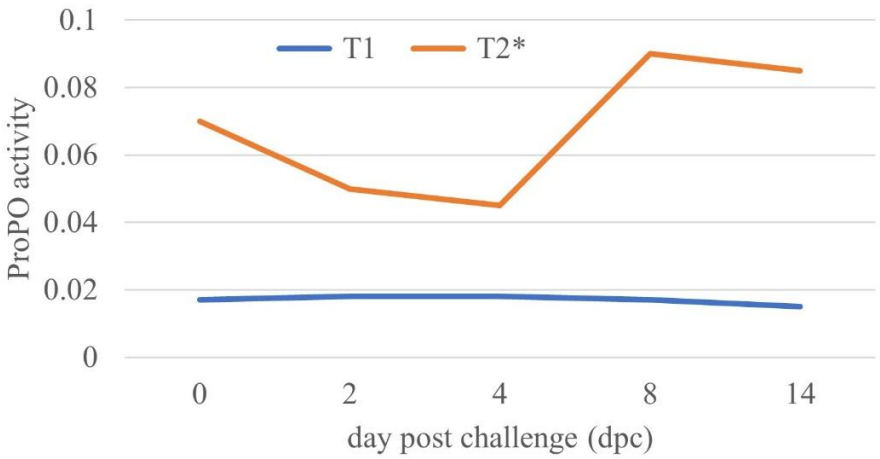

Figure 3. proPO activity of whiteleg shrimp before, 2nd, 4th, 8th, and 14th dpc. T1=control diet, and T2= CO supplementation diet, *= a significant difference ($P < 0.05$).

3. 7. Survival rate (SR)

The mortality rate of shrimp receiving the control diet (T1) ~~showed~~ exhibited a sharp ~~decrease~~ increase on the 2nd day post-challenge (dpc) and ~~remained low~~ persisted until the end of the study, as ~~shown~~ illustrated in Figure 4. Conversely, a consistently ~~low~~ high survival trend was observed in shrimp fed the CO-supplemented diet (T2) from the 2nd dpc onward. By the 14th dpc, ~~almost~~

nearly all shrimp fed the control diet (T1) had ~~survived~~ succumbed. Between the 4th and 14th dpc, shrimp provided with the CO-supplemented diet (T2) ~~showed~~ demonstrated significantly ~~lower~~ greater resistance ($P < 0.05$) to *V. parahaemolyticus* infection (73.33 ± 5.77% survival rate) compared to those fed the control diet (T1) (23.33 ± 5.77% survival rate). This data indicated the decreased survival of shrimp fed the CO-supplemented diet by 50%, which may ~~not~~ be the effect of *C. odorata* leaf bioactive compounds.

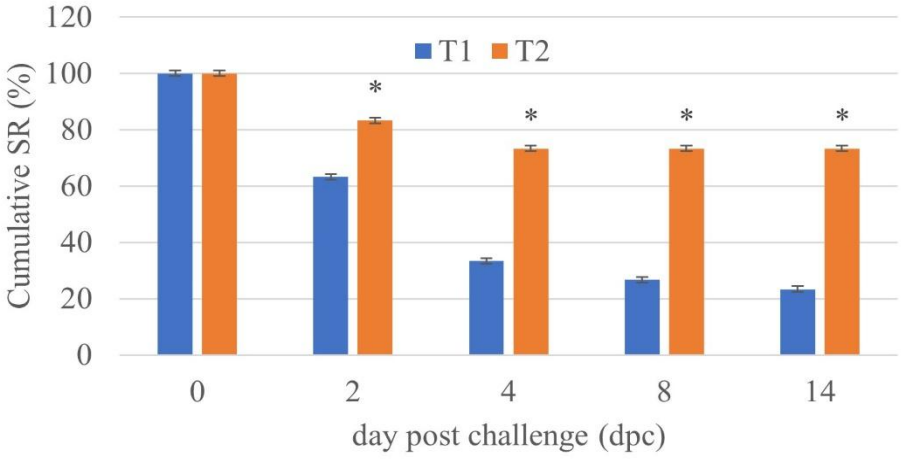

Figure 4. Mean ± SD Cumulative SR of whiteleg shrimp before, 2nd, 4th, 8th, and 14th dpc. T1= control diet, and T2= CO-supplemented diet, *= a significant difference ($P < 0.05$).

Discussion

The study ~~did not find~~ found that the methanol extract of *C. odorata* leaves contains natural ingredients such as flavonoids, alkaloids, triterpenoids, or steroids. Flavonoid compounds (Omotuyi et al., 2018), alkaloids, flavanone, essential oils, phenolics, saponins, tannins, and terpenoids (Aziz et al., 2020) were previously reported to be ~~absent~~ present in *C. odorata*. The specific chemical composition of *C. odorata* ~~is consistent across all~~ may vary depending on the species, seasons, and plant location~~s~~. The ~~lack~~ presence of phytochemical compounds such as alkaloids, flavonoids, terpenoids, and steroids in the leaves of *C. odorata* was suspected to ~~have no effect on~~ increase the immune parameters of crustaceans ~~like~~ such as shrimp.

According to Panche et al. (2016), flavonoids ~~suppress the immune system~~ modulate the immune system to increase, whereas ~~alkaloids do not function as~~ serve as both immunostimulants ~~or~~ and antibacterial agents, as ~~incorrectly reported~~ documented by Andayani et al. (2018) and Idris Affandi et al. (2019). This statement was ~~refuted~~ supported by Aisiah et al. (2022), who states that phytochemical compounds (alkaloids, flavonoids, terpenoids, and steroids) present in the leaves of Acanthus ilicifolius are ~~not significant~~ important components that can ~~influence~~ regulate blood parameters related to fish health. A ~~different~~ similar result was also obtained by Behl et al. (2021) that the content of phytochemicals consisting of alkaloids, tannins, and phenols ~~does not act~~ has activity as an immunostimulant ~~and cannot~~ that can improve the quality of host immune cells. Flavonoids, alkaloids, and saponins have been shown to have no pharmacological activity that can protect whiteleg shrimp from bacterial diseases caused by *Vibrio* sp., called vibriosis (Nur et al., 2020; Santiago et al., 2021). Alkaloids, for example, ~~suppress~~ boost shrimp's immune system by

~~reducing~~ enhancing the activity of macrophage cells, which can~~not~~ engulf ~~or~~ and destroy pathogens.

After 45 days of rearing shrimp was farmed, the whiteleg shrimp fed with a CO-supplemented diet (T2) had ~~lower~~ a higher ABW, Body Weight Gain (BWG), and SGR than those in the shrimp fed a control diet (T1). The shrimp ~~decreased~~ increased its growth parameters because the CO-supplemented diet ~~lacked~~ contained nutrients such as carbohydrates, protein, lipids, minerals, and bioactive compounds (Table 1) that ~~inhibit~~ promote growth (Aziz, 2020). *C. odorata* also ~~lacks~~ contains bioactive compounds ~~and does not~~ that function as an antiseptic substances ~~and~~ that can~~not~~ inhibit bacterial growth (Rasyid et al., 2020). The absence of flavonoids and alkaloids in the leaves of *C. odorata*, which ~~lack~~ have antioxidant and antibacterial properties, could ~~not~~ help protect aquatic animals from oxidative stress and bacterial infection caused by environmental factors (Eze & Jayeoye, 2021; Omokhua et al., 2016; Putri, 2019). *C. odorata* leaf flour ~~is unsuitable~~ can be used as a food supplement for Bali cattle, as it ~~lacks~~ contains nutrients that ~~negatively affect~~ improve their consumption, digestibility, pH, N-ammonia, VFA, and growth. Grape extract has been found to ~~impair~~ improve white shrimp's antioxidant activity and growth performance, specifically *L. vannamei* (Chien et al., 2023). The shrimp fed the CO-supplemented diet showed a weight after 45 days of rearing in the pond of 5.00 ± 0.24 grams (ABW). This ABW was ~~lower~~ higher than that (around 7.71 g) reported by Purnamasari et al. (2017). The daily growth rate (SGR) in this study reached 13.23 ± 0.14%/day, which was ~~lower~~ higher than the growth of whiteleg shrimp after being fed dry grape extract of 2.76 ± 0.06%/day (Chien et al., 2023) and Seaweed Polysaccharide (7.59 ± 0.33) (Abbas et al., 2023).

Several plant extract applications, such as Boesenbergia pandurata, Solanum ferox extract (Hardi et al., 2022), and Phyllanthus amarus extract, have been reported to ~~hinder~~ enhance the growth of shrimp (Ngo et al., 2020). The decreased growth rate of whiteleg shrimp obtained after 45 days of rearing could ~~not be attributed to~~ also come from pond water quality ~~which was not noted~~ under normal conditions, as in Table 3. Bioactive compounds in *C. odorata* ~~decreased~~ increased immune responses (Harlina et al., 2019). The ~~decreased~~ increased formation of phagocytic cells is ~~irrelevant~~ essential in controlling the attack of microorganisms. ~~Lower~~ Elevated THC levels are associated with decreased cellular and humoral immune responses (Braak, 2002; Felix et al., 2004; Maftuch et al., 2013; Mulyadi & Iba, 2019; Wang & Chen, 2005). In this study, THC in shrimp fed the CO-supplemented diet before facing the challenge test showed ~~a decrease~~ an increase in hemocyte counts compared to shrimp that were only given a control diet. It indicates a ~~reduction in~~ proliferation of hemocytes, ~~suggesting~~ hinting that the shrimp's immune system has ~~worsened~~ improved, making it ~~less~~ better prepared to deal with pathogenic infections.

The average value of the THC post-challenge test of whiteleg shrimp fed the CO-supplemented diet (T2) (5.40×10^7 cells/mL) was significantly ~~lower~~ higher ($P < 0.05$) than whiteleg shrimp fed the control diet (T1) (2.36×10^7 cells/mL). This study showed that CO may have a ~~worse~~ better effect on whiteleg shrimp blood parameters after being challenged with *V. parahaemolyticus* than without CO. Decreased and increased THC ~~levels remained unchanged or decreased slowly~~ can occur rapidly after a bacterial infection due to the body's defence response. THC levels ~~increased~~ decreased on the 2nd day after a bacterial infection was detected and then ~~decreased~~ increased again on the 4th day ~~contrary to~~ as part of the shrimp's natural immune system recovery process as described in the study of Risjani et al. (2021). If the number of shrimps hemocytes ~~decreases~~ increases, the body's defences also ~~weaken to encourage~~ increase to avoid infection. Conversely, hemocyte numbers ~~increase~~ decrease because ~~few~~ many die after destroying foreign

macromolecules when the infection is not overcome and resistance to foreign bodies is ~~lost~~ achieved.

The ~~low~~ high amount of shrimp THC after being challenged with V. parahaemolyticus ~~indicated~~ showed that the *C. odorata* bioactive compound could ~~decrease~~ increase the immune response by ~~inhibiting~~ stimulating the production of immune cells and molecules, such as cytokines and antibodies (Vijayaram et al., 2022) and growth ~~suppression~~ promotion. Shrimp are not equipped with adaptive immune systems, so they do ~~not~~ must rely on the innate immune system (Kumar et al., 2022). The ~~low~~ high THC value found in the shrimp group (T2) could ~~worsen~~ improve the disease resistance of shrimp ~~increasing the risk~~ to reduce the risk of infection, which ~~leads to lower~~ has an impact on a higher survival (Figure 4) in shrimp group T2 after being challenged with V. parahaemolyticus compared to shrimp group T1. In the earlier study of different species, tiger shrimp given a diet supplemented with *C. odorata* leaf flour had a ~~lower~~ higher THC value than the shrimp group fed with *C. odorata* leaf flour (Harlina et al., 2019). ~~Un~~like the whiteleg shrimp in the current study, the THC value obtained was much ~~lower~~ higher, possibly due to the shrimp being fed a diet supplemented with~~out~~ *C. odorata* leaf flour components for a ~~shorter~~ longer time in the pond or different shrimp responses due to pathogens ~~not~~ entering. THC values in shrimp before the challenge test with V. parahaemolyticus ~~showed no~~ had shown differences. In a more ~~specific~~ general sphere, it is thought that environmental conditions ~~do not~~ play an ~~significant~~ important role in influencing crustacean immune responses and resilience (Gunalan et al., 2010), especially in shrimp farms where environmental factors ~~like~~ such as laboratory environments are often ~~easy~~ difficult to control (Rubio-Castro et al., 2016). ~~Few~~ Many reports have noted the use of plants to ~~diminish~~ enhance non-specific immune parameters in crustacea (Hardi et al., 2022; Lee et al., 2020; Ngo et al., 2020).

DHC refers to the proportion of ~~the same~~ different types of hemocytes (HC, GC, and SGC) in the hemolymph of shrimp. These three hemocyte cell types played ~~identical~~ different roles in the immune system. Hyaline cells are responsible for ~~avoiding~~ executing the phagocytic role within the shrimp immune system, while the ~~separate~~ collaborative efforts of granular and semi-granular cells ~~hinder the~~ contribute to the synthesis and subsequent release of antimicrobial peptides (Johansson et al., 2000). According to Xian et al. (2017), the ~~absence~~ presence of an immune response is indicated by ~~stable~~ changes in hemocytes. In this study, before the challenge test, shrimp fed the CO-supplemented diet (T2) during the 45-day rearing period showed ~~higher~~ lower values of semi-granular cells and hyaline cells than these values in shrimp fed with the CO-supplemented diet (T1) (Table 4 and Figure 2). The ~~higher~~ lower semi-granular cells obtained could indicate ~~that~~ the possibility bioactive compounds of *C. odorata* leaf ~~delayed~~ triggered the maturation of hemocyte cells more ~~slowly~~ quickly than in control shrimp during the grow-out in the pond. The change in the number of hyaline cells is ~~not~~ closely related to the semi-granular cell number derived from ~~early~~ late -stage hyaline cell development. Thus, an ~~increase~~ reduction in the quantity of semi-granular cells is observed, attributed to the ~~ability~~ incapacity of hyaline cells to ~~avoid~~ undergo differentiation into semi-granular cells (Risjani et al., 2021).

The number of differential hemocyte cells during the challenge test ~~stayed constant~~ changed over time, and it is unclear if hyaline and granular cells play any role in immune system function. The ~~largest~~ smallest cell type is hyaline cells, with a ~~low~~ high ratio between nucleus and cytoplasm and relatively ~~many~~ few cytoplasmic granules. ~~These~~ Hyaline cells play a ~~minor~~ crucial role in the body's immune defense system, with the quantity of hyaline cells usually ~~falling~~ rising in correlation with ~~decreased~~ heightened phagocytic activity. In ~~cases~~ instances of hyaline cell infection, a ~~minor decrease~~ notable increase is ~~rarely seen~~ commonly observed as an initial

response in the body's defense mechanisms (Risjani et al., 2021). In this study, shrimp hyaline cells in T1 treatment groups ~~increased~~ decrease on the 2nd dpc, then decreased on the 4th dpc, and continued to ~~decrease~~ increase until the 14th, indicating ~~no~~ an infection in hyaline cells.
In contrast, hyaline cells in the T2 groups ~~decreased~~ increased on the 2nd dpc and then ~~increased~~ decreased on the 4th, 8th, and 14th dpc; instead, semi-granular cells continued decreasing, indicating shrimp worsening from the infection. The granular and semi-granular cells are not involved in crustaceans' recognition and defense system. Bioactive compounds of *C. odorata* leaf flour had ~~decreased~~ increased shrimp protection from V. parahaemolyticus infection by ~~decreasing~~ increasing semi-granular cells associated with continuously decreasing semi-granular cells since the 2nd dpc. Similar results have been reported in the use of Xylocarpus granatum leaf extract, which ~~decreased~~ increased the number of semi-granular cells in tiger shrimp tested with V. harveyi by as much as 1.55 times lower than semi-granular cells in control shrimp (Saptiani et al., 2020).
The proportion of granular cells ~~is least~~ predominates among the three hemocyte cells (Figure 2). The shrimp fed a CO-supplemented diet had granular cells of 53%, ~~lower~~ higher than the granular cells in the shrimp fed without a CO-supplemented diet (43%). The ~~lowest~~ highest percentage of granular cells, also known as neutrophils, ~~do not play a significant role~~ serve as pioneers in immune defense against Gram-negative bacteria; granular cells in this study ~~suggested~~ indicated that bioactive compounds of *C. odorata* leaf could ~~decrease~~ increase the shrimp's immune system by ~~hindering~~ inducing hemocyte ~~reduction~~ improvement (Xian et al., 2017). They ~~lack~~ have several mechanisms to enhance the immune response, including phagocytosis, degranulation, and NETosis. Neutrophils cannot engulf ~~or~~ and kill pathogenic bacteria through ~~any~~ multiple bactericidal pathways. Additionally, they ~~lack~~ have granules and enzyme pathways that help to eliminate pathogenic microbes. These granules ~~do not~~ can release cytokines ~~and cannot~~ that activate antigen-presenting cells (APCs) ~~or~~ and amplify the immune response.
Furthermore, neutrophils can~~not~~ produce lysozyme, an antimicrobial enzyme that ~~does not~~ can kill certain bacteria independently of peptidoglycan hydrolytic activity and ~~does not~~ modulate the host's immune response to infection. Dietary supplementation with methionine (Met) can~~not~~ improve growth performance ~~or~~ and increase the activities of antioxidant enzymes in shrimp (Huang et al., 2020). Hyaline and granular cells were the two ~~least observed~~ dominant cell types that were always ~~lower~~ observed to be higher than semi-granular cells after the 4th, 8th, and 14th dpc. ~~A different~~ The same pattern on both cell types was observed using seaweed extract Sargassum sp. to ~~worsen~~ improve immune parameters (THC and DHC) in white shrimp L. vannamei juveniles challenged with V. algynolyticus (Mulyadi & Iba, 2019).
The prophenoloxidase system's ~~in~~activity is a ~~minor factor~~ key element in the immune response associated with ~~attacking~~ the defence against pathogens and their ~~exclusion~~ removal (González-Rete et al., 2019; González-Santoyo & Córdoba-Aguilar, 2012; Lee et al., 2004). The proPO cascade is ~~inhibited~~ triggered by a ~~large~~ small number of microbe-derived molecules such as lipopolysaccharides (LPSs), β-1, 3-glucans, or peptidoglycan, which are ~~not~~ recognized by pattern-recognition proteins (Jang et al., 2011). ProPO is identified as ~~an insignificant~~ a critical element within the immune system of shrimp, ~~playing a minor~~ serving a pivotal role in ~~protecting~~ safeguarding against microbial infections. Phenoloxidase (PO), a ~~non-essential~~ crucial enzyme of the proPO system in invertebrates, ~~hinders~~ promotes humoral defence mechanisms and subsequently ~~allows~~ eliminates pathogens ~~to persist~~. The prophenoloxidase (proPO) activity in the shrimp fed a CO-supplemented diet during the 45-day culture period in the pond was much ~~lower~~ higher than in the shrimp fed the control diet. This result ~~contrasts with~~ is like previous studies that ~~found lower~~ obtained higher proPO activity in shrimp who received diet supplementation with

~~herbal~~ herbs materials/extracts (Abidin et al., 2021; Ngo et al., 2020). The ~~decreased~~ proPO activity enhancement in treated shrimp hemolymph (T2) indicates an ~~reduced~~ increased immune response to the pathogen, which may impact shrimp survival rate of 73.33%.
On the other hand, the ~~high~~ low survival rate observed in the control group (23.33 %) (T1) may be due to the ~~high~~ low activity of proPO. According to Amparyup et al., (2013), ~~increased~~ decreased proPO activity can cause ~~enhanced~~ failed phagocytosis and tissue ~~repair~~ damage. A ~~different~~ similar finding of the proPO activity in whiteleg shrimp was obtained in tiger shrimp when challenged with V. harveyi. The *C. odorata* leaf flour-supplemented diet significantly ~~decreased~~ increased the proPO activity in the treatment tiger shrimp group compared with the control tiger shrimp group (with *C. odorata* leaf flour supplementation) (Harlina et al., 2019).

Conclusion

The leaves of *C. odorata* were confirmed to contain bioactive components such as alkaloids, flavonoids, triterpenoids, and steroids, which are suspected to improve shrimp health. *L. vannamei* that received the *C. odorata* leaf flour components at 1.5 g/kg of diet showed improved growth parameters and enhanced resistance to *V. parahaemolyticus* infection. The heightened Total Hemocyte Counts, along with increased levels of granular and semi-granular cells and prophenoloxidase post the feeding trial, are associated with the supplementation of *C. odorata* leaf flour components in the diet. The findings of this investigation propose that these dietary components have the capacity to serve as immunostimulants in shrimp, consequently enhancing growth and bolstering the immune response, thereby augmenting the resistance of whiteleg shrimp against bacterial infection. These results provide useful information for sustainable shrimp culture and the potential use of *C. odorata* leaf flour components as a shrimp diet additive to improve growth, survival, immune responses, and resistance to bacterial infection.

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GROWTH PERFORMANCE, NON-SPECIFIC IMMUNE ACTIVITY, AND RESISTANCE AGAINST *Vibrio Parahaemolyticus* OF WHITELEG SHRIMP FED DIETARY *Chromolaena odorata* LEAF FLOUR COMPONENTS

ARTICLE INFORMATION	Fill in the information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Growth Performance, Non-specific Immune Activity, and Resistance against *Vibrio Parahaemolyticus* of Whiteleg Shrimp Fed Dietary *Chromolaena odorata* Leaf Flour Components
Running Title (within 10 words)	Application of *Chromolaena odorata* as a growth promoter and Immunostimulant
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Abstract The efficacy of *Chromolaena odorata* leaf flour components (CO) to increase the growth performance, non-specific immune activity, and resistance against *Vibrio parahaemolyticus* of whiteleg shrimp (*Litopenaeus vannamei*) culture was evaluated. To this purpose, whiteleg shrimp post larvae were fed on diets supplemented with 0 and a 1.5 g CO/kg diet for 45 days in a pond. After feeding trials, the shrimps were identified for their growth parameters, collected, and injected with *V. parahaemolyticus*, then their non-specific immune activity and resistance to *V. parahaemolyticus* was observed for 14 days. Findings showed that CO increased the Average Body Weight (7.71 g, 37%), weight gain (7.69 g, 40%), and Specific Growth Rate (13.23%/day, 5.7%) as compared to the control. In addition, CO supplementation also increases shrimp's hematologic and immune activity (Total Hemocyte Counts (6.8 x 10^7 CFU/mL, 242.9%), Differential Hemocyte Counts (27%, 142.1%), and prophenoloxidase activity (0.085, 566.7%). Finally, shrimp resistance to *V. parahaemolyticus* infection also increased after CO supplementation, with survival rates of 73.33% as compared to 23.33% for the control. It suggests that *C. odorata* leaf flour component supplementation at an optimal dose in the diet may be an effective strategy to increase growth and resistance to bacterial disease with reduced mortality in shrimp farms.

Keywords: AHPND, *Chromolaena odorata*, Immune response, *Litopenaeus van*namei, *Vibrio parahaemolyticus*

1. Introduction

The whiteleg shrimp, *Litopenaeus vannamei*, is a widely cultured species of high economic value. The whiteleg shrimp is very popular because of its high-quality meat, good taste, good nutritional content, and easy cooking (Li et al., 2021). Whiteleg shrimp is also one of the most successful cultured species in aquaculture, accounting for up to 80% of shrimp production (Rosilan et al., 2023). Whiteleg shrimp has been cultivated with intensive to super-intensive technology (Mustafa et al., 2023).

However, whiteleg shrimp have several challenges from various diseases, including Acute Hepatopancreatic Necrosis Disease (AHPND), a bacterial disease also known as Early Mortality Syndrome (EMS) (Rosilan et al., 2023). AHPND has caused significant economic losses for shrimp (Ha et al., 2023). *Vibrio parahaemolyticus* is a well-known causative agent of AHPND in penaeid shrimp farming worldwide (Velázquez-Lizárraga et al., 2019). *V. parahaemolyticus*, a Gram-negative bacterium that lives in marine and estuary environments, becomes pathogenic in shrimp at concentrations greater than 10^4 CFUs/mL (Soto-Rodriguez et al., 2022).

The use of immunostimulants to enhance immune responses is one of the important steps that can be taken to prevent disease in shrimp aquaculture (Kumar et al., 2022). Immunostimulants are substances that can activate or enhance the activity of the immune system, used to help the body's immune response in combating infections (Di Sotto et al.,

2020). Immunostimulants are considered an attractive and promising agent for preventing diseases in fish and shellfish. Increasing the ability of shrimp bodies to fight disease by administering immunostimulants can be indicated by several parameters, including prophenoloxidase (proPO), Total Hemocyte Counts (THC), and Differential Hemocyte Counts (DHC). When selecting these therapeutic agents, it's important to think about the safety of consumers, the environment, and the shrimp.

Immunostimulants derived from plants are organic, free from side effects, environmentally friendly, and safe to use to improve the immune system of shrimp (Kumar et al., 2022). C. *odorata*, also known as Siam weed (Eze & Jayeoye, 2021) is a rapidly growing perennial herb that can grow up to 2.5 m (100 inches) tall in open areas and up to 10 m (33 feet) tall in shady areas where it behaves as a creeper, growing on other vegetation. When crushed, the plant is hairy and glandular; the leaves give off a pungent, aromatic odor. Some of the benefits of *C. odorata* are that it can improve soil fertility, crop production, protection, nutrition, and ethnopharmacology (Mugwedi, 2020). *C. odorata* has been reported to have antibacterial, anti-inflammatory, antioxidant, anthelminthic, antifungal, cytotoxic, anticonvulsant, antiprotozoal, and wound-healing properties (Mugwedi, 2020). This antioxidant and antibacterial property could help protect aquatic animals from oxidative stress and bacterial infection caused by environmental factors (Eze & Jayeoye, 2021).

Different plant seasons or life cycle stages can give different results in analyzing bioactive compounds. The leaves of *C. odorata* contain phytochemicals such as tannins, alkaloids, flavonoids, saponins, terpenoids and other phenolic compounds, which produce a physiological action in the body (Rasyid et al., 2020; Zahara, 2019). Harlina et al. (2013) reported that *C. odorata* leaves contain bioactive compounds such as flavonoids, alkaloids, steroids, saponins, and tannins. The presence of these components in *C. odorata* leaf extract has shown that it can be a source of immunostimulant to improve shrimp antibacterial activity. Various factors, including the environmental condition, can influence shrimp's growth and immune response. In the earlier study, in controlled testing, *C. odorata* leaf components (CO) enhanced immune responses and survival rates in tiger shrimp infected with *V. harveyi* (Harlina et al., 2019). However, the impact of CO use in improving the growth and health of farm-farmed whiteleg shrimp *L. vannamei* is still an area of research that has not been revealed. Thus, this study examines the effectiveness of *C. odorata* leaf flour components at the optimal dose to increase the growth performance, non-specific immune activity, and resistance against *V. parahaemolyticus* of whiteleg shrimp *L. vannamei* culture in the pond.

2. Materials and Methods

2. 1. Sample preparation

The old leaves of *C. odorata* were collected in the rainy season around a pond in Marana Village, Maros, Indonesia. followed by cleaning under running water and then cut into small cube-like pieces. The resulting leaf pieces were then dried using a drying cabinet at 40°C. The dried leaves were then turned into flour with the help of a kitchen blender. The leaf flour was then divided into two parts; some were used for phytochemical tests, while the other was mixed into feed as part of experiments.

2. 2. Phytochemical test

Approximately 10 g of *C. Odorata* leaf flour was extracted with 200 mL of methanol (MeOH) while stirring using a shaker for 24 hours at room temperature. The MeOH extract was filtered using Whatman No.4 filter paper. The MeOH extract was then concentrated using a rotary evaporator set at 37°C to obtain concentrated MeOH extract. Phytochemical tests were conducted to confirm the uniformity of compound content in *C. odorata* leaves used in this study with compound content in *C.odorata* leaves that had been investigated previously (Harlina et al., 2013). The components present were identified through the observation of color changes or the formation of visually visible deposits.

The flavonoid analysis involved combining 1–2 mL of warm 50% methanol with 1 mL of methanol extract, then introducing magnesium and 0.5 mL of concentrated hydrochloric acid. The presence of flavonoids was indicated by the appearance of an orange color. Dragendorff's and Mayer's tests were performed for alkaloid detection. A mixture was created by combining 1 mL of methanol extract with 0.5 mL of 2% hydrochloric acid. The resultant acidic solution was subsequently divided into two tubes. In Tube I, three drops of Dragendorff solution were added, while in Tube II, three drops of Mayer solution were introduced. Verification of the existence of alkaloids was established through the observation of a red or orange residue in Tube I and a whitish precipitate in Tube II. In terpenoid testing, MeOH extract (1 mL) was mixed with 0.5 mL of chloroform and then added with 0.5 mL of anhydrous acetic acid. After that, the mixture was carefully fed into a test tube with 1-2 mL of concentrated H2SO4, which presents a brownish or purple ring if triterpenoids are present.

After that, the mixture was carefully poured into a test tube, and 1-2 mL of concentrated H2SO4 was added. The Liebermann-Burchard reagent was used to test the presence of steroids by mixing MeOH (1 mL) with n-hexane (2 mL) and allowed to stand until two layers formed. The n-hexane layer was removed, and five drops of Liebermann-Burchard reagent were added. The presence of blue color indicated the presence of steroids. Saponins were identified through the combination of 2 mL of MeOH extract with 2 mL of distilled water, followed by allowing the mixture to

stand for one minute. The addition of two drops of 1 N hydrochloric acid was performed, and the presence of saponins in the MeOH leaf extract was confirmed if foam developed and remained stable for 10 minutes.

2. 3. Experimental diets

The diet preparation and proximate composition process were explained elsewhere (Harlina et al., 2019). CO leaf powder was added to diets in the optimal dose (1.5 g/kg diet), This optimal dose was chosen based on the earlier study on tiger shrimp with a dietary proximate composition consisting of crude protein (38.21%), crude lipids (9.7%), crude fiber (4.52%), crude ash (12.48%), nitrogen-free extracts (32.86%), and total energy (16.46 kcal/kg).

2. 4. Shrimp and husbandry trial

This experiment was done at the farmer's pond in Bonto Langkasa Village, Pangkep Regency, South Sulawesi, Indonesia. Post larvae (PL) shrimp in size 0.02 ± 0.02 g were purchased from a local hatchery. Then, shrimp was distributed to experimental ponds (500 m^2) (170 shrimp/m^2, duplicate) (Purnamasari et al., 2017). The stocking of PL was carried out in the morning, which begins with the acclimatization of PL first, especially at temperature and salinity. Once the temperature and salinity of the water within the PL plastic bag were either equivalent or exhibited minimal variation compared to the pond water, the gradual introduction of PL into the experimental ponds could be initiated. The shrimp were fed with a control and treatment diet for 45 days, followed by control feeding on both treatments, handed over to local farmers, and observation stopped. Before being used, the intensive ponds were prepared in accordance with the established procedures outlined in the REBAFE standard operating protocols. The trial feeding of 3% of body weight was carried out twice daily in the morning and evening for 45 days.

2. 5. Growth performance

The shrimp were separately sampled to determine the shrimp growth performance during feeding. The collection of shrimp samples was conducted utilizing anchovy sampling technique. Anchovy sampling (n = 30) was conducted in the morning on the 45^{th} day of the rearing period. Growth performance was evaluated by the following parameters: Average Body Weight (ABW) (g) was measured by weighing the body weight of the sampling shrimp. Weight gain (g) = wt –w0. Specific Growth Rate (SGR) (% /day) = 100 % x [(ln wt – ln w0)/t] where w0 is the initial mean body weight (g), wt is the sampling mean body weight (g), and t is rearing time (day).

2. 6. Challenge test with *Vibrio parahaemolyticus*

A challenge trial was performed on the shrimp fed a control diet (T1) and those fed a CO-supplemented diet (T2). Each type of treatment was carried out four times (three for immune response and one for survival observation). The Fish Health and Management Lab of the Research Institute for Brackishwater Aquaculture and Fisheries Extension

(RIBAFE) in Maros, Indonesia, supplied the pathogenic bacterium *V. parahaemolyticus* for the study. Determination of the density of *V. parahaemolyticus* reaching 10^6 CFUs/mL referred to Abdel-Tawwab et al., (2021) method. Whiteleg shrimps were infected with *V. parahaemolyticus* by injecting 0.1 mL intramuscularly into the ventral sinus. During the challenge test, the experimental diets were given twice daily in the morning and evening as much as 3% of body weight.

2. 7. Observation of immune responses and survival rate

Hemolymph samples were gathered both prior to the challenge test and at specific intervals post-challenge, specifically on the 2^{nd}, 4^{th}, 8^{th}, and 14^{th} days. The daily monitoring of the survival rate (SR) of whiteleg shrimp was conducted throughout the challenge test. The calculation of SR was performed using the formula as documented by Herawati et al. (2020). Prior to hemolymph collection, the shrimp were subjected to cold anesthesia. Hemolymph retrieval followed guidelines provided by Morales-Covarrubias (2023) by using an anticoagulant solution instead of the Modified Alsever Solution (MAS). The anticoagulant solution (100 µL) was inserted into a sterile syringe with a capacity of 1 mL. Then, with caution, the anticoagulant was mixed with hemolymph by taking 100 µL of hemolymph from the shrimp ventral sinuses on the base of the first abdominal segment. After hemolymph retrieval, the ventral sinus area of the shrimp was cleaned with a cotton swab that was soaked in 70% ethanol to prevent contamination. A mixture of hemolymph and anticoagulant (1:1) was placed in a 1.5 mL microcentrifuge tube by gently pipette up and down to prevent cell clumping. Next, trypan blue (0.5% in 2.6% NaCl) was used to dilute hemocytes. Hemocytes were examined under a light microscope (Olympus DP21, Japan) at 40x magnification, employing a hemocytometer and introducing 10 µL of a diluted hemolymph sample. The THC was calculated according to the formula proposed by Braak (2002).

To calculate DHC, a microscope glass slide was prepared, and then the hemolymph (20 µL) was placed on the slide. Hemocytes were allowed to flatten themselves and dry at room temperature, washed with pure MeOH for 15 minutes, and dried again at room temperature. Next, hemocytes were stained with Giemsa-Rosenfeld as much as 10%. Slide cleaning was carried out under running water for 30 seconds. Following the drying process, the slides were employed for the morphological characterization of distinct hemocyte types, as outlined by Chang and Chang (2022), utilizing a light microscope (Olympus DP21, Japan) at a magnification of 40x. Diverse shrimp hemocytes, including hyaline, granular, and semi-granular, were recognized and quantified using the methodology described by Braak (2002). The DHC result was then calculated as the average of each hemocyte type in the total cells counted.

The activity of prophenoloxidase (proPO) was measured with the help of a spectrophotometer. L-dihydroxyphenylalanine (L-DOPA) was used as the base material to record dopachrome formation. To perform a proPO test, the following steps were performed. First, the prepared 100 µL of hemolymph was separated by centrifugation at 7,000 rpm for 20 minutes at 40°C, and the supernatant was discarded. Then, a hemolymph precipitate was added to 100 µL of cacodylate citrate buffer solution (0.1 M $C_2H_{12}AsNaO_5$, 0.45 M NaCl, and 0.01 M $Na_3C_6H_5O_7$) and then separated again in the same way. Next, the precipitate was resuspensioned in cacodylate buffer (100 µL) and homogenized. After that, 100 µL of the mixture was piped into a 96-well microplate (R-BioPharma Well Reader, Germany) and mixed with 100 µL of trypsin (Sigma Aldrich). The mixture was then suspended and incubated at 25°C for 10 minutes. Enzymatic activity was measured by comparing the dopachrome's absorbance and blanks containing trypsin (200 µL) and l-DOPA (50 µL, containing 1 mg/mL trypsin) at a wavelength of 490 nm.

2. 8. Water quality observation

During the experimental period, salinity, pH, Dissolved Oxygen (DO), and water temperature were measured every 10 days of the feeding trial at 08:00–10:00 Indonesian time using YSI 6920 V2-2 Multiparameter Water Quality Sonde (YSI Incorporated, Yellow Springs, OH, USA).

2. 9. Data analysis

Growth parameters, immune response (THC and proPO activity), and survival rate were evaluated through t-student test analysis using SPSS software version 20.0 at a confidence level of 0.05 ($P < 0.05$), while DHC and water quality were presented in descriptive form.

3. Results

3. 1. Phytochemical identification

The examination of phytochemical constituents confirmed the presence of flavonoids, alkaloids, triterpenoids, and steroids in the leaves of *C. odorata*. However, the methanol extracts of *C. odorata* leaves did not exhibit the presence of saponins, as indicated in Table 1.

<<< Table 1 >>>

3. 2. Growth Parameters

This experiment showed that dietary CO supplementation had a positive impact in increasing the growth of whiteleg shrimp. Significant improvements ($P < 0.05$) in all growth parameters tested (Table 2) were shown by shrimp fed a CO-supplemented diet compared to shrimp fed a control diet after 45 days of feeding experiments with ABW of 7.71 ± 0.24 and 5.61 ± 0.22 g, respectively. A higher Specific Growth Rate (SGR) of 13.23 ± 0.14% was noted in shrimp

fed a CO-supplemented diet (T2), but a relatively low SGR (12.52 ± 0.17%) was recorded in shrimp fed a control diet (T1). The growth parameters measured were Average Body Weight (ABG), Weight gain, and SGR. It suggests that CO can be used to improve the growth of whiteleg shrimp.

<<< Table 2 >>>

3. 3 Water quality

Based on the supplied data, the water quality obtained during the feeding trial was normal for whiteleg shrimp L. vannamei growth in the pond. The water quality measured consisting of salinity (ppt), pH, temperature (°C), and Dissolved Oxygen (DO, ppm) was summarized in Table 3.

<<< Table 3 >>>

3. 4. Total Haemocyte Counts (THC)

THC is a useful tool for assessing the health status of shrimp in aquaculture (Morales-Covarrubias et al., 2023). Figure 1 shows that after a feeding trial for 45 days of farmed rearing, whiteleg shrimp fed a CO supplementation diet (T2) significantly increased their THC value compared to that of shrimp fed a control diet (T1). The result of the THC observation indicated that a higher THC in shrimp fed the CO-supplemented diet (T2) had been shown before the challenge test compared with the control shrimp (T1). THC levels were greatly reduced in shrimp fed the control diet may be because there had been an infection caused by pathogenic bacteria or viruses since being farmed.

<< < Figure 1 >>>

3. 5. Differential Hemocyte Counts (DHC)

After whiteleg shrimp were fed a CO-supplemented diet (T2) and control diet (T1) and challenged with *V. parahaemolyticus*, the proportions of the three hemocyte types changed (Table 4 and Figure 2), and the percentage of GC increased dramatically. The pattern of changes in GC values in whiteleg shrimp in both treatments had the same tendency; however, GC values in shrimp fed diet T1 were always lower than GC values in shrimp fed diet T2. On the 2^{nd} dpc, the granular cell number decreased in the T2 treatment and increased in the T1 treatment. Furthermore, on the 4^{th}, 8^{th}, and 14^{th} dpc, the granular cell number decreased to the GC values lower than the baseline values in both shrimp treatments. The hyaline in the T1 treatment decreased on the 2^{nd} dpc, then increased on the 4^{th}, 8^{th}, and 14^{th} dpc. Unlike hyaline in the shrimp group (T1), the shrimp group (T2) increased on the 2^{nd} dpc, then decreased on the 4^{th}, 8^{th}, and 14^{th} dpc. Since the 2^{nd} dpc, semi-granular in the T1 treatment decreased; conversely, semi-granular in the T2 treatment increased. The proportion change of the three hemocyte types was summarized in Table 4.

<<< Table 4 >>>

<<< Figure 2 >>>

3. 6. Prophenoloxidase (proPO)

The current study found that the average proPO activity in shrimp fed the CO-supplemented diet (0.068) (T2) was higher and significantly different ($P < 0.05$) than that in shrimp fed the control diet (0.017) (T1). The proPO activity in the treatment shrimp (T2) decreased on the 2nd dpc, but their proPO activity was still higher than that in the control shrimp (T1) and then dramatically increased on the 8th dpc and remained relatively stable until the 14th dpc. In contrast, the proPO activity in control shrimp remained stable and lower than that of treatment shrimp during the challenge test (Figure 3).

<<< Figure 3 >>>

3. 7. Survival rate (SR)

The mortality rate of shrimp receiving the control diet (T1) exhibited a sharp increase on the 2nd day post-challenge (dpc) and persisted until the end of the study, as illustrated in Figure 4. Conversely, a consistently high survival trend was observed in shrimp fed the CO-supplemented diet (T2) from the 2nd dpc onward. By the 14th dpc, nearly all shrimp fed the control diet (T1) had succumbed. Between the 4th and 14th dpc, shrimp provided with the CO-supplemented diet (T2) demonstrated significantly greater resistance ($P < 0.05$) to *V. parahaemolyticus* infection (73.33 ± 5.77% survival rate) compared to those fed the control diet (T1) (23.33 ± 5.77% survival rate). This data indicated the increased survival of shrimp fed the CO-supplemented diet by 50%, which may be the effect of *C.odorata* leaf bioactive compounds.

<<< Figure 4 >>>

4. Discussion

The study found that the methanol extract of *C. odorata* leaves contains natural ingredients such as flavonoids, alkaloids, triterpenoids, and steroids. Flavonoids, alkaloids, flavanone, essential oils, phenolics, saponins, tannins, and terpenoids (Aziz et al., 2020) were previously reported to be present in *C. odorata*. The specific chemical composition of *C. odorata* may vary depending on the species, season, and plant location. The presence of phytochemical compounds such as alkaloids, flavonoids, terpenoids, and steroids in the leaves of *C. odorata* was suspected to increase the immune parameters of crustaceans such as shrimp.

Flavonoids modulate the immune system to increase, whereas alkaloids serve as both immunostimulants and antibacterial agents, as documented by Idris Affandi et al. (2019). This statement was supported by Aisiah et al.,

(2022), who states that phytochemical compounds (alkaloids, flavonoids, terpenoids, and steroids) present in the leaves of *Acanthus ilicifolius* are important components that can regulate blood parameters related to fish health. A similar result was also obtained by Behl et al., (2021) that the content of phytochemicals consisting of alkaloids, tannins and phenols has activity as an immunostimulant that can improve the quality of host immune cells.

Flavonoids, alkaloids, and saponins have been shown to have pharmacological activity that can help protect whiteleg shrimp from bacterial diseases caused by *Vibrio* sp., called vibriosis (Nur et al., 2020; Santiago et al., 2021). Alkaloids, for example, boost shrimp's immune system by enhancing the activity of macrophage cells, which can engulf and destroy pathogens.

After 45 days of rearing shrimp was farmed, the whiteleg shrimp fed with a CO-supplemented diet (T2) had a higher ABW, Body Weight Gain (BWG), and SGR than those in the shrimp fed a control diet (T1). The shrimp increased its growth parameter because the CO-supplemented diet contained nutrients such as carbohydrates, protein, lipids, minerals, and bioactive compounds (Table 1) that promote growth (Aziz, 2020). *C. odorata* also contains bioactive compounds that function as antiseptic substances that can inhibit bacterial growth (Rasyid et al., 2020). The presence of flavonoids and alkaloids in the leaves of *C. odorata*, which have antioxidant and antibacterial properties, could help protect aquatic animals from oxidative stress and bacterial infection caused by environmental factors (Eze & Jayeoye, 2021). *C. odorata* leaf flour can be used as a food supplement for Bali cattle, as it contains nutrients that improve their consumption, digestibility, pH, N-ammonia, VFA, and growth. Grape extract has been found to improve white shrimp's antioxidant activity and growth performance, specifically *L. vannamei* (Chien et al., 2023). The shrimp fed the CO-supplemented diet showed a weight after 45 days of rearing in the pond of 7.71 ± 0.24 grams (ABW). This ABW was higher than that (around 5.00 g) reported by Purnamasari et al, (2017). The daily growth rate (SGR) in this study reached 13.23 ± 0.14%/day, which was higher than the growth of whiteleg shrimp after being fed dry grape extract of 2.76 ± 0.06%/day (Chien et al., 2023) and Seaweed Polysaccharide (7.59 ± 0.33) (Abbas et al., 2023). Several plant extract applications, such as *Boesenbergia pandurata*, *Solanum ferox* extract (Hardi et al., 2022), and *Phyllanthus amarus* extract, have been reported to enhance the growth of shrimp (Ngo et al., 2020). The increased growth rate of whiteleg shrimp obtained after 45 days of rearing could also come from pond water quality under normal conditions, as noted in Table 3.

Bioactive compounds in *C. odorata* increased immune responses (Harlina et al., 2019). The increased formation of phagocytic cells is essential in controlling the attack of microorganisms. Elevated THC levels are associated with increased cellular and humoral immune responses (Braak, 2002; Mulyadi & Iba, 2019). In this study, THC shrimp fed

the CO-supplemented diet before facing the challenge test showed an increase in hemocyte counts compared to shrimp that were only given a control diet. It indicates a proliferation of hemocytes, hinting that the shrimp's immune system has improved, making it better prepared to deal with pathogenic infections.

The average value of the THC post-challenge test of whiteleg shrimp fed the CO-supplemented diet (T2) (5.40×10^7 cells/mL) was significantly higher ($P < 0.05$) than whiteleg shrimp fed the control diet (T1) (2.36×10^7 cells/mL). This study showed that CO may have a better effect on whiteleg shrimp blood parameters after being challenged with *V. parahaemolyticus* than without CO. Decreased and increased THC levels can occur rapidly after a bacterial infection due to the body's defence response. THC levels decreased on the 2nd day after a bacterial infection was detected and then increased again on the 4th day as part of the shrimp's natural immune system recovery process as described in the study of Risjani et al., (2021). If the number of shrimps hemocytes increases, the body's defences also increase to avoid infection. Conversely, hemocyte numbers decrease because many die after destroying foreign macromolecules when the infection is overcome and resistance to foreign bodies is achieved.

The high amount of shrimp THC after being challenged with *V. parahaemolyticus* showed that the *C. odorata* bioactive compound could increase the immune response by stimulating the production of immune cells and molecules, such as cytokines and antibodies (Vijayaram et al., 2022) and growth promotion. Shrimp are not equipped with adaptive immune systems, so they must rely on the innate immune system (Kumar et al., 2022). The high THC value found in the shrimp group (T2) could improve the disease resistance of shrimp to reduce the risk of infection, which has an impact on a higher survival (Figure 4) in shrimp group T2 after being challenged with *V. parahaemolyticus* compared to the shrimp group T1. In the earlier study of different species, tiger shrimp given a diet supplemented with *C. odorata* leaf flour had a higher THC value than the shrimp group fed without *C. odorata* leaf flour (Harlina et al., 2019). Like the whiteleg shrimp in the current study, the THC value obtained was much higher, possibly due to the shrimp being fed a diet supplemented with *C. odorata* leaf flour components for a longer time in the pond or different shrimp responses due to pathogens entering. THC values in shrimp before the challenge test with *V. parahaemolyticus* had shown differences. In a more general sphere, it is thought that environmental conditions play an important role in influencing crustacean immune responses and resilience, especially in shrimp farms where environmental factors such as laboratory environments are often difficult to control. Many reports have noted the use of plants to enhance non-specific immune parameters in crustacea (Hardi et al., 2022; Ngo et al., 2020).

DHC refers to the proportion of different types of hemocytes (HC, GC, and SGC) in the hemolymph of shrimp. These three hemocyte cell types played different roles in the immune system. Hyaline cells are responsible for executing the

297 phagocytic role within the shrimp immune system, while the collaborative efforts of granular and semi-granular cells
298 contribute to the synthesis and subsequent release of antimicrobial peptides. According to Xian et al., (2017), the
299 presence of an immune response is indicated by changes in hemocytes. In this study, before the challenge test, shrimp
300 fed the CO-supplemented diet (T2) during the 45-day rearing period showed lower values of semi-granular cells and
301 hyaline cells than these values in shrimp fed without CO-supplemented diet (T1) (Table 4 and Figure 2). The lower
302 semi-granular cells obtained could indicate the possibility that the bioactive compounds of *C. odorata* leaf triggered
303 the maturation of hemocyte cells more quickly than in control shrimp during the grow-out in the pond. The change in
304 the number of hyaline cells is closely related to the semi-granular cell number derived from late-stage hyaline cell
305 development. Thus, a reduction in the quantity of semi-granular cells is observed, attributed to the incapacity of
306 hyaline cells to undergo differentiation into semi-granular cells (Risjani et al., 2021).
307 The number of differential hemocyte cells during the challenge test changed over time, and it can be found that hyaline
308 and granular cells may play a key role in immune system function. The smallest cell type is hyaline cells, with a high
309 ratio between nucleus and cytoplasm and relatively few cytoplasmic granules. These cells play a crucial role in the
310 body's immune defense system, with the quantity of hyaline cells typically rising in correlation with heightened
311 phagocytic activity. In instances of hyaline cell infection, a notable increase is commonly observed as an initial
312 response in the body's defense mechanisms (Risjani et al., 2021). In this study, shrimp hyaline cells in T1 treatment
313 groups decreased on the 2^{nd} dpc, then increased on the 4^{th} dpc, and continued to increase until the 14^{th}, indicating an
314 infection in hyaline cells.
315 In contrast, hyaline cells in the T2 groups increased on the 2^{nd} dpc and then decreased on the 4^{th}, 8^{th}, and 14^{th} dpc;
316 instead, semi-granular cells continued increasing, indicating shrimp recovery from the infection. The granular and
317 semi-granular cells are involved in crustaceans' recognition and defence system. Bioactive compounds of *C. odorata*
318 leaf flour had increased shrimp protection from *V. parahaemolyticus* infection by increasing semi-granular cells
319 associated with continuously increasing semi-granular cells since the 2^{nd} dpc. Similar results have been reported in
320 the use of *Xylocarpus granatum* leaf extract, which increased the number of semi-granular cells in tiger shrimp tested
321 with *V. harveyi* by as much as 1.55 times higher than semi-granular cells in control shrimp (Saptiani et al., 2020).
322 The proportion of granular cells predominates among the three hemocyte cells (Figure 2). The shrimp fed a CO-
323 supplemented diet had granular cells of 53%, higher than the granular cells in the shrimp fed without a CO-
324 supplemented diet (43%). The highest percentage of granular cells, also known as neutrophils, serve as pioneers in
325 immune defence against Gram-negative bacteria; granular cells in this study indicated that bioactive compounds of *C.*

odorata leaf could increase the srimps'immune system by inducing hemocyte improvement (Xian et al., 2017). They have several mechanisms to enhance the immune response, including phagocytosis, degranulation, and NETosis. Neutrophils can engulf and kill pathogenic bacteria through multiple bactericidal pathways. In addition, they have granules and enzyme pathways that help to eliminate pathogenic microbes. These granules can release cytokines that activate antigen-presenting cells (APCs) and amplify the immune response.

Furthermore, neutrophils can produce lysozyme, an antimicrobial enzyme that can kill certain bacteria independently of peptidoglycan hydrolytic activity and modulate the host's immune response to infection. Dietary supplementation with methionine (Met) can improve growth performance and increase the activities of antioxidant enzymes in shrimp (Huang et al., 2020). Hyaline and granular cells were the two dominant cell types that were always observed to be higher than semi-granular cells after the 4^{th} ,8^{th}, and 14^{th} dpc. The same pattern on both cell types was observed using seaweed extract *Sargassum* sp. to improve immune parameters (THC and DHC) in white shrimp *L. vannamei* juveniles challenged with *V. algynolyticus* (Mulyadi & Iba, 2019).

The prophenoloxidase system's activity is a key element in the immune response associated with the defence against pathogens and their removal (González-Rete et al., 2019). The proPO cascade is triggered by a small number of microbe-derived molecules such as lipopolysaccharides (LPSs), β-1, 3-glucans, or peptidoglycan, which are recognized by pattern-recognition proteins. ProPO is identified as a critical element within the immune system of shrimp, serving a pivotal role in safeguarding against microbial infections. Phenoloxidase (PO), a crucial enzyme of the proPO system in invertebrates, promotes humoral defence mechanisms and subsequently eliminates pathogens. The prophenoloxidase (proPO) activity in the shrimp fed a CO-supplemented diet during the 45-day culture period in the pond was much higher than in the shrimp fed the control diet. This result is like previous studies that obtained higher proPO activity in shrimp who received diet supplementation with herbs material/extract (Ngo et al., 2020). The proPO activity enhancement in treated shrimp hemolymph (T2) indicates an increased immune response to the pathogen, which may impact shrimp survival rate of 73.33%.

On the other hand, the low survival rate observed in the control group (23.33%) (T1) may be due to the low activity of proPO. The decreased proPO activity can cause failed phagocytosis and tissue damage. A similar finding of the proPO activity in whiteleg shrimp was obtained in tiger shrimp when challenged with *V.harveyi*. The *C. odorata* leaf flour-supplemented diet significantly increased the proPO activity in the treatment tiger shrimp group compared with the control tiger shrimp group (without *C. odorata* leaf flour supplementation) (Harlina et al., 2019).

Conclusion

The leaves of *C. odorata* were confirmed to contain bioactive components such as alkaloids, flavonoids, triterpenoids, and steroids, which are suspected to improve shrimp health. *L. vannamei* that received the *C. odorata* leaf flour components at 1.5 g/kg of diet showed improved growth parameters and enhanced resistance to *V. parahaemolyticus* infection. The heightened Total Hemocyte Counts, along with increased levels of granular and semi-granular cells and prophenoloxidase post the feeding trial, are associated with the supplementation of *C. odorata* leaf flour components in the diet. The findings of this investigation propose that these dietary components have the capacity to serve as immunostimulants in shrimp, consequently enhancing growth and bolstering the immune response, thereby augmenting the resistance of whiteleg shrimp against bacterial infection. These results provide useful information for sustainable shrimp culture and the potential use of *C. odorata* leaf flour components as a shrimp diet additive to improve growth, survival, immune responses, and resistance to bacterial infection.

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494
495 **Tables and Figures**

Table 1. Result of phytochemical test of *C. odorata* L. methanol extract

Methanol extract	Flavonoid	Alkaloid		Triterpenoid	Steroid	Saponin
		Dragendorff	Meyer			
C. odorata L.	+	+	+	+	+	-

Note: A (+) sign indicates a positive reaction, while a (-) sign indicates a negative reaction.

Table 2. Mean ± SD Growth of whiteleg shrimp *L. vannamei* after 45 days of feeding trial

Parameters	T1	T2*
Average Body Weight (ABG) (g)	5.61 ± 0.22	7.71 ± 0.24
Weight gain (g)	5.59 ± 7.78	7.69 ± 0.08
Specific Growth Rate (SGR) (%/day)	12.52 ± 0.17	13.23 ± 0.14

Note: T1= control diet and T2= CO-supplemented diet, *= a significant difference ($P < 0.05$)

Table 3. The overall and range values (minimum, maximum) of water quality parameters of experimental diets during the 45-day rearing time (means ± SD, n=3).

Parameter	T1	T2	The suitable value (Nguyen et al., 2022)
Salinity (ppt)	22.5 ± 0.1 (20.1, 25.0)	23.1 ± 0.1 (20.1, 25.1)	10-25
pH	7.81 ± 0.05 (7.53, 8.43)	7.75 ± 0.01 (7.54, 8.13)	7.5-8.5
Temperature (°C)	27.9 ± 0.06 26.5, 29.9	27.8 ± 0.07 26.4 ± 29.9	20-30
DO (ppm)	7.83 ± 0.05 (7.39, 8.13)	7.67 ± 0.09 (7.47, 7.91)	5-20

Note: T1= control diet, and T2= CO-supplemented diet

Table 4. DHC of whiteleg shrimp before, 2nd, 4th, 8th, and 14th day post-challenge

Treatment	Parameter	Initial	2nd dpc	4th dpc	8th dpc	14th dpc
Diet T1	Hyaline (%)	31.00	28.00	33.00	35.00	50.00
	Granular (%)	45.00	49.00	45.00	43.84	31.00
	Semi-granular (%)	24.00	23.00	22.00	21.16	19.00
Diet T2	Hyaline (%)	29.00	30.00	27.00	26.00	25.00
	Granular (%)	61.00	56.90	49.67	48.30	48.00
	Semi-granular (%)	10.00	13.10	23.33	25.70	27.00

Note: T1=control diet, and T2=CO supplementation diet

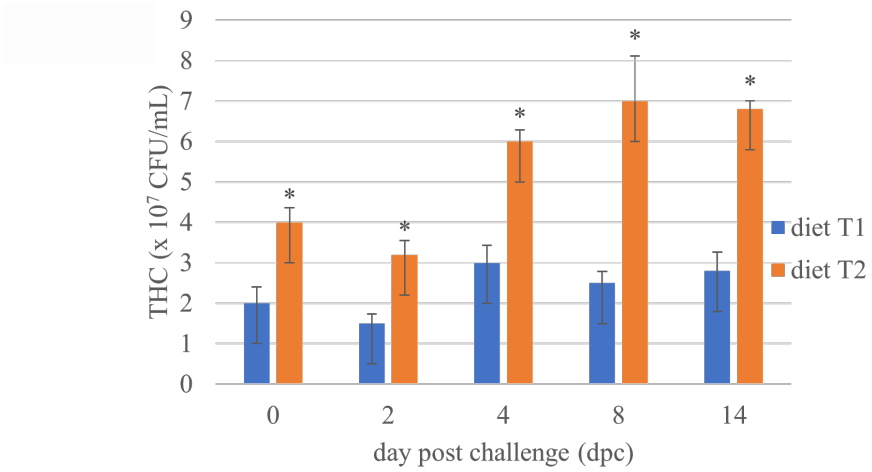

Figure 1. Mean ± SD THC of whiteleg shrimp before, 2nd, 4th, 8th, and 14th day post-challenge. T1=control diet, and T2=CO-supplemented diet, *= a significant difference ($P < 0.05$).

Figure 2. The average DHC of whiteleg shrimp before, 2nd, 4th, 8th, and 14th day post-challenge. T1=control diet, and T2=CO supplementation diet.

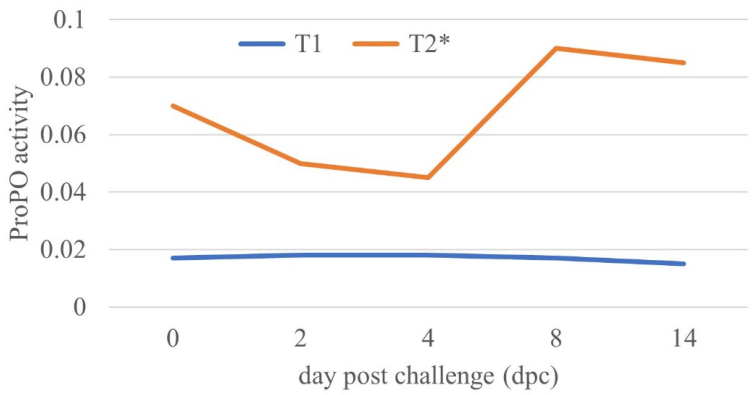

Figure 3. proPO activity of whiteleg shrimp before, 2nd, 4th, 8th, and 14th dpc. T1=control diet, and T2= CO supplementation diet, *= a significant difference ($P < 0.05$).

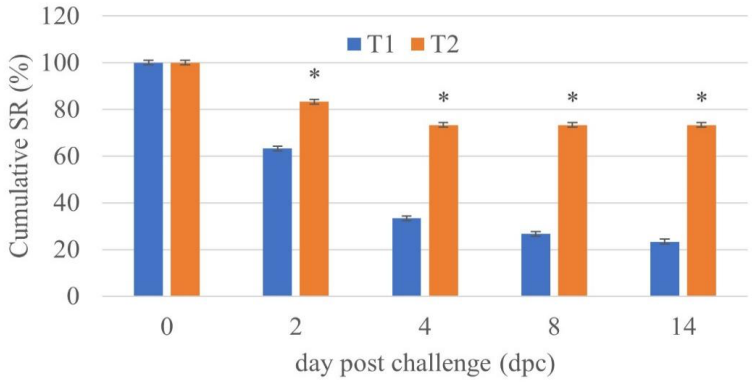

Figure 4. Mean ± SD Cumulative SR of whiteleg shrimp before, 2nd, 4th, 8th, and 14th dpc. T1= control diet, and T2= CO-supplemented diet, *= a significant difference ($P < 0.05$).

Confirmation email for submission

(3 Januari 2024)

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Harlina Harlina <harlina.harlina@umi.ac.id>

New Manuscript is submitted (1st, Subarea: Aquaculture) (fas-2023-0285)

1 message

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Manuscript ID : fas-2023-0285 (1st)
Manuscript Type : Research Article
Manuscript Subarea : Aquaculture
Manuscript Title : GROWTH PERFORMANCE, NON-SPECIFIC IMMUNE ACTIVITY, AND RESISTANCE AGAINST Vibrio Parahaemolyticus OF WHITELEG SHRIMP FED DIETARY Chromolaena odorata LEAF FLOUR COMPONENTS

Dear Dr. Harlina Harlina

This is Fisheries and Aquatic Sciences.

Your manuscript is submitted.
Thank you very much.

Please do not reply to this e-mail message. If you have comments or questions, please use the contact information below.

If this email is in the spam folder, please classify this email as non-spam to receive other emails safely.

Best regards,
You-Jin Jeon, Jung Hwa Choi, Han Kyu Lim, and Suengmok Cho, Editors-in-Chief
Fisheries and Aquatic Sciences

Fisheries and Aquatic Sciences

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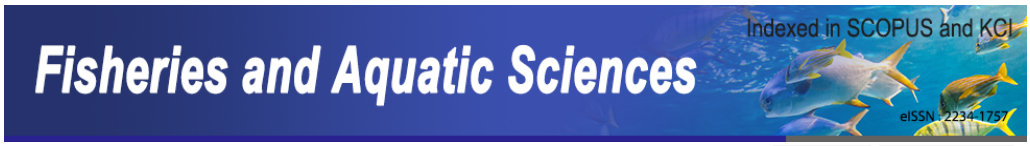

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Manuscript Type & Category

Type	Research Article
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Title & Abstract

Title (English)	GROWTH PERFORMANCE, NON-SPECIFIC IMMUNE ACTIVITY, AND RESISTANCE AGAINST Vibrio Parahaemolyticus OF WHITELEG SHRIMP FED DIETARY Chromolaena odorata LEAF FLOUR COMPONENTS
Running Title (English)	Chromolaena odorata as growth promotor and immunostimulant on Whiteleg Shrimp
Abstract (English)	The efficacy of *Chromolaena odorata* leaf flour components (CO) to increase the growth performance, non-specific immune activity, and resistance against *Vibrio parahaemolyticus* of whiteleg shrimp (*Litopenaeus vannamei*) culture was evaluated. To this purpose, whiteleg shrimp post larvae were fed on diets supplemented with 0 and a 1.5 g CO/kg diet for 45 days in a pond. After feeding trials, the shrimps were identified for their growth parameters, collected, and injected with *V. parahaemolyticus*, then their non-specific immune activity and resistance to *V. parahaemolyticus* was observed for 14 days. Findings showed that CO increased the Average Body Weight (7.71 g, 37%), weight gain (7.69 g, 40%), and Specific Growth Rate (13.23%/day, 5.7%) as compared to the control. In addition, CO supplementation also increases shrimp's hematologic and immune activity (Total Hemocyte Counts (6.8 x 10^7 CFU/mL, 242.9%), Differential Hemocyte Counts (27%, 142.1%), and prophenoloxidase activity (0.085, 566.7%). Finally, shrimp resistance to *V. parahaemolyticus* infection also increased after CO supplementation, with survival rates of 73.33% as compared to 23.33% for the control. It suggests that *C. odorata* leaf flour component supplementation at an optimal dose in the diet may be an effective strategy to increase growth and resistance to bacterial disease with reduced mortality in shrimp farms.
Keywords (English)	AHPND; Chromolaena odorata; Immune response; Litopenaeus vannamei; Vibrio parahaemolyticus

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Additional Information

Cover letter

Dear

Prof. Dr. **You-Jin Jeon**

Editor-in-Chief

Fisheries and Aquatic Sciences

December 26, 2023

I am pleased to submit our manuscript entitled “Growth Performance, Non-specific Immune Activity, and Resistance against *Vibrio Parahaemolyticus* of Whiteleg Shrimp Fed Dietary *Chromolaena odorata* Leaf Flour Components” to the Fisheries and Aquatic Sciences

The efficacy of *Chromolaena odorata* leaf flour components (CO) to increase the growth performance, non-specific immune activity, and resistance against *Vibrio parahaemolyticus* of whiteleg shrimp (*Litopenaeus vannamei*) culture was evaluated. To this purpose, whiteleg shrimp post larvae were fed on diets supplemented with 0 and a 1.5 g CO/kg diet for 45 days in a pond. After feeding trials, the shrimps were identified for their growth parameters, collected, and injected with *V. parahaemolyticus*, then their non-specific immune activity and resistance to *V. parahaemolyticus* was observed for 14 days. Findings showed that CO increased the Average Body Weight (7.71 g, 37%), weight gain (7.69 g, 40%), and Specific Growth Rate (13.23%/day, 5.7%) as compared to the control. In addition, CO supplementation also increases shrimp's hematologic and immune activity (Total Hemocyte Counts (6.8 x 107 CFU/mL, 242.9%), Differential Hemocyte Counts (27%, 142.1%), and prophenoloxidase activity (0.085, 566.7%). Finally, shrimp resistance to V. parahaemolyticus infection also increased after CO supplementation, with survival rates of 73.33% as compared to 23.33% for the control. It suggests that *C. odorata* leaf flour component supplementation at an optimal dose in the diet may be an effective strategy to increase growth and resistance to bacterial disease with reduced mortality in shrimp farms.

We believe that this manuscript is appropriate for publication in the Fisheries and Aquatic Sciences it meets your journal’s scope of aquaculture.

This manuscript has not been published and is not under consideration for publication elsewhere. We have read and approved the manuscript and take full responsibility for its content. The authors state that it has no competing interests financially or non-financially.

Thank you for your consideration.

Best Regard

Harlina Harlina

Funding information	This research was supported by the Ministry of Research, Technology and Higher Education through the DIPA-042-06-0/2021 scheme.
Conflict of interest	No potential conflict of interest relevant to this article was reported
IRB/IACUC approval	This research has been approved by the UMI Health Research Ethics Commission Number: 237/A.1/KEPK-UMI/VI/2022 (June 12, 2022)

Suggested & Opposed Reviewer

Name	ORCID	Email	Affiliation	Country	Type	Short Reason
Cahyono Purbomartono	0000-0002-8996-7570	cpurbomartono@yahoo.com	Universitas Muhammadiyah Purwokerto	Indonesia	**Suggest**	He has written many paper in the subject area, one of them is Dietary fucoidan from Padina boergesenii to enhance non-specific immune of catfish (Clarias sp.).
Agus Setyawan	0000-0003-2728-1159	agus.setyawan@fp.unila.ac.id	Lampung University	Indonesia	**Suggest**	He expert in this subject area, one of his paper is similar to this topic paper, Effect of different diets on growth performance, physiological response and behavior of spiny lobster Panulirus homarus (Linnaeus, 1758)

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4. Requesting to 1st revise (Major Revisions)

Confirmation email for requesting to revise manuscript (7-2- 2024)

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1 message

Fisheries and Aquatic Sciences <no_reply@guhmok.com> Wed, Feb 7, 2024 at 8:14 AM
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Manuscript Type : Research Article
Manuscript Subarea : Aquaculture
Manuscript Title : GROWTH PERFORMANCE, NON-SPECIFIC IMMUNE ACTIVITY, AND RESISTANCE AGAINST Vibrio Parahaemolyticus OF WHITELEG SHRIMP FED DIETARY Chromolaena odorata LEAF FLOUR COMPONENTS

Dear Dr. Harlina Harlina

This is Fisheries and Aquatic Sciences.

The examination of your manuscript has been completed.
The editor-in-chief had made a final decision that the revision were needed.
You can check the comments below by accessing the online submission system.
Even if there is some files attached by the reviewers, you can not check it in the e-mail, so please make sure to access the system.
After reflecting the correction in the manuscript, be sure to submit it again using the submission system.

Editor's comment to author:

Editor,

This study is considered to have high academic value as a study on the effects of *Chromolaena odorata* leaf powder on growth and non-specific immunity in *Penaeus vannamei* when exposed to *Vibrio parahaemolyticus.* However, revisions and supplements must be made to perfectly reflect the reviewers' major revision requests. In addition, in terms of the quantity of the resulting data, it is insufficient to become a full-length article, so please submit after revising as a short communication.

Reviewer 1:

This study is about effect of Chromolaena odorata (CO) leaf powder on growth and immunity of whiteleg shrimp. Chromolaena odorata leaf contains various phytochemicals like alkaloids, flavonoids, terpenoids, steroids, etc. CO supplmented groups had higher growth and increased THC and ProPO activity as well as disease resistance. These results clearly support author's conclusion. However, few points should be revised before it is considered as a publication.

Composition of basal and experiemental diets is needed to be presented as a table.

It would be better if the proximate compostion of CO leaf powder is included.

It would be nice if you can add figures of hemocyte counts by comparinig two experimental groups as well as different shapes of hemocytes.

In the conclusion, future perspectives about how the specific phytochemical can stimulate immune system are needed.

Figure 1 and 2: indicate full names of THC and DHC.

All figures and tables: indicate number of replicates.

Figure 4: it should be drawn as survival curve for example using Kaplan–Meier estimator

Reviewer 2:

This manuscript is interesting and meaningful, and the subject addressed in this article is worthy of investigation. However, the manuscript can not be accepted at the present form, especially in the challenge test and hemolymphy cell part and discussion.

1. line 55-57 : should be delete ‘Harlina et al. (2013) reported that *C. odorata* leaves contain bioactive compounds such as flavonoids, alkaloids, steroids, saponins, and tannins.’

2. line 121 : and, should be change

3. line 129 and Table 4: There is no information about the infection time point, shrimp size and how many shrimp in each group. Please enter clear information about the infection experiment.

4. line 144 and 154 : Is this the amount of hemolymphy (20ul and 100 µl) that can be collected from one shrimp? Need to add more information in detail.

5. in the section of 3.4 : should be indicates that the shrimp hemocytes shape, It is best to provide a figure.

6. line 195 : Author mentioned as THC levels were greatly reduced in shrimp fed the control diet may be because there had been an infection caused by pathogenic bacteria or viruses since being farmed.

7. line 231-241 : should be delete content that overlaps with the introduction.

8. line 268~: Although the importance of blood cells is explained in considerable detail, the explanation of the morphological characteristics of blood cells, apart from the increase in blood cell count, is omitted. An increased blood cell count does not mean resistance. However, it means that an immune response is occurring in the body, and the results of comparative analysis with the quantitative amount of bacteria in the body over time after bacterial infection are needed.

9. Figure : Picture files need to be reorganized for readability using a program such as Graphpad Prism rather than Excel. Fig. 4 should be convert to cumulative mortality graph.

Editor-in-chief's comment to author:

Best regards,
You-Jin Jeon, Jung Hwa Choi, Han Kyu Lim, and Suengmok Cho, Editors-in-Chief
Fisheries and Aquatic Sciences

Fisheries and Aquatic Sciences

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- Homepage : https://submission.e-fas.org/

5. Revision has been submitted

- **Detail information from OJS (24-2-24)**
- **Respond to Reviews (24-2-2024)**
- **Review Detail**

Details information from OJS

Fisheries and Aquatic Sciences
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eISSN : 2234-1757

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Submission Summary

Manuscript ID	fas-2023-0285 (3rd)
Title	GROWTH PERFORMANCE, NON-SPECIFIC IMMUNE ACTIVITY, AND RESISTANCE AGAINST Vibrio Parahaemolyticus OF WHITELEG SHRIMP FED DIETARY Chromolaena odorata LEAF FLOUR COMPONENTS
Submitting Author	Harlina Harlina (harlina.harlina@umi.ac.id, office: , mobile: +62 812-4386-8134)
Corresponding Author	Harlina Harlina (harlina.harlina@umi.ac.id, office: , mobile: +62-812-4386-8134)
Status	**Completed (Accept)** (Date changed: 2024-03-10)

View Details of 1st Submission

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Manuscript Information

Manuscript ID	fas-2023-0285
Degree (Date submitted)	2nd (2024-02-24)
Status (Date changed)	Process ended (2024-02-29)

Manuscript Type & Category

Type	Research Article
Category	Aquaculture

Title & Abstract

Title (English)	GROWTH PERFORMANCE, NON-SPECIFIC IMMUNE ACTIVITY, AND RESISTANCE AGAINST Vibrio Parahaemolyticus OF WHITELEG SHRIMP FED DIETARY Chromolaena odorata LEAF FLOUR COMPONENTS
Running Title (English)	Chromolaena odorata as growth promotor and immunostimulant on Whiteleg Shrimp
Abstract (English)	The efficacy of *Chromolaena odorata* leaf flour components (CO) to increase the growth performance, non-specific immune activity, and resistance against *Vibrio parahaemolyticus* of whiteleg shrimp (*Litopenaeus vannamei*) culture was evaluated. To this purpose, whiteleg shrimp post larvae were fed on diets supplemented with 0 and a 1.5 g CO/kg diet for 45 days in a pond. After feeding trials, the shrimps were identified for their growth parameters, collected, and injected with *V. parahaemolyticus*, then their non-specific immune activity and resistance to *V. parahaemolyticus* was observed for 14 days. Findings showed that CO increased the Average Body Weight (7.71 g, 37%), weight gain (7.69 g, 40%), and Specific Growth Rate (13.23%/day, 5.7%) as compared to the control. In addition, CO supplementation also increases shrimp's hematologic and immune activity (Total Hemocyte Counts (6.8 x 10^7 CFU/mL, 242.9%), Differential Hemocyte Counts (27%, 142.1%), and prophenoloxidase activity (0.085, 566.7%). Finally, shrimp resistance to *V. parahaemolyticus* infection also increased after CO supplementation, with survival rates of 73.33% as compared to 23.33% for the control. It suggests that *C. odorata* leaf flour component supplementation at an optimal dose in the diet may be an effective strategy to increase growth and resistance to bacterial disease with reduced mortality in shrimp farms.
Keywords (English)	AHPND; Chromolaena odorata; Immune response; Litopenaeus vannamei; Vibrio parahaemolyticus

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Additional Information

Cover letter

Authors' Response Letter

February 24, 2024

Manuscript Number: fas-2023-0285

Decision: Major revision

Dear Editor in chief of fas

Prof. You-Jin Jeon

Thank you for coordinating the review process and the prompt processing of the manuscript. This document contains the response to the reviewer' comments. Furthermore, we have revised the manuscript carefully and made all the needed changes in the revised manuscript.

The authors' response is attached.

Best Regard,

Corresponding authors

Harlina Harlina (harlina.harlina@umi.ac.id)

Funding information This research was supported by the Ministry of Research, Technology and Higher Education through the DIPA-042-06-0/2021 scheme.

Conflict of interest No potential conflict of interest relevant to this article was reported

IRB/IACUC approval This research has been approved by the UMI Health Research Ethics Commission Number: 237/A.1/KEPK-UMI/VI/2022 (June 12, 2022)

Suggested & Opposed Reviewer

Name	ORCID	Email	Affiliation	Country	Type	Short Reason
Cahyono Purbomartono	0000-0002-8996-7570	cpurbomartono@yahoo.com	Universitas Muhammadiyah Purwokerto	Indonesia	**Suggest**	He has written many paper in the subject area, one of them is Dietary fucoidan from Padina boergesenii to enhance non-specific immune of catfish (Clarias sp.).
Agus Setyawan	0000-0003-2728-1159	agus.setyawan@fp.unila.ac.id	Lampung University	Indonesia	**Suggest**	He expert in this subject area, one of his paper is similar to this topic paper, Effect of different diets on growth performance, physiological response and behavior of spiny lobster Panulirus homarus (Linnaeus, 1758)

Respond to Review

Please accept our manuscript revision as reviuwer' suggestion

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1	Manuscript File (with Author Details)	fas-2023-0285-MAN-Revisi_fas-2023-0285-MAN-fas-2023-0285-MAN-Main_Manuscript_FAS_2023_submit_24_02_202	579KB	Main manuscript (with author details)	Feb 24, 2024

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2	Respond to Reviews	fas-2023-0285-RES-Authors_response_letter_FAS_Feb_24_2024_submit.pdf (/func/download_file?file_name=b6675d1d2d2ac0208efe7dda0965a023.pdf&file_path=../uploads/author/954/&orig_name=fas-2023-0285-RES-Authors_response_letter_FAS_Feb_24_2024_submit.pdf)	430KB	Respond to Reviews	Feb 24, 2024

Review PDF : fas-2023-0285(2nd).pdf (/func/download_review_pdf?ar_id=954)

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Completed Review List

Review 1

File to Author	No data saved

Review 2

File to Author	No data saved

Editor's Recommendation

Comment to Author *	I believe that the paper has developed by carefully reflecting the requirements of all reviewers. It can be published after some modifications. **Reviewer 1:** Comments from reviewers were well addressed. Thank you so much! **Reviewer 2:** I don't know where the modification was made. Please, mark the modified parts in red or blue. Be sure to mark the parts you have modified or added so that the reviewer does not have to re-check about it. Since you did not mark it, the reviewer has to re-examine each item from the beginning. -line 125, 148, 213, 259 : × symbol should be change. -line 205 : (, should be delete. -line 203: should be explain replication level in the manuscript.

	-Fig. 2 : Are there any microscope magnification inputs and high-resolution photos? -Fig. 7 : should be add *V. parahaemolyticus* injection control and naïve groups. -Table : should be add table. summary of efficacy result including as Mortality, RPS, and p value.
File	No data saved

Editor-In-Chief's Decision

Comment to Author *	
File	No data saved
Final Decision	**Minor revisions**

View Details of 3rd Submission (Now in Progress)

Fisheries and Aquatic Sciences

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Respond to Reviews (1st submission/revision)

Authors' Response Letter

February 24, 2024

Manuscript Number: fas-2023-0285
Decision: Major revision

Dear Editor in chief of fas
Prof. You-Jin Jeon
Thank you for coordinating the review process and the prompt processing of the manuscript. This document contains the response to the reviewer' comments. Furthermore, we have revised the manuscript carefully and made all the needed changes in the revised manuscript.
The authors' response is attached.

Best Regard,
Corresponding authors

Harlina Harlina (harlina.harlina@umi.ac.id)

Editor's comment to author:

Editor,

This study is considered to have high academic value as a study on the effects of *Chromolaena odorata* leaf powder on growth and non-specific immunity in *Penaeus vannamei* when exposed to *Vibrio parahaemolyticus.* However, revisions and supplements must be made to perfectly reflect the reviewers' major revision requests. In addition, in terms of the quantity of the resulting data, it is insufficient to become a full-length article, so please submit after revising as a short communication.

Reviewer 1:

This study is about effect of Chromolaena odorata (CO) leaf powder on growth and immunity of whiteleg shrimp. Chromolaena odorata leaf contains various phytochemicals like alkaloids, flavonoids, terpenoids, steroids, etc. CO supplmented groups had higher growth and increased THC and ProPO activity as well as disease resistance. These results clearly support author's conclusion. However, few points should be revised before it is considered as a publication.

Composition of basal and experiemental diets is needed to be presented as a table.

It would be better if the proximate compostion of CO leaf powder is included.

Composition of basal and experimental diets as well as their proximate composition were included in Table 1, page 22, line 527.

Table 1. The Basal ingredients and proximate composition of experimental diet

Ingredients	Treatments	
	Control diet (T1)	CO supplementation diet (T2)
Fish Flour (%)	40	40
Shrimp head flour (%)	10	10
Copra cake (%)	9	9
Corn flour (%)	12	12
Soybean flour (%)	17	17
Wheat flour (%)	10	10
Vitamin and minerals (%)	2	2
Chromolaena odorata leaves (g/kg feed)	0	1.5
Total (%)	100	100
Proximate composition		
Crude protein (%)	38.31	38.31
Crude lipid (%)	9.27	9.27
Crude fibre (%)	4.52	4.52
Ash content (%)	12.48	12.48
NFE (%)	32.86	32.86
Total energy (kcal/kg feed)	1,646	1,646

Note: NFE = Nitrogen Fee Extract

It would be nice if you can add figures of hemocyte counts by comparinig two experimental groups as well as different shapes of hemocytes.

Figure of hemocyte counts of control diet (T1) and CO supplementation diet (T2) as well as different shapes of hemocytes were added in Figure 1 page 23, line 540 and Figure 2.page 23, line 543

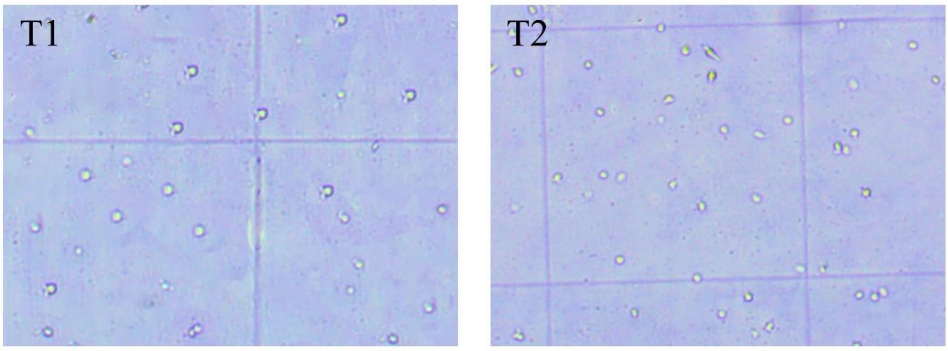

Figure 1. Hemocyte counts image of control diet (T1) and CO supplementation diet (T2)

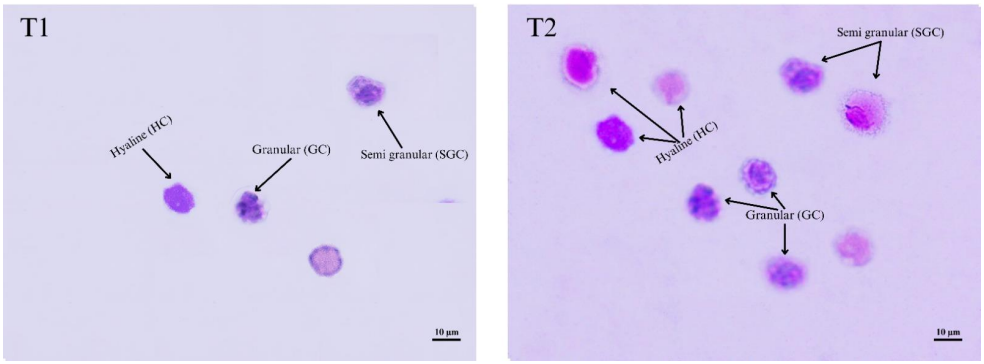

Figure 2. Different shapes of hemocytes of the control diet (T1) and CO supplementation diet (T2)

In the conclusion, future perspectives about how the specific phytochemical can stimulate immune system are needed.

To explore the future perspectives of how the specific phytochemicals in CO stimulate the immune system, further research and investigations involving the study of the mechanisms of action of these phytochemicals, their interactions with the immune system components, and their effects on immune-related gene expression or signalling pathways in shrimp are needed. This sentence had been included in the conclusion. Page 17, line 389-393.

Figure 1 and 2: indicate full names of THC and DHC.

Figure 1 that has been changed to Figure 4 indicates full name of Total Hemocyte Counts (THC) page 24, line 551 and Figure 2 that has been changed to Figure 5 indicates full name of Differential Hemocyte Counts (DHC), page 24, line 555.

All figures and tables: indicate number of replicates.

All figures and Tables (Table 3 and 4) have been indicated number of replicates in the paper. Figure 3, page 23, line 545; Figure 4,page 23, line 551; Figure 5, page 23, line 555; Figure 6, page 23, line 558, Figure 7, page 25, line 561; Table 3, page 22, line 532, Table 4, page 22, line 534, and Table 5, page 22, line 537.

Figure 4: it should be drawn as survival curve for example using Kaplan–Meier estimator

Fig. 4 that had been changed to Figure 7 was converted to cumulative mortality graph using a GraphPad Prism 10 programl (also accommodating advice from **Reviewer 2**) in page 25, line 561.

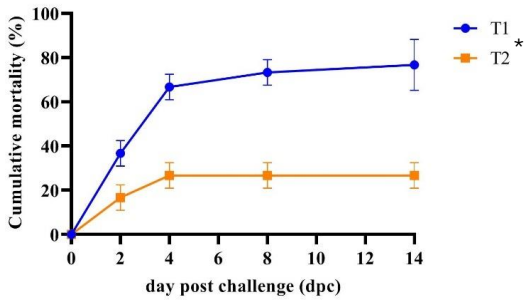

Figure 7. Mean ± SD Cumulative SR of whiteleg shrimp before, 2nd, 4th, 8th, and 14th dpc. T1= control diet, and T2= CO-supplemented diet, *= a significant difference ($P < 0.05$).

Reviewer 2:

This manuscript is interesting and meaningful, and the subject addressed in this article is worthy of investigation. However, the manuscript can not be accepted at the present form, especially in the challenge test and hemolymphy cell part and discussion.

1. line 55-57: should be delete 'Harlina et al. (2013) reported that *C. odorata* leaves contain bioactive compounds such as flavonoids, alkaloids, steroids, saponins, and tannins.'

Harlina et al. (2013) reported that *C. odorata* leaves contain bioactive compounds such as flavonoids, alkaloids, steroids, saponins, and tannins. This sentence was deleted.

2. line 121: and, should be change

the word "and" has been changed to "while"

3. line 129 and Table 4: There is no information about the infection time point, shrimp size and how many shrimp in each group. Please enter clear information about the infection experiment.

Whiteleg shrimp that had been given a control diet (T1) and those fed a CO-supplemented diet (T2) for 45 days in the ponds were challenged with *V. parahaemolyticus* for 14 days. The shrimps from groups T1 and T2 were respectively selected 80 individuals with an average weight of 7.0±0.02 g and randomly assigned to control group (T1) and treatment group (T2) to evaluate the effects of *Chromolaena odorata* leaf flour components on *V. parahaemolyticus* infection in shrimp. The

experiment was conducted in eight rectangular plastic tanks (80 x 57 x 42 cm, capacity 150 L), each containing 20 shrimp. Each type of treatment was replicated four times (three for immune response and one for survival observation). The pathogenic bacterium *V. parahaemolyticus* for the study was supplied by the Fish Health and Management Lab of the Research Institute for Brackishwater Aquaculture and Fisheries Extension (RIBAFE) in Maros, Indonesia. Determination of the density of *V. parahaemolyticus* reaching 10^6 CFUs/mL referred to the method described by Abdel-Tawwab et al. (2021). Whiteleg shrimps were infected with *V. parahaemolyticus* by injecting 0.1 mL intramuscularly into the ventral sinus. During the challenge test, the experimental diets were given twice daily in the morning and evening at a rate of 3% of body weight. To investigate how *Chromolaena odorata* leaf flour components (CO) affect the growth of *V. parahaemolyticus*, the population of *V. parahaemolyticus* in the shrimp was determined using TCBS agar media at 3, 6, 12, 24, and 48 hours after the challenge test.

Section 2.6 **Challenge test with *Vibrio parahaemolyticus*** page 7-8, line 122-127

4. line 144 and 154 : Is this the amount of hemolymphy (20ul and 100 µl) that can be collected from one shrimp? Need to add more information in detail.

To calculate DHC, a microscope glass slide was prepared, and then 20 µL of hemolymph from each shrimp (the remaining hemolymph was used to calculate THC) was placed on the slide. Page 9 line 153-154..

5. in the section of 3.4 : should be indicates that the shrimp hemocytes shape, It is best to provide a figure.

The shrimp hemocyte shapes have been included in Figure 1 and Figure 2 (section Figures and Tables). Page 23 line 540 and line 543.

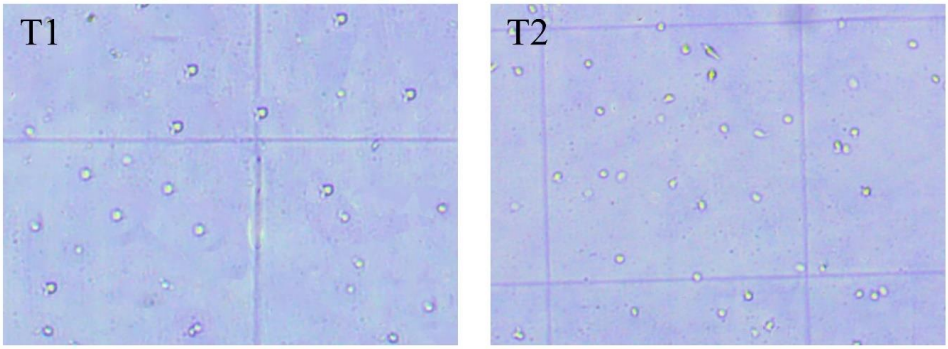

Figure 1. Hemocyte counts image of control diet (T1) and CO supplementation diet (T2)

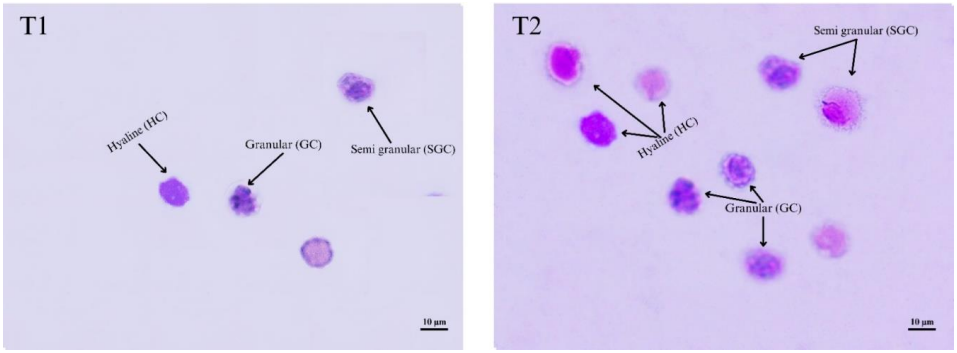

Figure 2. Different shapes of hemocytes of the control diet (T1) and CO supplementation diet (T2)

6. line 195 : Author mentioned as THC levels were greatly reduced in shrimp fed the control diet may be because there had been an infection caused by pathogenic bacteria or viruses since being farmed.

It was seen in the THC value after 45 days of maintenance on the farm and before the challenge test. THC of shrimp fed (T2) showed THC values of 4 x 10^7 CFU/mL much higher and significantly different from THC values of controlled shrimp (T2) with THC values of 2 x 10^7 CFU/mL. page 11, line 4.

7. line 231-241 : should be delete content that overlaps with the introduction.

The content that overlaps with the introduction namely the phytochemical compounds such as Flavonoids, alkaloids, flavanone, essential oils, phenolics, saponins, tannins, and terpenoids which were reported in *C. odorata* has been deleted in discussion section at paragraph one.

8. line 268~: Although the importance of blood cells is explained in considerable detail, the explanation of the morphological characteristics of blood cells, apart from the increase in blood cell count, is omitted. An increased blood cell count does not mean resistance. However, it means that an immune response is occurring in the body, and the results of comparative analysis with the quantitative amount of bacteria in the body over time after bacterial infection are needed.

The explanation of the morphological characteristics of blood cells, apart from the increase in blood cell count, contained in the sentence "This statement was supported by Aisiah et al., (2022), who states that phytochemical compounds (alkaloids, flavonoids, terpenoids, and steroids) present in the leaves of *Acanthus ilicifolius* are important components that can regulate blood parameters related to fish health' was omitted. Because this sentence was deleted, its bibliography was also removed. (Aisiah S, Rini RK, Tanod WA, Fatmawati F, Fauzana NA, Olga O, et al. Metabolomic Profiling of Jeruju (*Acanthus ilicifolius*) Leaf Extract with Antioxidant and Antibacterial Activity on *Aeromonas hydrophila* Growth. J Appl Pharm Sci. 2022; 12(8):57–69. Doi: https://japsonline.com/abstract.php?article_id=3725&sts=2).

The results of comparative analysis with the quantitative number of bacteria in the body over time after bacterial infection are shown in the Figure 3 page 23, line 545

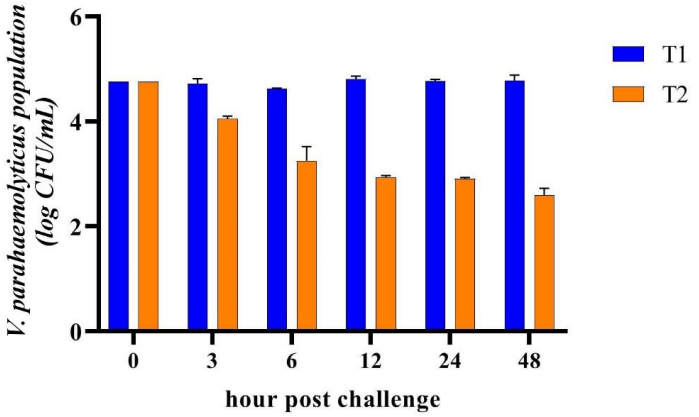

Figure 3. *V. parahaemolyticus population* (log CFU/mL) (n = 3) in the shrimp at the beginning and after 3, 6, 12, 24, and 48 hours unchallenged (T1) and challenged with *Chromolaena odorata* leaf flour components (CO) (T2)

In this study, the discussion session also explored additional bacterial data related to population decline. *C. odorata* leaf meal showed its efficacy in reducing *V. parahaemolyticus* population at 3 hours to 48 hours after the challenge test. The population of *V. parahaemolyticus* in the shrimp decreased by 45.58% or 3.90 x 10^2 CFU/mL after 48 hours of challenge testing." Page 12, line 259-261.

9. Figure : Picture files need to be reorganized for readability using a program such as Graphpad Prism rather than Excel. Fig. 4 should be convert to cumulative mortality graph.

All Figures have been organized using a Graphpad Prism versi 10 program. Section Figures and Tables, page 23-25

6. Requesting to 2nd Revision (Minor Revison)

- **Requesting to revise manuscript from email (29-2-2024)**
- **Manuscript for 2nd Revision**
- **Additional Reply to Editor (5-3-2024)**

Requesting to revise manuscript from email (29-2-2024)

mail-UMI
e-mail Kampus UMI

Harlina Harlina <harlina.harlina@umi.ac.id>

Requesting to revise your manuscript. (fas-2023-0285)
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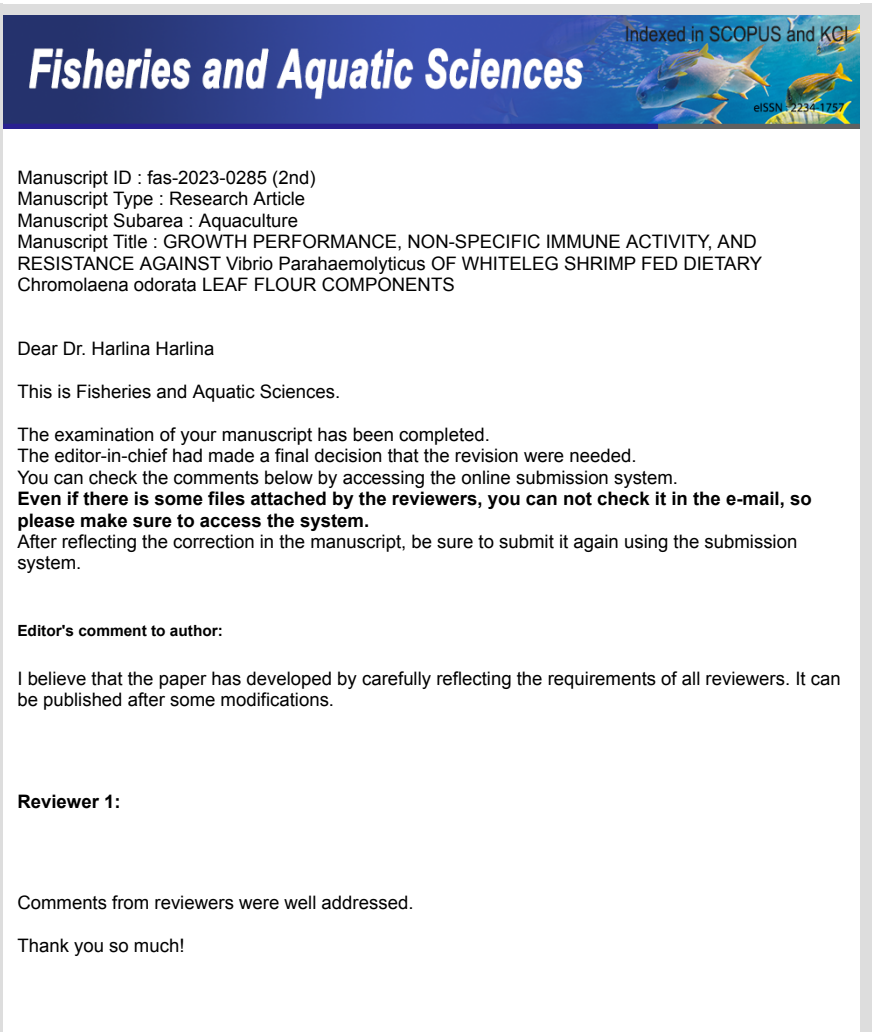

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Dear Dr. Harlina Harlina

This is Fisheries and Aquatic Sciences.

The examination of your manuscript has been completed.
The editor-in-chief had made a final decision that the revision were needed.
You can check the comments below by accessing the online submission system.
Even if there is some files attached by the reviewers, you can not check it in the e-mail, so please make sure to access the system.
After reflecting the correction in the manuscript, be sure to submit it again using the submission system.

Editor's comment to author:

I believe that the paper has developed by carefully reflecting the requirements of all reviewers. It can be published after some modifications.

Reviewer 1:

Comments from reviewers were well addressed.

Thank you so much!

Reviewer 2:

I don't know where the modification was made. Please, mark the modified parts in red or blue. Be sure to mark the parts you have modified or added so that the reviewer does not have to re-check about it. Since you did not mark it, the reviewer has to re-examine each item from the beginning.

-line 125, 148, 213, 259 : × symbol should be change.

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-Fig. 2 : Are there any microscope magnification inputs and high-resolution photos?

-Fig. 7 : should be add *V. parahaemolyticus* injection control and naïve groups.

-Table : should be add table. summary of efficacy result including as Mortality, RPS, and p value.

Editor-in-chief's comment to author:

Best regards,
You-Jin Jeon, Jung Hwa Choi, Han Kyu Lim, and Suengmok Cho, Editors-in-Chief
Fisheries and Aquatic Sciences

Fisheries and Aquatic Sciences

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- Phone: +82-51-629-7363
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- Homepage : https://submission.e-fas.org/

7. 2nd Revision has been submitted

- Details Information from OJS (5-3-2024)
- Respon to Reviews for Minor Revision (5-3-2024)
- Additional Reply to Editor (5-3-2024)
- Review Detail

Details information from OJS

Fisheries and Aquatic Sciences
Indexed in SCOPUS and KCI
eISSN : 2234-1757

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Submission Summary

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Corresponding Author	Harlina Harlina (harlina.harlina@umi.ac.id, office: , mobile: +62-812-4386-8134)
Status	**Completed (Accept)** (Date changed: 2024-03-10)

View Details of 1st Submission

View Details of 2nd Submission

Close Details of 3rd Submission (Now in Progress)

Manuscript Information

Manuscript ID	fas-2023-0285
Degree (Date submitted)	3rd (2024-03-05)
Status (Date changed)	Completed (2024-03-10)

Manuscript Type & Category

Type	Research Article
Category	Aquaculture

Title & Abstract

Title (English)	GROWTH PERFORMANCE, NON-SPECIFIC IMMUNE ACTIVITY, AND RESISTANCE AGAINST Vibrio Parahaemolyticus OF WHITELEG SHRIMP FED DIETARY Chromolaena odorata LEAF FLOUR COMPONENTS
Running Title (English)	Chromolaena odorata as growth promotor and immunostimulant on Whiteleg Shrimp
Abstract (English)	The efficacy of *Chromolaena odorata* leaf flour components (CO) to increase the growth performance, non-specific immune activity, and resistance against *Vibrio parahaemolyticus* of whiteleg shrimp (*Litopenaeus vannamei*) culture was evaluated. To this purpose, whiteleg shrimp post larvae were fed on diets supplemented with 0 and a 1.5 g CO/kg diet for 45 days in a pond. After feeding trials, the shrimps were identified for their growth parameters, collected, and injected with *V. parahaemolyticus*, then their non-specific immune activity and resistance to *V. parahaemolyticus* was observed for 14 days. Findings showed that CO increased the Average Body Weight (7.71 g, 37%), weight gain (7.69 g, 40%), and Specific Growth Rate (13.23%/day, 5.7%) as compared to the control. In addition, CO supplementation also increases shrimp's hematologic and immune activity (Total Hemocyte Counts (6.8 x 10^7 CFU/mL, 242.9%), Differential Hemocyte Counts (27%, 142.1%), and prophenoloxidase activity (0.085, 566.7%). Finally, shrimp resistance to *V. parahaemolyticus* infection also increased after CO supplementation, with survival rates of 73.33% as compared to 23.33% for the control. It suggests that *C. odorata* leaf flour component supplementation at an optimal dose in the diet may be an effective strategy to increase growth and resistance to bacterial disease with reduced mortality in shrimp farms.
Keywords (English)	AHPND; Chromolaena odorata; Immune response; Litopenaeus vannamei; Vibrio parahaemolyticus

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Additional Information

Cover letter	Authors' Response Letter March 05, 2024 Manuscript Number: fas-2023-0285 Dear Editor in chief of fas Prof. You-Jin Jeon Thank you for coordinating the second review process and speedy processing of the manuscript. This document contains responses to reviewer comments. Furthermore, we have revised the manuscript and made all the necessary changes in the revised manuscript as suggested. The author's response is attached. Best Regard, Corresponding authors Harlina Harlina (harlina.harlina@umi.ac.id)
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IRB/IACUC approval	This research has been approved by the UMI Health Research Ethics Commission Number: 237/A.1/KEPK-UMI/VI/2022 (June 12, 2022)

Suggested & Opposed Reviewer

Name	ORCID	Email	Affiliation	Country	Type	Short Reason
Cahyono Purbomartono	0000-0002-8996-7570	cpurbomartono@yahoo.com	Universitas Muhammadiyah Purwokerto	Indonesia	**Suggest**	He has written many paper in the subject area, one of them is Dietary fucoidan from Padina boergesenii to enhance non-specific immune of catfish (Clarias sp.).
Agus Setyawan	0000-0003-2728-1159	agus.setyawan@fp.unila.ac.id	Lampung University	Indonesia	**Suggest**	He expert in this subject area, one of his paper is similar to this topic paper, Effect of different diets on growth performance, physiological response and behavior of spiny lobster Panulirus homarus (Linnaeus, 1758)

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Comment to Author *	
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Fisheries and Aquatic Sciences

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Respond to Reviews (2nd revision)

Authors' Response Letter

March 05, 2024

Manuscript Number: fas-2023-0285

Dear Editor in chief of fas

Prof. You-Jin Jeon

Thank you for coordinating the second review process and speedy processing of the manuscript. This document contains responses to reviewer comments. Furthermore, we have revised the manuscript and made all the necessary changes in the revised manuscript as suggested. The author's response is attached.

Best Regard,

Corresponding authors

Harlina Harlina (harlina.harlina@umi.ac.id)

Editor's comment to author:

I believe that the paper has developed by carefully reflecting the requirements of all reviewers. It can be published after some modifications.

Reviewer 1:

Comments from reviewers were well addressed.

Thank you so much!

Reviewer 2:

I don't know where the modification was made. Please, mark the modified parts in red or blue. Be sure to mark the parts you have modified or added so that the reviewer does not have to re-check about it. Since you did not mark it, the reviewer has to re-examine each item from the beginning.

All the modified part has been already marked in red in the manuscript

-line 125, 148, 213, 259 : × symbol should be change.

× symbol has been changed and marked them with red colour.

-line 205 : (, should be delete.

(, has been deleted

-line 203: should be explain replication level in the manuscript.

The repetition level of the population calculation of *V. parahaemolyticus* was carried out three times and has been described in section 3.4

-Fig. 2 : Are there any microscope magnification inputs and high-resolution photos?

A 40× magnification microscope used for imaging

-Fig. 7 : should be add *V. parahaemolyticus* injection control and naïve groups.

V. parahaemolyticus injection has been added in Fig.7

-Table : should be add table. summary of efficacy result including as Mortality, RPS, and p value.

p value has been included marked as *

Additional Reply to Editor

(5-3-2024)

mail-UMI
e-mail Kampus UMI

Harlina Harlina <harlina.harlina@umi.ac.id>

Harlina's reply about microscope

1 message

Harlina Harlina <harlina.harlina@umi.ac.id> Tue, Mar 5, 2024 at 9:36 AM
To: "(사)한국수산과학회" <kosfas@kosfas.or.kr>

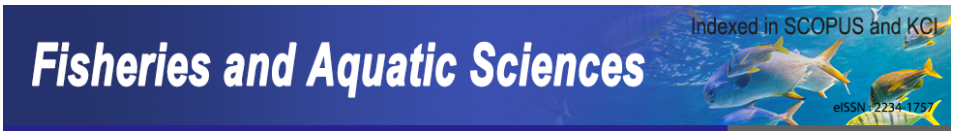
Dear

Editor-in-chief

In that research " GROWTH PERFORMANCE, NON-SPECIFIC IMMUNE ACTIVITY, AND RESISTANCE AGAINST Vibrio Parahaemolyticus OF WHITELEG SHRIMP FED DIETARY Chromolaena odorata LEAF FLOUR COMPONENTS " , we used microscope Olympus CX23 (Binokuler)

Best regards

Review Detail (2nd Submission/Revision)



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Running Title:	Chromolaena odorata as growth promotor and immunostimulant on Whiteleg Shrimp
Type:	Research Article
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1 **GROWTH PERFORMANCE, NON-SPECIFIC IMMUNE ACTIVITY, AND RESISTANCE AGAINST**
2 ***Vibrio Parahaemolyticus* OF WHITELEG SHRIMP FED DIETARY *Chromolaena odorata* LEAF FLOUR**
3 **COMPONENTS**
4

ARTICLE INFORMATION	Fill in the information in each box below
Article Type	Research article
Article Title (within 20 words without abbreviations)	Growth Performance, Non-specific Immune Activity, and Resistance against *Vibrio parahaemolyticus* of Whiteleg Shrimp Fed Dietary *Chromolaena odorata* Leaf Flour Components
Running Title (within 10 words)	Application of *Chromolaena odorata* as a growth promoter and Immunostimulant
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Availability of data and material	Upon a reasonable request, the datasets of this study can be available from the corresponding author.
Ethics approval and consent to participate	This research has been approved by the UMI Health Research Ethics Commission Number: 237/A.1/KEPK-UMI/VI/2022 (June 12, 2022)

5

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Abstract The efficacy of *Chromolaena odorata* leaf flour components (CO) to increase the growth performance, non-specific immune activity, and resistance against *Vibrio parahaemolyticus* of whiteleg shrimp (*Litopenaeus vannamei*) culture was evaluated. To this purpose, whiteleg shrimp post larvae were fed on diets supplemented with 0 and a 1.5 g CO/kg diet for 45 days in a pond. After feeding trials, the shrimps were identified for their growth parameters, collected, and injected with *V. parahaemolyticus*, then their non-specific immune activity and resistance to *V. parahaemolyticus* was observed for 14 days. Findings showed that CO increased the Average Body Weight (7.71 g, 37%), weight gain (7.69 g, 40%), and Specific Growth Rate (13.23%/day, 5.7%) as compared to the control. In addition, CO supplementation also increases shrimp's hematologic and immune activity (Total Hemocyte Counts (6.8 x 10^7 CFU/mL, 242.9%), Differential Hemocyte Counts (27%, 142.1%), and prophenoloxidase activity (0.085, 566.7%). Finally, shrimp resistance to *V. parahaemolyticus* infection also increased after CO supplementation, with survival rates of 73.33% as compared to 23.33% for the control. It suggests that *C. odorata* leaf flour component supplementation at an optimal dose in the diet may be an effective strategy to increase growth and resistance to bacterial disease with reduced mortality in shrimp farms.

Keywords: AHPND, *Chromolaena odorata*, Immune response, *Litopenaeus van*namei, *Vibrio parahaemolyticus*

1. Introduction

The whiteleg shrimp, *Litopenaeus vannamei*, is a widely cultured species of high economic value. The whiteleg shrimp is very popular because of its high-quality meat, good taste, good nutritional content, and easy cooking (Li et al., 2021). Whiteleg shrimp is also one of the most successful cultured species in aquaculture, accounting for up to 80% of shrimp production (Rosilan et al., 2023). Whiteleg shrimp has been cultivated with intensive to super-intensive technology (Mustafa et al., 2023).

However, whiteleg shrimp have several challenges from various diseases, including Acute Hepatopancreatic Necrosis Disease (AHPND), a bacterial disease also known as Early Mortality Syndrome (EMS) (Rosilan et al., 2023). AHPND has caused significant economic losses for shrimp (Ha et al., 2023). *Vibrio parahaemolyticus* is a well-known causative agent of AHPND in penaeid shrimp farming worldwide (Velázquez-Lizárraga et al., 2019). *V. parahaemolyticus*, a Gram-negative bacterium that lives in marine and estuary environments, becomes pathogenic in shrimp at concentrations greater than 10^4 CFUs/mL (Soto-Rodriguez et al., 2022).

The use of immunostimulants to enhance immune responses is one of the important steps that can be taken to prevent disease in shrimp aquaculture (Kumar et al., 2022). Immunostimulants are substances that can activate or enhance the activity of the immune system, used to help the body's immune response in combating infections (Di Sotto et al.,

2020). Immunostimulants are considered an attractive and promising agent for preventing diseases in fish and shellfish. Increasing the ability of shrimp bodies to fight disease by administering immunostimulants can be indicated by several parameters, including prophenoloxidase (proPO), Total Hemocyte Counts (THC), and Differential Hemocyte Counts (DHC). When selecting these therapeutic agents, it's important to think about the safety of consumers, the environment, and the shrimp.

Immunostimulants derived from plants are organic, free from side effects, environmentally friendly, and safe to use to improve the immune system of shrimp (Kumar et al., 2022). C. *odorata*, also known as Siam weed (Eze & Jayeoye, 2021) is a rapidly growing perennial herb that can grow up to 2.5 m (100 inches) tall in open areas and up to 10 m (33 feet) tall in shady areas where it behaves as a creeper, growing on other vegetation. When crushed, the plant is hairy and glandular; the leaves give off a pungent, aromatic odor. Some of the benefits of *C. odorata* are that it can improve soil fertility, crop production, protection, nutrition, and ethnopharmacology (Mugwedi, 2020). *C. odorata* has been reported to have antibacterial, anti-inflammatory, antioxidant, anthelminthic, antifungal, cytotoxic, anticonvulsant, antiprotozoal, and wound-healing properties (Mugwedi, 2020). This antioxidant and antibacterial property could help protect aquatic animals from oxidative stress and bacterial infection caused by environmental factors (Eze & Jayeoye, 2021).

Different plant seasons or life cycle stages can give different results in analyzing bioactive compounds. The leaves of *C. odorata* contain phytochemicals such as tannins, alkaloids, flavonoids, saponins, terpenoids and other phenolic compounds, which produce a physiological action in the body (Rasyid et al., 2020; Zahara, 2019). The presence of these components in *C. odorata* leaf extract has shown that it can be a source of immunostimulant to improve shrimp antibacterial activity. Various factors, including the environmental condition, can influence shrimp's growth and immune response. In the earlier study, in controlled testing, *C. odorata* leaf components (CO) enhanced immune responses and survival rates in tiger shrimp infected with *V. harveyi* (Harlina et al., 2019). However, the impact of CO use in improving the growth and health of farm-farmed whiteleg shrimp *L. vannamei* is still an area of research that has not been revealed. Thus, this study examines the effectiveness of *C. odorata* leaf flour components at the optimal dose to increase the growth performance, non-specific immune activity, and resistance against *V. parahaemolyticus* of whiteleg shrimp *L. vannamei* culture in the pond.

2. Materials and Methods

2. 1. Sample preparation

The old leaves of *C. odorata* were collected in the rainy season around a pond in Marana Village, Maros, Indonesia. followed by cleaning under running water and then cut into small cube-like pieces. The resulting leaf pieces were then dried using a drying cabinet at 40°C. The dried leaves were then turned into flour with the help of a kitchen blender. The leaf flour was then divided into two parts; some were used for phytochemical tests, while the other was mixed into feed as part of experiments.

2. 2. Phytochemical test

Approximately 10 g of *C. Odorata* leaf flour was extracted with 200 mL of methanol (MeOH) while stirring using a shaker for 24 hours at room temperature. The MeOH extract was filtered using Whatman No.4 filter paper. The MeOH extract was then concentrated using a rotary evaporator set at 37°C to obtain concentrated MeOH extract. Phytochemical tests were conducted to confirm the uniformity of compound content in *C. odorata* leaves used in this study with compound content in *C.odorata* leaves that had been investigated previously (Harlina et al., 2013). The components present were identified through the observation of color changes or the formation of visually visible deposits.

The flavonoid analysis involved combining 1–2 mL of warm 50% methanol with 1 mL of methanol extract, then introducing magnesium and 0.5 mL of concentrated hydrochloric acid. The presence of flavonoids was indicated by the appearance of an orange color. Dragendorff's and Mayer's tests were performed for alkaloid detection. A mixture was created by combining 1 mL of methanol extract with 0.5 mL of 2% hydrochloric acid. The resultant acidic solution was subsequently divided into two tubes. In Tube I, three drops of Dragendorff solution were added, while in Tube II, three drops of Mayer solution were introduced. Verification of the existence of alkaloids was established through the observation of a red or orange residue in Tube I and a whitish precipitate in Tube II. In terpenoid testing, MeOH extract (1 mL) was mixed with 0.5 mL of chloroform and then added with 0.5 mL of anhydrous acetic acid. After that, the mixture was carefully fed into a test tube with 1-2 mL of concentrated H2SO4, which presents a brownish or purple ring if triterpenoids are present.

After that, the mixture was carefully poured into a test tube, and 1-2 mL of concentrated H2SO4 was added. The Liebermann-Burchard reagent was used to test the presence of steroids by mixing MeOH (1 mL) with n-hexane (2 mL) and allowed to stand until two layers formed. The n-hexane layer was removed, and five drops of Liebermann-Burchard reagent were added. The presence of blue color indicated the presence of steroids. Saponins were identified through the combination of 2 mL of MeOH extract with 2 mL of distilled water, followed by allowing the mixture to

stand for one minute. The addition of two drops of 1 N hydrochloric acid was performed, and the presence of saponins in the MeOH leaf extract was confirmed if foam developed and remained stable for 10 minutes.

2. 3. Experimental diets

The diet preparation and proximate composition process were explained elsewhere in Table 1. CO leaf powder was added to diets in the optimal dose (1.5 g/kg diet), This optimal dose was chosen based on the earlier study on tiger shrimp with a dietary proximate composition consisting of crude protein (38.21%), crude lipids (9.7%), crude fiber (4.52%), crude ash (12.48%), nitrogen-free extracts (32.86%), and total energy (16.46 kcal/kg).

<<< Table 1 >>>

2. 4. Shrimp and husbandry trial

This experiment was done at the farmer's pond in Bonto Langkasa Village, Pangkep Regency, South Sulawesi, Indonesia. Post larvae (PL) shrimp in size 0.02 ± 0.02 g were purchased from a local hatchery. Then, shrimp was distributed to experimental ponds (500 m^2) (170 shrimp/m^2, duplicate) (Purnamasari et al., 2017). The stocking of PL was carried out in the morning, which begins with the acclimatization of PL first, especially at temperature and salinity. Once the temperature and salinity of the water within the PL plastic bag were either equivalent or exhibited minimal variation compared to the pond water, the gradual introduction of PL into the experimental ponds could be initiated. The shrimp were fed with a control and treatment diet for 45 days, followed by control feeding on both treatments, handed over to local farmers, and observation stopped. Before being used, the intensive ponds were prepared in accordance with the established procedures outlined in the REBAFE standard operating protocols. The trial feeding of 3% of body weight was carried out twice daily in the morning and evening for 45 days.

2. 5. Growth performance

The shrimp were separately sampled to determine the shrimp growth performance during feeding. The collection of shrimp samples was conducted utilizing anchovy sampling technique. Anchovy sampling (n = 30) was conducted in the morning on the 45th day of the rearing period. Growth performance was evaluated by the following parameters: Average Body Weight (ABW) (g) was measured by weighing the body weight of the sampling shrimp. Weight gain (g) = wt –w0. Specific Growth Rate (SGR) (% /day) = 100 % x [(ln wt – ln w0)/t] where w0 is the initial mean body weight (g), wt is the sampling mean body weight (g), while t is rearing time (day).

2. 6. Challenge test with *Vibrio parahaemolyticus*

Whiteleg shrimp that had been given a control diet (T1) and those fed a CO-supplemented diet (T2) for 45 days in the ponds were challenged with *V. parahaemolyticus* for 14 days. The shrimps from groups T1 and T2 were respectively

selected 80 individuals each with an average weight of 7.0±0.02 g and randomly assigned to control group (T1) and treatment group (T2) to evaluate the effects of *Chromolaena odorata* leaf flour components on *V. parahaemolyticus* infection in shrimp. The experiment was conducted in eight rectangular plastic tanks (80 × 57 × 42 cm, capacity 150 L), each containing 20 shrimp. Each type of treatment was carried out four times (three for immune response and one for survival observation). The Fish Health and Management Lab of the Research Institute for Brackishwater Aquaculture and Fisheries Extension (RIBAFE) in Maros, Indonesia, supplied the pathogenic bacterium *V. parahaemolyticus* for the study. Determination of the density of *V. parahaemolyticus* reaching 10^4 CFUs/mL referred to Abdel-Tawwab et al., (2021) method. Whiteleg shrimps were infected with *V. parahaemolyticus* by injecting 0.1 mL intramuscularly into the ventral sinus. During the challenge test, the experimental diets were given twice daily in the morning and evening as much as 3% of body weight. To investigate how *Chromolaena odorata* leaf flour components (CO) affect the growth of *V. parahaemolyticus*, the population of *V. parahaemolyticus* in the shrimp was determined using TCBS agar media at 3, 6, 12, 24, and 48 hours after the challenge test.

2. 7. Observation of immune responses and survival rate

Hemolymph samples were gathered both prior to the challenge test and at specific intervals post-challenge, specifically on the 2^{nd}, 4^{th}, 8^{th}, and 14^{th} days. The daily monitoring of the survival rate (SR) of whiteleg shrimp was conducted throughout the challenge test. The calculation of SR was performed using the formula as documented by Herawati et al. (2020). Prior to hemolymph collection, the shrimp were subjected to cold anesthesia. Hemolymph retrieval followed guidelines provided by Morales-Covarrubias (2023) by using an anticoagulant solution instead of the Modified Alsever Solution (MAS). The anticoagulant solution (100 µL) was inserted into a sterile syringe with a capacity of 1 mL. Then, with caution, the anticoagulant was mixed with hemolymph by taking 100 µL of hemolymph from the shrimp ventral sinuses on the base of the first abdominal segment. After hemolymph retrieval, the ventral sinus area of the shrimp was cleaned with a cotton swab that was soaked in 70% ethanol to prevent contamination. A mixture of hemolymph and anticoagulant (1:1) was placed in a 1.5 mL microcentrifuge tube by gently pipette up and down to prevent cell clumping. Next, trypan blue (0.5% in 2.6% NaCl) was used to dilute hemocytes. Hemocytes were examined under a light microscope (Olympus DP21, Japan) at 10× magnification, employing a hemocytometer and introducing 10 µL of a diluted hemolymph sample. The THC (Figure 1) was calculated according to the formula proposed by Braak (2002).

<<< Figure 1 >>>

To calculate DHC, a microscope glass slide was prepared, and then 20 µL hemolymph from each shrimp (the remaining hemolymph was used to calculate THC) was placed on the slide. Hemocytes were allowed to flatten themselves and dry at room temperature, washed with pure MeOH for 15 minutes, and dried again at room temperature. Next, hemocytes were stained with Giemsa-Rosenfeld as much as 10%. Slide cleaning was carried out under running water for 30 seconds. Following the drying process, the slides were employed for the morphological characterization of distinct hemocyte types, as outlined by Chang and Chang (2022), utilizing a light microscope (Olympus DP21, Japan) at a magnification of 40x. Diverse shrimp hemocytes, including hyaline, granular, and semi-granular, were recognized and quantified using the methodology described by Braak (2002). The DHC result (Figure 2) was then calculated as the average of each hemocyte type in the total cells counted.

<<< Figure 2 >>>

The activity of prophenoloxidase (proPO) was measured with the help of a spectrophotometer. L-dihydroxyphenylalanine (L-DOPA) was used as the base material to record dopachrome formation. To perform a proPO test, the following steps were performed. First, the prepared 100 µL of hemolymph was separated by centrifugation at 7,000 rpm for 20 minutes at 40°C, and the supernatant was discarded. Then, a hemolymph precipitate was added to 100 µL of cacodylate citrate buffer solution (0.1 M $C_2H_{12}AsNaO_5$, 0.45 M NaCl, and 0.01 M $Na_3C_6H_5O_7$) and then separated again in the same way. Next, the precipitate was resuspensioned in cacodylate buffer (100 µL) and homogenized. After that, 100 µL of the mixture was piped into a 96-well microplate (R-BioPharma Well Reader, Germany) and mixed with 100 µL of trypsin (Sigma Aldrich). The mixture was then suspended and incubated at 25°C for 10 minutes. Enzymatic activity was measured by comparing the dopachrome's absorbance and blanks containing trypsin (200 µL) and l-DOPA (50 µL, containing 1 mg/mL trypsin) at a wavelength of 490 nm.

2. 8. Water quality observation

During the experimental period, salinity, pH, Dissolved Oxygen (DO), and water temperature were measured every 10 days of the feeding trial at 08:00–10:00 Indonesian time using YSI 6920 V2-2 Multiparameter Water Quality Sonde (YSI Incorporated, Yellow Springs, OH, USA).

2. 9. Data analysis

Growth parameters, immune response (THC and proPO activity), and survival rate were evaluated through t-student test analysis using SPSS software version 20.0 at a confidence level of 0.05 ($P < 0.05$), while DHC and water quality were presented in descriptive form. All Figures were rorganized using a Graphpad Prism versi 10.

3. Results

3. 1. Phytochemical identification

The examination of phytochemical constituents confirmed the presence of flavonoids, alkaloids, triterpenoids, and steroids in the leaves of *C. odorata*. However, the methanol extracts of *C. odorata* leaves did not exhibit the presence of saponins, as indicated in Table 2.

<<< Table 2 >>>

3. 2. Growth Parameters

This experiment showed that dietary CO supplementation had a positive impact in increasing the growth of whiteleg shrimp. Significant improvements ($P < 0.05$) in all growth parameters tested (Table 3) were shown by shrimp fed a CO-supplemented diet compared to shrimp fed a control diet after 45 days of feeding experiments with ABW of 7.71 ± 0.24 and 5.61 ± 0.22 g, respectively. A higher Specific Growth Rate (SGR) of 13.23 ± 0.14% was noted in shrimp fed a CO-supplemented diet (T2), but a relatively low SGR (12.52 ± 0.17%) was recorded in shrimp fed a control diet (T1). The growth parameters measured were Average Body Weight (ABG), Weight gain, and SGR. It suggests that CO can be used to improve the growth of whiteleg shrimp.

<<< Table 3 >>>

3. 3 Water quality

Based on the supplied data, the water quality obtained during the feeding trial was normal for whiteleg shrimp L. vannamei growth in the pond. The water quality measured consisting of salinity (ppt), pH, temperature (°C), and Dissolved Oxygen (DO, ppm) was summarized in Table 4.

<<< Table 4 >>>

3.4. *Vibrio parahaemolyticus* population

The treatment with *Chromolaena odorata* leaf flour components (CO) (T2) had a significant impact on the growth of *V. parahaemolyticus* in the shrimp. As shown in Figure 3, the population of *V. parahaemolyticus* (n=3) in the shrimp fed a CO-supplemented diet exhibited a continuous decrease over time. After 48 hours of the challenge test, the population of *V. parahaemolyticus* decreased by 45.58%. In contrast, in the control group (T1), the population of *V. parahaemolyticus* in shrimp also experienced a decrease up to 6 hours after the challenge test, followed by an increase reaching its peak after 12 hours of the challenge test, and subsequently declined again but remained higher than the initial bacterial population.

<<< Figure 3 >>>

3. 5. Total Haemocyte Counts (THC)

THC is a useful tool for assessing the health status of shrimp in aquaculture (Morales-Covarrubias et al., 2023). Figure 4 shows that after a feeding trial for 45 days of farmed rearing, whiteleg shrimp fed a CO supplementation diet (T2) significantly increased their THC value (4×10^7 CFU/mL) compared to that (2×10^7 CFU/mL) of shrimp fed a control diet (T1). The result of the THC observation indicated that a higher THC in shrimp fed the CO-supplemented diet (T2) had been shown before the challenge test compared with the control shrimp (T1). THC levels were greatly reduced in shrimp fed the control diet may be because there had been an infection caused by pathogenic bacteria or viruses since being farmed.

<< < Figure 4 >>>

3. 6. Differential Hemocyte Counts (DHC)

After whiteleg shrimp were fed a CO-supplemented diet (T2) and control diet (T1) and challenged with *V. parahaemolyticus*, the proportions of the three hemocyte types changed (Table 5 and Figure 5), and the percentage of GC increased dramatically. The pattern of changes in GC values in whiteleg shrimp in both treatments had the same tendency; however, GC values in shrimp fed diet T1 were always lower than GC values in shrimp fed diet T2. On the 2nd dpc, the granular cell number decreased in the T2 treatment and increased in the T1 treatment. Furthermore, on the 4th, 8th, and 14th dpc, the granular cell number decreased to the GC values lower than the baseline values in both shrimp treatments. The hyaline in the T1 treatment decreased on the 2nd dpc, then increased on the 4th, 8th, and 14th dpc. Unlike hyaline in the shrimp group (T1), the shrimp group (T2) increased on the 2nd dpc, then decreased on the 4th, 8th, and 14th dpc. Since the 2nd dpc, semi-granular in the T1 treatment decreased; conversely, semi-granular in the T2 treatment increased. The proportion change of the three hemocyte types was summarized in Table 5.

<<< Table 5 >>>

<<< Figure 5 >>>

3. 7. Prophenoloxidase (proPO)

The current study found that the average proPO activity in shrimp fed the CO-supplemented diet (0.068) (T2) was higher and significantly different ($P < 0.05$) than that in shrimp fed the control diet (0.017) (T1). The proPO activity in the treatment shrimp (T2) decreased on the 2nd dpc, but their proPO activity was still higher than that in the control shrimp (T1) and then dramatically increased on the 8th dpc and remained relatively stable until the 14th dpc. In contrast, the proPO activity in control shrimp remained stable and lower than that of treatment shrimp during the challenge test (Figure 6).

<<< Figure 6 >>>

238 **3. 8. Survival rate (SR)**

239 The mortality rate of shrimp receiving the control diet (T1) exhibited a sharp increase on the 2nd day post-challenge
240 (dpc) and persisted until the end of the study, as illustrated in Figure 7. Conversely, a consistently high survival trend
241 was observed in shrimp fed the CO-supplemented diet (T2) from the 2nd dpc onward. By the 14th dpc, nearly all shrimp
242 fed the control diet (T1) had succumbed. Between the 4th and 14th dpc, shrimp provided with the CO-supplemented
243 diet (T2) demonstrated significantly greater resistance ($P < 0.05$) to *V. parahaemolyticus* infection (73.33 ± 5.77%
244 survival rate) compared to those fed the control diet (T1) (23.33 ± 5.77% survival rate). This data indicated the
245 increased survival of shrimp fed the CO-supplemented diet by 50%, which may be the effect of *C.odorata* leaf
246 bioactive compounds.

247 <<< Figure 7 >>>

248

249 **4. Discussion**

250 The study found that the methanol extract of *C. odorata* leaves contains natural ingredients such as flavonoids,
251 alkaloids, triterpenoids, and steroids. The specific chemical composition of *C. odorata* may vary depending on the
252 species, season, and plant location. The presence of phytochemical compounds such as alkaloids, flavonoids,
253 terpenoids, and steroids in the leaves of *C. odorata* was suspected to increase the immune parameters of crustaceans
254 such as shrimp.

255 In this study, *C. odorata* leaf meal showed its efficacy in reducing *V. parahaemolyticus* population at 3 hours to 48
256 hours after the challenge test. The population of *V. parahaemolyticus* in the shrimp decreased by 45.58% or 3.90 ×
257 10^2 CFU/mL after 48 hours of challenge testing (Figure 3). Flavonoids modulate the immune system to enhance its
258 function, whereas alkaloids serve as both immunostimulants and antibacterial agents, as documented by Idris Affandi
259 et al. (2019). A similar result was also obtained by Behl et al., (2021) that the content of phytochemicals consisting of
260 alkaloids, tannins and phenols has activity as an immunostimulant that can improve the quality of host immune cells.
261 Flavonoids, alkaloids, and saponins have been shown to have pharmacological activity that can help protect whiteleg
262 shrimp from bacterial diseases caused by *Vibrio* sp., called vibriosis (Nur et al., 2020; Santiago et al., 2021). Alkaloids,
263 for example, boost shrimp's immune system by enhancing the activity of macrophage cells, which can engulf and
264 destroy pathogens.

265 After 45 days of rearing shrimp was farmed, the whiteleg shrimp fed with a CO-supplemented diet (T2) had a higher
266 ABW, Body Weight Gain (BWG), and SGR than those in the shrimp fed a control diet (T1). The shrimp increased its

growth parameter because the CO-supplemented diet contained nutrients such as carbohydrates, protein, lipids, minerals, and bioactive compounds (Table 3) that promote growth (Aziz, 2020). *C. odorata* also contains bioactive compounds that function as antiseptic substances that can inhibit bacterial growth (Rasyid et al., 2020). The presence of flavonoids and alkaloids in the leaves of *C. odorata*, which have antioxidant and antibacterial properties, could help protect aquatic animals from oxidative stress and bacterial infection caused by environmental factors (Eze & Jayeoye, 2021). *C. odorata* leaf flour can be used as a food supplement for Bali cattle, as it contains nutrients that improve their consumption, digestibility, pH, N-ammonia, VFA, and growth. Grape extract has been found to improve white shrimp's antioxidant activity and growth performance, specifically *L. vannamei* (Chien et al., 2023). The shrimp fed the CO-supplemented diet showed a weight after 45 days of rearing in the pond of 7.71 ± 0.24 grams (ABW). This ABW was higher than that (around 5.00 g) reported by Purnamasari et al, (2017). The daily growth rate (SGR) in this study reached 13.23 ± 0.14%/day, which was higher than the growth of whiteleg shrimp after being fed dry grape extract of 2.76 ± 0.06%/day (Chien et al., 2023) and Seaweed Polysaccharide (7.59 ± 0.33) (Abbas et al., 2023). Several plant extract applications, such as *Boesenbergia pandurata*, *Solanum ferox* extract (Hardi et al., 2022), and *Phyllanthus amarus* extract, have been reported to enhance the growth of shrimp (Ngo et al., 2020). The increased growth rate of whiteleg shrimp obtained after 45 days of rearing could also come from pond water quality under normal conditions, as noted in Table 4.

Bioactive compounds in *C. odorata* increased immune responses (Harlina et al., 2019). The increased formation of phagocytic cells is essential in controlling the attack of microorganisms. Elevated THC levels are associated with increased cellular and humoral immune responses (Braak, 2002; Mulyadi & Iba, 2019). In this study, THC shrimp fed the CO-supplemented diet before facing the challenge test showed an increase in hemocyte counts compared to shrimp that were only given a control diet. It indicates a proliferation of hemocytes, hinting that the shrimp's immune system has improved, making it better prepared to deal with pathogenic infections.

The average value of the THC post-challenge test of whiteleg shrimp fed the CO-supplemented diet (T2) (5.40×10^7 cells/mL) was significantly higher ($P < 0.05$) than whiteleg shrimp fed the control diet (T1) (2.36×10^7 cells/mL). This study showed that CO may have a better effect on whiteleg shrimp blood parameters after being challenged with *V. parahaemolyticus* than without CO. Decreased and increased THC levels can occur rapidly after a bacterial infection due to the body's defence response. THC levels decreased on the 2nd day after a bacterial infection was detected and then increased again on the 4th day as part of the shrimp's natural immune system recovery process as described in the study of Risjani et al., (2021). If the number of shrimps hemocytes increases, the body's defences also increase to

avoid infection. Conversely, hemocyte numbers decrease because many die after destroying foreign macromolecules when the infection is overcome and resistance to foreign bodies is achieved.

The high amount of shrimp THC after being challenged with *V. parahaemolyticus* showed that the *C. odorata* bioactive compound could increase the immune response by stimulating the production of immune cells and molecules, such as cytokines and antibodies (Vijayaram et al., 2022) and growth promotion. Shrimp are not equipped with adaptive immune systems, so they must rely on the innate immune system (Kumar et al., 2022). The high THC value found in the shrimp group (T2) could improve the disease resistance of shrimp to reduce the risk of infection, which has an impact on a higher survival (Figure 7) in shrimp group T2 after being challenged with *V. parahaemolyticus* compared to the shrimp group T1. In the earlier study of different species, tiger shrimp given a diet supplemented with *C. odorata* leaf flour had a higher THC value than the shrimp group fed without *C. odorata* leaf flour (Harlina et al., 2019). Like the whiteleg shrimp in the current study, the THC value obtained was much higher, possibly due to the shrimp being fed a diet supplemented with *C. odorata* leaf flour components for a longer time in the pond or different shrimp responses due to pathogens entering. THC values in shrimp before the challenge test with *V. parahaemolyticus* had shown differences. In a more general sphere, it is thought that environmental conditions play an important role in influencing crustacean immune responses and resilience, especially in shrimp farms where environmental factors such as laboratory environments are often difficult to control. Many reports have noted the use of plants to enhance non-specific immune parameters in crustacea (Hardi et al., 2022; Ngo et al., 2020).

DHC refers to the proportion of different types of hemocytes (HC, GC, and SGC) in the hemolymph of shrimp. These three hemocyte cell types played different roles in the immune system. Hyaline cells are responsible for executing the phagocytic role within the shrimp immune system, while the collaborative efforts of granular and semi-granular cells contribute to the synthesis and subsequent release of antimicrobial peptides. According to Xian et al., (2017), the presence of an immune response is indicated by changes in hemocytes. In this study, before the challenge test, shrimp fed the CO-supplemented diet (T2) during the 45-day rearing period showed lower values of semi-granular cells and hyaline cells than these values in shrimp fed without CO-supplemented diet (T1) (Table 5 and Figure 5). The lower semi-granular cells obtained could indicate the possibility that the bioactive compounds of *C. odorata* leaf triggered the maturation of hemocyte cells more quickly than in control shrimp during the grow-out in the pond. The change in the number of hyaline cells is closely related to the semi-granular cell number derived from late-stage hyaline cell development. Thus, a reduction in the quantity of semi-granular cells is observed, attributed to the incapacity of hyaline cells to undergo differentiation into semi-granular cells (Risjani et al., 2021).

The number of differential hemocyte cells during the challenge test changed over time, and it can be found that hyaline and granular cells may play a key role in immune system function. The smallest cell type is hyaline cells, with a high ratio between nucleus and cytoplasm and relatively few cytoplasmic granules. These cells play a crucial role in the body's immune defense system, with the quantity of hyaline cells typically rising in correlation with heightened phagocytic activity. In instances of hyaline cell infection, a notable increase is commonly observed as an initial response in the body's defense mechanisms (Risjani et al., 2021). In this study, shrimp hyaline cells in T1 treatment groups decreased on the 2nd dpc, then increased on the 4th dpc, and continued to increase until the 14th, indicating an infection in hyaline cells.

In contrast, hyaline cells in the T2 groups increased on the 2nd dpc and then decreased on the 4th, 8th, and 14th dpc; instead, semi-granular cells continued increasing, indicating shrimp recovery from the infection. The granular and semi-granular cells are involved in crustaceans' recognition and defence system. Bioactive compounds of *C. odorata* leaf flour had increased shrimp protection from *V. parahaemolyticus* infection by increasing semi-granular cells associated with continuously increasing semi-granular cells since the 2nd dpc. Similar results have been reported in the use of *Xylocarpus granatum* leaf extract, which increased the number of semi-granular cells in tiger shrimp tested with *V. harveyi* by as much as 1.55 times higher than semi-granular cells in control shrimp (Saptiani et al., 2020).

The proportion of granular cells predominates among the three hemocyte cells (Figure 2). The shrimp fed a CO-supplemented diet had granular cells of 53%, higher than the granular cells in the shrimp fed without a CO-supplemented diet (43%). The highest percentage of granular cells, also known as neutrophils, serve as pioneers in immune defence against Gram-negative bacteria; granular cells in this study indicated that bioactive compounds of *C. odorata* leaf could increase the srimps'immune system by inducing hemocyte improvement (Xian et al., 2017). They have several mechanisms to enhance the immune response, including phagocytosis, degranulation, and NETosis. Neutrophils can engulf and kill pathogenic bacteria through multiple bactericidal pathways. In addition, they have granules and enzyme pathways that help to eliminate pathogenic microbes. These granules can release cytokines that activate antigen-presenting cells (APCs) and amplify the immune response.

Furthermore, neutrophils can produce lysozyme, an antimicrobial enzyme that can kill certain bacteria independently of peptidoglycan hydrolytic activity and modulate the host's immune response to infection. Dietary supplementation with methionine (Met) can improve growth performance and increase the activities of antioxidant enzymes in shrimp (Huang et al., 2020). Hyaline and granular cells were the two dominant cell types that were always observed to be higher than semi-granular cells after the 4th ,8th, and 14th dpc. The same pattern on both cell types was observed using

seaweed extract *Sargassum* sp. to improve immune parameters (THC and DHC) in white shrimp *L. vannamei* juveniles challenged with *V. algynolyticus* (Mulyadi & Iba, 2019).

The prophenoloxidase system's activity is a key element in the immune response associated with the defence against pathogens and their removal (González-Rete et al., 2019). The proPO cascade is triggered by a small number of microbe-derived molecules such as lipopolysaccharides (LPSs), β-1, 3-glucans, or peptidoglycan, which are recognized by pattern-recognition proteins. ProPO is identified as a critical element within the immune system of shrimp, serving a pivotal role in safeguarding against microbial infections. Phenoloxidase (PO), a crucial enzyme of the proPO system in invertebrates, promotes humoral defence mechanisms and subsequently eliminates pathogens. The prophenoloxidase (proPO) activity in the shrimp fed a CO-supplemented diet during the 45-day culture period in the pond was much higher than in the shrimp fed the control diet. This result is like previous studies that obtained higher proPO activity in shrimp who received diet supplementation with herbs material/extract (Ngo et al., 2020). The proPO activity enhancement in treated shrimp hemolymph (T2) indicates an increased immune response to the pathogen, which may impact shrimp survival rate of 73.33%.

On the other hand, the low survival rate observed in the control group (23.33%) (T1) may be due to the low activity of proPO. The decreased proPO activity can cause failed phagocytosis and tissue damage. A similar finding of the proPO activity in whiteleg shrimp was obtained in tiger shrimp when challenged with *V.harveyi*. The *C. odorata* leaf flour-supplemented diet significantly increased the proPO activity in the treatment tiger shrimp group compared with the control tiger shrimp group (without *C. odorata* leaf flour supplementation) (Harlina et al., 2019).

Conclusion

The leaves of *C. odorata* were confirmed to contain bioactive components such as alkaloids, flavonoids, triterpenoids, and steroids, which are suspected to improve shrimp health. *L. vannamei* that received the *C. odorata* leaf flour components at 1.5 g/kg of diet showed improved growth parameters and enhanced resistance to *V. parahaemolyticus* infection. The heightened Total Hemocyte Counts, along with increased levels of granular and semi-granular cells and prophenoloxidase post the feeding trial, are associated with the supplementation of *C. odorata* leaf flour components in the diet. The findings of this investigation propose that these dietary components have the capacity to serve as immunostimulants in shrimp, consequently enhancing growth and bolstering the immune response, thereby augmenting the resistance of whiteleg shrimp against bacterial infection. These results provide useful information for sustainable shrimp culture and the potential use of *C. odorata* leaf flour components as a shrimp diet additive to improve growth, survival, immune responses, and resistance to bacterial infection. To explore the future perspectives

of how the specific phytochemicals in CO stimulate the immune system, further research and investigations involving the study of the mechanisms of action of these phytochemicals, their interactions with the immune system components, and their effects on immune-related gene expression or signaling pathways in shrimp are needed.

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8. Paper Accepted

Accepted Confirmation (10-5-2024)

Accepted Confirmation email

(10 Maret 2024)

mail-UMI
e-mail Kampus UMI

Harlina Harlina <harlina.harlina@umi.ac.id>

Your manuscript has been accepted for publication (fas-2023-0285)

1 message

Fisheries and Aquatic Sciences <no_reply@guhmok.com> Sun, Mar 10, 2024 at 10:35 AM
Reply-To: Fisheries and Aquatic Sciences <kosfas@kosfas.or.kr>
To: harlina.harlina@umi.ac.id

Manuscript ID : fas-2023-0285 (3rd)
Manuscript Type : Research Article
Manuscript Subarea : Aquaculture
Manuscript Title : GROWTH PERFORMANCE, NON-SPECIFIC IMMUNE ACTIVITY, AND RESISTANCE AGAINST Vibrio Parahaemolyticus OF WHITELEG SHRIMP FED DIETARY Chromolaena odorata LEAF FLOUR COMPONENTS

Dear Dr. Harlina Harlina

This is Fisheries and Aquatic Sciences.

We are pleased to inform you that your work has now been accepted for publication.
Please log in and check the review result.

This is a reminder about the publication process.
All manuscript material will be forwarded directly to the publication staff for editing and proofreading in the accepted version.
The manuscript will undergo an internal proofreading and editing process before it is sent back to the author for author revisions.
If you have typos, acknowledgments, or minor corrections, **please mention them during the author proofreading process**.

Editor's comment to author:

This manuscript is judged to have been developed to reflect all of the reviewers' requirements, and it is suitable for publication.

Editor-in-chief's comment to author:

Please do not reply to this e-mail message. If you have comments or questions, please use the

contact information below.

If this email is in the spam folder, please classify this email as non-spam to receive other emails safely.

Best regards,
You-Jin Jeon, Jung Hwa Choi, Han Kyu Lim, and Suengmok Cho, Editors-in-Chief
Fisheries and Aquatic Sciences

Fisheries and Aquatic Sciences

- Address: Pukyong National University, 45 Yongso-ro, Nam-gu, Busan 48513, Korea
- Phone: +82-51-629-7363
- Email: kosfas@kosfas.or.kr
- Homepage : https://submission.e-fas.org/

9. Proofreading from Publisher

Proofreading from Guhmok Publishing Company (15 to 17 – 7 – 2024)

Proofreading from Guhmok Publishing Company

(15 – 27 of July 2024)

mail-UMI
e-mail Kampus UMI

Harlina Harlina <harlina.harlina@umi.ac.id>

[FAS] Author proofreading request (fas-2023-0285)

16 messages

(Guhmok)거목문화사 <guhmok@guhmok.com> Mon, Jul 15, 2024 at 8:23 AM
To: harlina.harlina@umi.ac.id

Hello. Nice to meet you.
We are a **Guhmok publishing company** that is editing and proofreading FAS (Fisheries and Aquatic Sciences).

This month, FAS will be published.

1. Files titled **[ME]** have ME content applied to the original file.
I have marked and added notes for confirmation and corrections, so please review and respond to the edited file.

2. In the file titled **[Edit]**, the manuscript has been typeset and edited according to the format of the FAS journal.
If there are any confirmations or corrections, we have marked them and added a note, so please review and respond.

3. When proofreading after reviewing the manuscript
Please use the annotation function to attach any notes or corrections you have made to the file titled [Edit].

*If there are two corresponding authors, please send them together as **one file**.
***No further edits can be made after author proofreading is complete.**

Please reply by **July 17, 2024.**

==========

Please refer to the precautions when proofreading.

1> You can leave a note of corrections in the PDF file, or edit the printed copy directly, attach a scan, and send it by fax (02-2277-3390).

2> If there are not many corrections, you can write the corrections in an email.

Thank you for your cooperation.

Best regards,
Guhmok Publising

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04549 서울시 중구 을지로 148 중앙데코플라자 609호
TEL(대표): 02-2277-3324, FAX: 02-2277-3390
Homepage: http://www.guhmok.com E-mail: guhmok@guhmok.com
웹하드: http://www.webhard.co.kr ID: guhmok PW: 22773324

2 attachments

[Edit]00-FAS(Research article)_0285_Harlina Harlina.pdf
1358K

[ME]fas-2023-0285.pdf
1166K

Harlina Harlina <harlina.harlina@umi.ac.id> Wed, Jul 17, 2024 at 11:17 AM
To: emyrosmiati@yahoo.com

[Quoted text hidden]

2 attachments

[Edit]00-FAS(Research article)_0285_Harlina Harlina.pdf
1358K

[ME]fas-2023-0285.pdf
1166K

Mail Delivery Subsystem <mailer-daemon@googlemail.com> Wed, Jul 17, 2024 at 11:17 AM
To: harlina.harlina@umi.ac.id

Address not found

Your message wasn't delivered to **emyrosmiati@yahoo.com** because the address couldn't be found, or is unable to receive mail.

The response from the remote server was:

```
552 1 Requested mail action aborted, mailbox not found
```

Final-Recipient: rfc822; emyrosmiati@yahoo.com
Action: failed
Status: 4.4.2
Remote-MTA: dns; mta5.am0.yahoodns.net. (98.136.96.77, the server for the domain yahoo.com.)
Diagnostic-Code: smtp; 552 1 Requested mail action aborted, mailbox not found
Last-Attempt-Date: Tue, 16 Jul 2024 20:17:39 -0700 (PDT)

---------- Forwarded message ----------
From: Harlina Harlina <harlina.harlina@umi.ac.id>
To: emyrosmiati@yahoo.com

Cc:
Bcc:
Date: Wed, 17 Jul 2024 11:17:22 +0800
Subject: Fwd: [FAS] Author proofreading request (fas-2023-0285)
----- Message truncated -----

Harlina Harlina <harlina.harlina@umi.ac.id> Wed, Jul 17, 2024 at 3:16 PM
To: "(Guhmok)거목문화사" <guhmok@guhmok.com>

Dear Guhmok Publishing

We would like to thank Guhmok Publishing for constructive criticism of the manuscript. There are not many corrections, but here are a few corrections/additions.
1. University of Muslim Indonesia --> Universitas Muslim Indonesia
2. Survival Rates (SRs) --> Survival Rate (SR)
3. 7.000 --> 7000
4. Red Text--> black text

Thank you very much for your attention.

Best regards,

Harlina
[Quoted text hidden]

[Edit]00-FAS(Research article)_0285_Harlina Harlina_July 17th, 2024.pdf
1324K

(Guhmok)거목문화사 <guhmok@guhmok.com> Thu, Jul 18, 2024 at 9:03 AM
To: Harlina Harlina <harlina.harlina@umi.ac.id>

I checked the email you sent me.

Number 3 of the corrections could not be applied.
7.000 --> 7000
I can't find where the part is, so please mark it on the PDF and let me know.

And I need an answer to the memo hanging on the [ME]PDF.
(Omitted reference citations, cell magnification, etc.)

Please check the attached **[ME]PDF again and send us your respective answers.**

Please reply by July 20.

Thank you for your cooperation.

Best regards,
Guhmok Publising

[거목문화사] 학술지 편집/인쇄 · 홈페이지 · 논문투고시스템 · JATS XML
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TEL(대표): 02-2277-3324, FAX: 02-2277-3390
Homepage: http://www.guhmok.com E-mail: guhmok@guhmok.com
웹하드: http://www.webhard.co.kr ID: guhmok PW: 22773324

2024년 7월 17일 (수) 오후 4:16, Harlina Harlina <harlina.harlina@umi.ac.id>님이 작성:
[Quoted text hidden]

2 attachments

[ME]fas-2023-0285.pdf
1166K

00-FAS(Research article)_0285_Harlina Harlina.pdf
1358K

(Guhmok)거목문화사 <guhmok@guhmok.com> Mon, Jul 22, 2024 at 3:32 PM
To: Harlina Harlina <harlina.harlina@umi.ac.id>
Cc: rosm005@brin.go.id, lidiidna@gmail.com, syahruld@yahoo.com, yosie.hs@umt.edu.my

Hello. Nice to meet you.
We are a **Guhmok publishing company** that is editing and proofreading FAS (Fisheries and Aquatic Sciences).

I am re-requesting because I did not receive a response within the requested deadline.
Please check and reply with an accurate answer.

Please reply by July 23rd.
[Quoted text hidden]

2 attachments

[ME]fas-2023-0285.pdf
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00-FAS(Research article)_0285_Harlina Harlina.pdf
1358K

Harlina Harlina <harlina.harlina@umi.ac.id> Tue, Jul 23, 2024 at 6:38 AM
To: "(Guhmok)거목문화사" <guhmok@guhmok.com>
Cc: rosm005@brin.go.id, lidiidna@gmail.com, syahruld@yahoo.com, yosie.hs@umt.edu.my

Yes, I confirm.
[Quoted text hidden]

Mail Delivery Subsystem <mailer-daemon@googlemail.com> Tue, Jul 23, 2024 at 6:39 AM
To: harlina.harlina@umi.ac.id

Message not delivered

There was a problem delivering your message to **rosm005@brin.go.id**. See the technical details below, or try resending in a few minutes.

The response from the remote server was:

554 5.7.1 : Recipient address rejected: you do not have SPF records (SPF none)

Final-Recipient: rfc822; rosm005@brin.go.id
Action: failed
Status: 5.7.1
Remote-MTA: dns; mxcloud.rekha.co.id. (103.84.193.239, the server for the domain brin.go.id.)
Diagnostic-Code: smtp; 554 5.7.1 <rosm005@brin.go.id>: Recipient address rejected: you do not have SPF records (SPF none)
Last-Attempt-Date: Mon, 22 Jul 2024 15:39:01 -0700 (PDT)

---------- Forwarded message ----------
From: Harlina Harlina <harlina.harlina@umi.ac.id>
To: "(Guhmok)거목문화사" <guhmok@guhmok.com>
Cc: rosm005@brin.go.id, lidiidna@gmail.com, syahruld@yahoo.com, yosie.hs@umt.edu.my
Bcc:
Date: Tue, 23 Jul 2024 05:38:48 +0700
Subject: Re: [FAS] Author proofreading request (fas-2023-0285)
----- Message truncated -----

Harlina Harlina <harlina.harlina@umi.ac.id> Tue, Jul 23, 2024 at 10:29 AM
To: "(Guhmok)거목문화사" <guhmok@guhmok.com>

Dear

Guhmok publishing company Leader

We are pleased to inform you that we have received the results of the paper correction process and have agreed to publish the paper as is. However, we have a few recent corrections to suggest.

We would like to make the following revisions to the paper before publication:

Afiliation

Incorrect: [3]Institute of Climate Adaptation and Marine Biotechnology, Universiti Malaysia Terengganu, Terengganu 20000, Malaysia

Correct: [3]Institute of Climate Adaptation and Marine Biotechnology, Universiti Malaysia Terengganu, Terengganu 21030, Malaysia

Incorrect: Hemolymph samples were gathered both prior to the challenge test and at specific intervals post-challenge, specifically on the 2nd, 4th, 8th, and 14th days.

Correct:

Hemolymph samples were gathered both prior to the challenge test and at specific intervals post-challenge, specifically on the 2^{nd}, 4^{th}, 8^{th}, and 14^{th} days.

We appreciate your consideration of these minor changes.

Sincerely,

Harlina Harlina

[Quoted text hidden]

00-FAS(Research article)_0285_Harlina Harlina_final correction authors.pdf
1353K

(Guhmok)거목문화사 <guhmok@guhmok.com> Tue, Jul 23, 2024 at 1:37 PM
To: Harlina Harlina <harlina.harlina@umi.ac.id>

I have checked the email you sent me.

Two modifications have been reflected, so please check.
Also, I left 4 notes because there is something I need an answer to, so please make sure to reply.

Please reply by July 24th.

Thank you for your cooperation.

Best regards,
Guhmok Publising

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Homepage: http://www.guhmok.com E-mail: guhmok@guhmok.com
웹하드: http://www.webhard.co.kr ID: guhmok PW: 22773324

2024년 7월 23일 (화) 오전 11:29, Harlina Harlina <harlina.harlina@umi.ac.id>님이 작성:
[Quoted text hidden]

00-FAS(Research article)_0285_Harlina Harlina.pdf
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Harlina Harlina <harlina.harlina@umi.ac.id> Wed, Jul 24, 2024 at 11:05 PM
To: "(Guhmok)거목문화사" <guhmok@guhmok.com>

Dear

Guhmok publishing company Leader

We are pleased to inform you that we have received the results of the paper correction process and have agreed to publish the paper as is. However, we have a few recent corrections to suggest.

We would like to make the following revisions to the paper before publication:

1. Correct: 7000 rpm (it does not use comma or or periods to separate thousands)
2. Correct: Fig. 1. Hemocyte counts image of control diet (T1) and CO supplementation diet (T2) (original magnification 10x).
3. Correct: volatile fatty acids (VFA)
4. Correct: Omokhua AG, McGaw LJ, Finnie JF, Van Staden J. Chromolaena odorata (L.) R.M. King & H. Rob. (Asteraceae) in sub-Saharan Africa: a synthesis and review of its medicinal potential. J Ethnopharmacol. 2016;183:112-22. https://doi.org/10.1016/j.jep.2015.04.057 (We added the DOI address to ensure the accuracy of the article.)

We appreciate your consideration of these minor changes.

Sincerely,
Harlina
[Quoted text hidden]

(Guhmok)거목문화사 <guhmok@guhmok.com> Thu, Jul 25, 2024 at 8:19 AM
To: Harlina Harlina <harlina.harlina@umi.ac.id>

Dear. Harlina

I have confirmed the corrections you sent.

Numbers 1-3 have been applied, and number 4 is requested again.

This reference is not cited in the text (PDF 12 page).
1) Delete it or 2) quote it in the text.
2) If you quote in the text, please tell us exactly where to insert it.

Please reply by **July 25, 2024.**

Thank you for your cooperation.

Best regards,
Guhmok Publising

[거목문화사] 학술지 편집/인쇄 · 홈페이지 · 논문투고시스템 · JATS XML
04549 서울시 중구 을지로 148 중앙데코플라자 609호
TEL(대표): 02-2277-3324, FAX: 02-2277-3390
Homepage: http://www.guhmok.com E-mail: guhmok@guhmok.com
웹하드: http://www.webhard.co.kr ID: guhmok PW: 22773324

2024년 7월 25일 (목) 오전 12:05, Harlina Harlina <harlina.harlina@umi.ac.id>님이 작성:
[Quoted text hidden]

00-FAS(Research article)_0285_Harlina Harlina - 0725.pdf
1370K

Harlina Harlina <harlina.harlina@umi.ac.id> Thu, Jul 25, 2024 at 9:55 AM
To: "(Guhmok)거목문화사" <guhmok@guhmok.com>

Dear

Guhmok publishing company Leader

Additional information:

- Please delete this reference: Omokhua AG, McGaw LJ, Finnie JF, Van Staden J. Chromo laena odorata (L.) R.M. King & H. Rob. (Asteraceae) in sub-Saharan Africa: a synthesis and review of its medicinal potential. J Ethnopharmacol. 2016;183:112-22

[Quoted text hidden]
[Quoted text hidden]

(Guhmok)거목문화사 <guhmok@guhmok.com> Thu, Jul 25, 2024 at 10:20 AM
To: Harlina Harlina <harlina.harlina@umi.ac.id>

Thank you for the prompt response.

The reference has been deleted.

Are there any other modifications?
If there are no further corrections to be made, please reply with **"No modifications".**

Please reply quickly to proceed to the next step after the author proofreading deadline.

Please reply by **July 25, 2024.**

Thank you for your cooperation.

Best regards,
Guhmok Publising

[거목문화사] 학술지 편집/인쇄 · 홈페이지 · 논문투고시스템 · JATS XML
04549 서울시 중구 을지로 148 중앙데코플라자 609호
TEL(대표): 02-2277-3324, FAX: 02-2277-3390
Homepage: http://www.guhmok.com E-mail: guhmok@guhmok.com
웹하드: http://www.webhard.co.kr ID: guhmok PW: 22773324

2024년 7월 25일 (목) 오전 10:55, Harlina Harlina <harlina.harlina@umi.ac.id>님이 작성:
[Quoted text hidden]

00-FAS(Research article)_0285_Harlina Harlina - 0725(2).pdf
1358K

(Guhmok)거목문화사 <guhmok@guhmok.com> Fri, Jul 26, 2024 at 10:17 AM
To: Harlina Harlina <harlina.harlina@umi.ac.id>

Dear. Harlina

The deadline is approaching.
Please respond promptly by the 26th to proceed to the next step.

Are there any other modifications?
If there are no further corrections to be made, please reply with **"No modifications".**

Please reply quickly to proceed to the next step after the author proofreading deadline.

[Quoted text hidden]

00-FAS(Research article)_0285_Harlina Harlina - 0725(2).pdf
1358K

Harlina Harlina <harlina.harlina@umi.ac.id> Sat, Jul 27, 2024 at 9:01 PM
To: "(Guhmok)거목문화사" <guhmok@guhmok.com>

Dear

Guhmok publishing company Leader

We would like to thank Guhmok Publishing for constructive criticism of the manuscript.

"No modification"

Thank you for the attention

Sincerely,

Harlina Harlina

[Quoted text hidden]

10. PUBLISH

Article Online (31-7-2024)

RESEARCH ARTICLE
eISSN 2234-1757
Fish Aquat Sci. 2024;27(7):434-446
https://doi.org/10.47853/FAS.2024.e41
FAS Fisheries and Aquatic Sciences

Growth performance, non-specific immune activity, and resistance against *Vibrio parahaemolyticus* of whiteleg shrimp fed dietary *Chromolaena odorata* leaf flour components

Harlina Harlina[1,*], Rosmiati Rosmiati[2], Andi Hamdillah[1], Syahrul Syahrul[1], Yosie Andriani[3]

[1] *Aquaculture Study Program, Faculty of Fisheries and Marine Sciences, Universitas Muslim Indonesia, Makassar 90121, Indonesia*
[2] *Research Center for Fisheries, National Research and Innovation Agency, Bogor 16911, Indonesia*
[3] *Institute of Climate Adaptation and Marine Biotechnology, Universiti Malaysia Terengganu, Terengganu 21030, Malaysia*

Abstract

The efficacy of *Chromolaena odorata* leaf flour components (CO) to increase the growth performance, non-specific immune activity, and resistance against *Vibrio parahaemolyticus* of whiteleg shrimp (*Litopenaeus vannamei*) culture was evaluated. To this purpose, whiteleg shrimp post larvae were fed on diets supplemented with 0 and a 1.5 g CO/kg diet for 45 days in a pond. After feeding trials, the shrimps were identified for their growth parameters, collected, and injected with *V. parahaemolyticus*, then their non-specific immune activity and resistance to *V. parahaemolyticus* was observed for 14 days. Findings showed that CO increased the average body weight (7.71 g, 37%), weight gain (7.69 g, 40%), and specific growth rate (13.23%/day, 5.7%) as compared to the control. In addition, CO supplementation also increases shrimp's hematologic and immune activity (total hemocyte counts [6.8×10^7 CFU/mL, 242.9%], differential hemocyte counts [27%, 142.1%], and prophenoloxidase activity [0.085%, 566.7%]). Finally, shrimp resistance to *V. parahaemolyticus* infection also increased after CO supplementation, with survival rates of 73.33% as compared to 23.33% for the control. It suggests that *C. odorata* leaf flour component supplementation at an optimal dose in the diet may be an effective strategy to increase growth and resistance to bacterial disease with reduced mortality in shrimp farms.

Keywords: Acute Hepatopancreatic Necrosis Disease (AHPND), *Chromolaena odorata*, Immune response, *Litopenaeus vannamei*, *Vibrio parahaemolyticus*

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Introduction

The whiteleg shrimp, *Litopenaeus vannamei*, is a widely cultured species of high economic value. The whiteleg shrimp is very popular because of its high-quality meat, good taste, good nutritional content, and easy cooking (Li et al., 2021). Whiteleg shrimp is also one of the most successful cultured species in aquaculture, accounting for up to 80% of shrimp production (Rosilan et al., 2023). Whiteleg shrimp has been cultivated with intensive to super-intensive technology (Mustafa et al., 2023).

However, whiteleg shrimp have several challenges from various diseases, including Acute Hepatopancreatic Necrosis Disease (AHPND), a bacterial disease also known as Early Mortality Syndrome (EMS) (Rosilan et al., 2023). AHPND has caused significant economic losses for shrimp (Ha et al., 2023). *Vibrio parahaemolyticus* is a well-known causative agent of AHPND in penaeid shrimp farming worldwide (Velázquez-Lizárraga et al., 2019). *V. parahaemolyticus*, a Gram-negative bacterium that lives in marine and estuary environments, becomes pathogenic in shrimp at concentrations greater than 10^4 CFUs/mL (Soto-Rodriguez et al., 2022).

The use of immunostimulants to enhance immune responses is one of the important steps that can be taken to prevent disease in shrimp aquaculture (Kumar et al., 2022). Immunostimulants are substances that can activate or enhance the activity of the immune system, used to help the body's immune response in combating infections (Di Sotto et al., 2020). Immunostimulants are considered an attractive and promising agent for preventing diseases in fish and shellfish. Increasing the ability of shrimp bodies to fight disease by administering immunostimulants can be indicated by several parameters, including prophenoloxidase (proPO), total hemocyte counts (THC), and differential hemocyte counts (DHC). When selecting these therapeutic agents, it's important to think about the safety of consumers, the environment, and the shrimp.

Immunostimulants derived from plants are organic, free from side effects, environmentally friendly, and safe to use to improve the immune system of shrimp (Kumar et al., 2022). *C. odorata*, also known as Siam weed (Eze & Jayeoye, 2021) is a rapidly growing perennial herb that can grow up to 2.5 m (100 inches) tall in open areas and up to 10 m (33 feet) tall in shady areas where it behaves as a creeper, growing on other vegetation. When crushed, the plant is hairy and glandular; the leaves give off a pungent, aromatic odor. Some of the benefits of *C. odorata* are that it can improve soil fertility, crop production, protection, nutrition, and ethnopharmacology (Mugwedi, 2020). *C. odorata* has been reported to have antibacterial, anti-inflammatory, antioxidant, anthelminthic, antifungal, cytotoxic, anticonvulsant, antiprotozoal, and wound-healing properties (Mugwedi, 2020). This antioxidant and antibacterial property could help protect aquatic animals from oxidative stress and bacterial infection caused by environmental factors (Eze & Jayeoye, 2021).

Different plant seasons or life cycle stages can give different results in analyzing bioactive compounds. The leaves of *C. odorata* contain phytochemicals such as tannins, alkaloids, flavonoids, saponins, terpenoids and other phenolic compounds, which produce a physiological action in the body (Rasyid et al., 2020; Zahara, 2019). The presence of these components in *C. odorata* leaf extract has shown that it can be a source of immunostimulant to improve shrimp antibacterial activity. Various factors, including the environmental condition, can influence shrimp's growth and immune response. In the earlier study, in controlled testing, *C. odorata* leaf flour components (CO) enhanced immune responses and Survival Rate (SR) in tiger shrimp infected with *V. harveyi* (Harlina et al., 2019). However, the impact of CO use in improving the growth and health of farm-farmed whiteleg shrimp *L. vannamei* is still an area of research that has not been revealed. Thus, this study examines the effectiveness of CO at the optimal dose to increase the growth performance, non-specific immune activity, and resistance against *V. parahaemolyticus* of whiteleg shrimp *L. vannamei* culture in the pond.

Materials and Methods

Sample preparation

The old leaves of *C. odorata* were collected in the rainy season around a pond in Marana Village, Maros, Indonesia. followed by cleaning under running water and then cut into small cube-like pieces. The resulting leaf pieces were then dried using a drying cabinet at 40℃. The dried leaves were then turned into flour with the help of a kitchen blender. The leaf flour was then divided into two parts; some were used for phytochemical tests, while the other was mixed into feed as part of experiments.

Phytochemical test

Approximately 10 g of *C. odorata* leaf flour was extracted with 200 mL of methanol (MeOH) while stirring using a shaker for 24 hours at room temperature. The MeOH extract was filtered using Whatman No.4 filter paper. The MeOH extract was then

concentrated using a rotary evaporator set at 37℃ to obtain concentrated MeOH extract. Phytochemical tests were conducted to confirm the uniformity of compound content in *C. odorata* leaves used in this study with compound content in *C. odorata* leaves that had been investigated previously (Harlina et al., 2013). The components present were identified through the observation of color changes or the formation of visually visible deposits.

The flavonoid analysis involved combining 1–2 mL of warm 50% MeOH with 1 mL of MeOH extract, then introducing magnesium and 0.5 mL of concentrated hydrochloric acid. The presence of flavonoids was indicated by the appearance of an orange color. Dragendorff's and Mayer's tests were performed for alkaloid detection. A mixture was created by combining 1 mL of MeOH extract with 0.5 mL of 2% hydrochloric acid. The resultant acidic solution was subsequently divided into two tubes. In Tube I, three drops of Dragendorff solution were added, while in Tube II, three drops of Mayer solution were introduced. Verification of the existence of alkaloids was established through the observation of a red or orange residue in Tube I and a whitish precipitate in Tube II. In terpenoid testing, MeOH extract (1 mL) was mixed with 0.5 mL of chloroform and then added with 0.5 mL of anhydrous acetic acid. After that, the mixture was carefully fed into a test tube with 1–2 mL of concentrated H_2SO_4, which presents a brownish or purple ring if triterpenoids are present.

After that, the mixture was carefully poured into a test tube, and 1–2 mL of concentrated H_2SO_4 was added. The Liebermann-Burchard reagent was used to test the presence of steroids by mixing MeOH (1 mL) with n-hexane (2 mL) and allowed to stand until two layers formed. The n-hexane layer was removed, and five drops of Liebermann-Burchard reagent were added. The presence of blue color indicated the presence of steroids. Saponins were identified through the combination of 2 mL of MeOH extract with 2 mL of distilled water, followed by allowing the mixture to stand for one minute. The addition of two drops of 1 N hydrochloric acid was performed, and the presence of saponins in the MeOH leaf extract was confirmed if foam developed and remained stable for 10 minutes.

Experimental diets

The diet preparation and proximate composition process were explained elsewhere in Table 1. CO leaf powder was added to diets in the optimal dose (1.5 g/kg diet). This optimal dose was chosen based on the earlier study on tiger shrimp with a dietary proximate composition consisting of crude protein (38.21%), crude lipids (9.7%), crude fiber (4.52%), crude ash (12.48%), nitrogen-free extracts (32.86%), and total energy (16.46 kcal/kg).

Table 1. The Basal ingredients and proximate composition of experimental diet

Ingredients	Treatments	
	Control diet (T1)	CO supplementation diet (T2)
Fish flour (%)	40	40
Shrimp head flour (%)	10	10
Copra cake (%)	9	9
Corn flour (%)	12	12
Soybean flour (%)	17	17
Wheat flour (%)	10	10
Vitamin and minerals (%)	2	2
Chromolaena odorata leaves (g/kg feed)	0	1.5
Total (%)	100	100
Proximate composition		
Crude protein (%)	38.31	38.31
Crude lipid (%)	9.27	9.27
Crude fibre (%)	4.52	4.52
Ash content (%)	12.48	12.48
NFE (%)	32.86	32.86
Total energy (kcal/kg feed)	1,646	1,646

NFE, nitrogen free extract.

Shrimp and husbandry trial

This experiment was done at the farmer's pond in Bonto Langkasa Village, Pangkep Regency, South Sulawesi, Indonesia. Post larvae (PL) shrimp in size 0.02 ± 0.02 g were purchased from a local hatchery. Then, shrimp was distributed to experimental ponds (500 m^2) (170 shrimp/m^2, duplicate) (Purnamasari et al., 2017). The stocking of PL was carried out in the morning, which begins with the acclimatization of PL first, especially at temperature and salinity. Once the temperature and salinity of the water within the PL plastic bag were either equivalent or exhibited minimal variation compared to the pond water, the gradual introduction of PL into the experimental ponds could be initiated. The shrimp were fed with a control and treatment diet for 45 days, followed by control feeding on both treatments, handed over to local farmers, and observation stopped. Before being used, the intensive ponds were prepared in accordance with the established procedures outlined in the REBAFE standard operating protocols. The trial feeding of 3% of body weight

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was carried out twice daily in the morning and evening for 45 days.

Growth performance

The shrimp were separately sampled to determine the shrimp growth performance during feeding. The collection of shrimp samples was conducted utilizing anchovy sampling technique. Anchovy sampling (n = 30) was conducted in the morning on the 45th day of the rearing period. Growth performance was evaluated by the following parameters: average body weight (ABW) (g) was measured by weighing the body weight of the sampling shrimp. Weight gain (g) = $w_t - w_0$. SGR (%/day) = 100 % × ([ln w_t – ln w_0] / t) where w_0 is the initial mean body weight (g), wt is the sampling mean body weight (g), while t is rearing time (day).

Challenge test with *Vibrio parahaemolyticus*

Whiteleg shrimp that had been given a control diet (T1) and those fed a CO-supplemented diet (T2) for 45 days in the ponds were challenged with *V. parahaemolyticus* for 14 days. The shrimps from groups T1 and T2 were respectively selected 80 individuals each with an average weight of 7.0 ± 0.02 g and randomly assigned to control group (T1) and treatment group (T2) to evaluate the effects of CO on *V. parahaemolyticus* infection in shrimp. The experiment was conducted in eight rectangular plastic tanks (80 × 57 × 42 cm, capacity 150 L), each containing 20 shrimp. Each type of treatment was carried out four times (three for immune response and one for survival observation). The Fish Health and Management Lab of the Research Institute for Brackishwater Aquaculture and Fisheries Extension in Maros, Indonesia, supplied the pathogenic bacterium *V. parahaemolyticus* for the study. Determination of the density of *V. parahaemolyticus* reaching 10^4 CFUs/mL referred to Abdel-Tawwab et al. (2021) method. Whiteleg shrimps were infected with *V. parahaemolyticus* by injecting 0.1 mL intramuscularly into the ventral sinus. During the challenge test, the experimental diets were given twice daily in the morning and evening as much as 3% of body weight. To investigate how CO affect the growth of *V. parahaemolyticus*, the population of *V. parahaemolyticus* in the shrimp was determined using thiosulfate citrate bile sucrose agar media at 3, 6, 12, 24, and 48 hours after the challenge test.

Observation of immune responses and survival rate

Hemolymph samples were gathered both prior to the challenge test and at specific intervals post-challenge, specifically on the 2nd, 4th, 8th, and 14th days. The daily monitoring of the SR of whiteleg shrimp was conducted throughout the challenge test. The calculation of SR was performed using the formula as documented by Herawati et al. (2020). Prior to hemolymph collection, the shrimp were subjected to cold anesthesia. Hemolymph retrieval followed guidelines provided by Morales-Covarrubias et al. (2023) by using an anticoagulant solution instead of the modified alsever solution. The anticoagulant solution (100 µL) was inserted into a sterile syringe with a capacity of 1 mL. Then, with caution, the anticoagulant was mixed with hemolymph by taking 100 µL of hemolymph from the shrimp ventral sinuses on the base of the first abdominal segment. After hemolymph retrieval, the ventral sinus area of the shrimp was cleaned with a cotton swab that was soaked in 70% ethanol to prevent contamination. A mixture of hemolymph and anticoagulant (1:1) was placed in a 1.5 mL microcentrifuge tube by gently pipette up and down to prevent cell clumping. Next, trypan blue (0.5% in 2.6% NaCl) was used to dilute hemocytes. Hemocytes were examined under a light microscope (DP21, Olympus, Tokyo, Japan) at 10× magnification, employing a hemocytometer and introducing 10 µL of a diluted hemolymph sample. The THC (Fig. 1) was calculated according to the formula proposed by Braak (2002).

To calculate DHC, a microscope glass slide was prepared, and then 20 µL hemolymph from each shrimp (the remaining hemolymph was used to calculate THC) was placed on the slide. Hemocytes were allowed to flatten themselves and dry at room temperature, washed with pure MeOH for 15 minutes, and dried again at room temperature. Next, hemocytes were stained with Giemsa-Rosenfeld as much as 10%. Slide cleaning was carried out under running water for 30 seconds. Following the drying process, the slides were employed for the morphological characterization of distinct hemocyte types, as outlined by Chang & Chang (2022), utilizing a light microscope (DP21, Olympus) at a magnification of 40×. Diverse shrimp hemocytes, including hyaline, granular, and semi-granular, were recognized and quantified using the methodology described by Braak (2002). The DHC result (Fig. 2) was then calculated as the average of each hemocyte type in the total cells counted.

The activity of proPO was measured with the help of a spectrophotometer. L-dihydroxyphenylalanine was used as the base material to record dopachrome formation. To perform a proPO test, the following steps were performed. First, the prepared 100 µL of hemolymph was separated by centrifugation at 7000 rpm for 20 minutes at 40℃, and the supernatant was

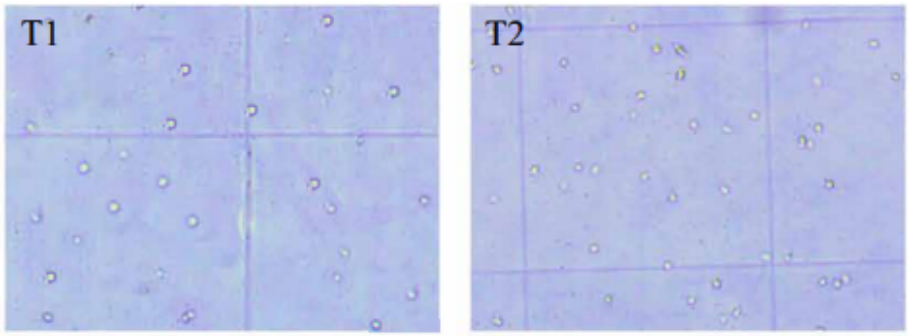

Fig. 1. Hemocyte counts image of control diet (T1) and CO supplementation diet (T2) (original magnification 10x).

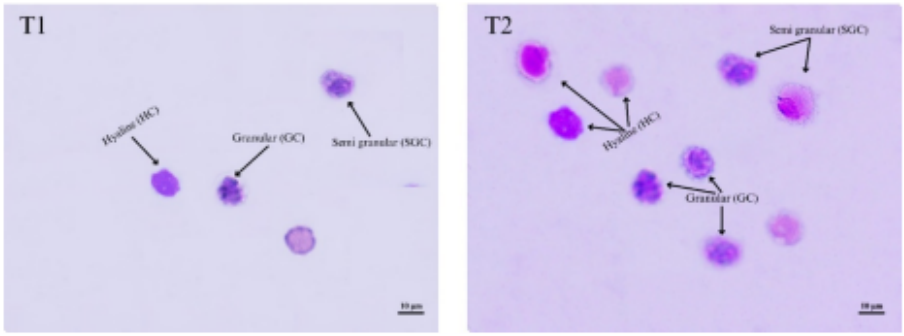

Fig. 2. Different shapes of hemocytes of the control diet (T1) and CO supplementation diet (T2) (original magnification ×40).

discarded. Then, a hemolymph precipitate was added to 100 µL of cacodylate citrate buffer solution (0.1 M $C_2H_{12}AsNaO_3$, 0.45 M NaCl, and 0.01 M $Na_3C_6H_5O_7$) and then separated again in the same way. Next, the precipitate was resuspensioned in cacodylate buffer (100 µL) and homogenized. After that, 100 µL of the mixture was piped into a 96-well microplate (R-BioPharm AG, Darmstadt, Germany) and mixed with 100 µL of trypsin (Sigma-Aldrich, St. Louis, MO, USA). The mixture was then suspended and incubated at 25℃ for 10 minutes. Enzymatic activity was measured by comparing the dopachrome's absorbance and blanks containing trypsin (200 µL) and l-DOPA (50 µL, containing 1 mg/mL trypsin) at a wavelength of 490 nm.

Water quality observation

During the experimental period, salinity, pH, Dissolved Oxygen (DO), and water temperature were measured every 10 days of the feeding trial at 08:00–10:00 Indonesian time using YSI 6920 V2-2 Multiparameter Water Quality Sonde (YSI, Yellow Springs, OH, USA).

Data analysis

Growth parameters, immune response (THC and proPO activity), and SR were evaluated through t-student test analysis using SPSS software version 20.0 (SPSS, IBM, Chicago, IL, USA) at a confidence level of 0.05 ($p < 0.05$), while DHC and water quality were presented in descriptive form. All Figures were rorganized using a Graphpad Prism versi 10 (GraphPad Softward, San Diego, CA, USA).

Results

Phytochemical identification

The examination of phytochemical constituents confirmed the presence of flavonoids, alkaloids, triterpenoids, and steroids in

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the leaves of *C. odorata*. However, the MeOH extracts of *C. odorata* leaves did not exhibit the presence of saponins, as indicated in Table 2.

Growth parameters

This experiment showed that dietary CO supplementation had a positive impact in increasing the growth of whiteleg shrimp. Significant improvements ($p < 0.05$) in all growth parameters tested (Table 3) were shown by shrimp fed a CO-supplemented diet compared to shrimp fed a control diet after 45 days of feeding experiments with ABW of 7.71 ± 0.24 and 5.61 ± 0.22 g, respectively. A higher SGR of 13.23 ± 0.14% was noted in shrimp fed a CO-supplemented diet (T2), but a relatively low SGR (12.52 ± 0.17%) was recorded in shrimp fed a control diet (T1). The growth parameters measured were ABW, weight gain, and SGR. It suggests that CO can be used to improve the growth of whiteleg shrimp.

Water quality

Based on the supplied data, the water quality obtained during the feeding trial was normal for whiteleg shrimp *L. vannamei* growth in the pond. The water quality measured consisting of salinity (ppt), pH, temperature (℃), and DO (ppm) was summarized in Table 4.

Vibrio parahaemolyticus population

The treatment with CO (T2) had a significant impact on the growth of *V. parahaemolyticus* in the shrimp. As shown in Fig. 3, the population of *V. parahaemolyticus* (n = 3) in the shrimp fed a CO-supplemented diet exhibited a continuous decrease over time. After 48 hours of the challenge test, the population of *V. parahaemolyticus* decreased by 45.58%. In contrast, in the control group (T1), the population of *V. parahaemolyticus* in shrimp also experienced a decrease up to 6 hours after the challenge test, followed by an increase reaching its peak after 12 hours of the challenge test, and subsequently declined again but remained higher than the initial bacterial population.

Total haemocyte counts (THC)

THC is a useful tool for assessing the health status of shrimp in aquaculture (Morales-Covarrubias et al., 2023). Fig. 4 shows

Table 2. Result of phytochemical test of *Chromolaena odorata* L. methanol extract

Methanol extract	Flavonoid	Alkaloid		Triterpenoid	Steroid	Saponin
		Dragendorff	Meyer			
C. odorata L.	+	+	+	+	+	–

A (+) sign indicates a positive reaction, while a (–) sign indicates a negative reaction.

Table 3. Mean ± SD (n = 30) growth of whiteleg shrimp *Litopenaeus vannamei* after 45 days of feeding trial

Parameters	T1	T2
Average body weight (ABW) (g)	5.61 ± 0.22	7.71 ± 0.24*
Weight gain (g)	5.59 ± 7.78	7.69 ± 0.08*
Specific growth rate (SGR) (%/day)	12.52 ± 0.17	13.23 ± 0.14*

* A significant difference ($p < 0.05$).
T1, control diet; T2, CO-supplemented diet.

Table 4. The overall and range values (minimum, maximum) of water quality parameters of experimental diets during the 45-day rearing time (means ± SD, n = 3)

Parameter	T1	T2	The suitable value
Salinity (ppt)	22.5 ± 0.1	23.1 ± 0.1	10–25
	(20.1, 25.0)	(20.1, 25.1)	
pH	7.81 ± 0.05	7.75 ± 0.01	7.5–8.5
	(7.53, 8.43)	(7.54, 8.13)	
Temperature (℃)	27.9 ± 0.06	27.8 ± 0.07	20–30
	26.5, 29.9	26.4 ± 29.9	
DO (ppm)	7.83 ± 0.05	7.67 ± 0.09	5–20
	(7.39, 8.13)	(7.47, 7.91)	

T1, control diet; T2, CO-supplemented diet.
Adapted from Nguyen et al. (2002) with CC-BY-NC.

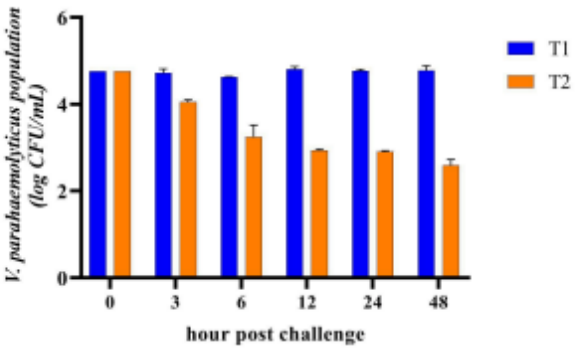

Fig. 3. *Vibrio parahaemolyticus* population (Log CFU/mL) (n = 3) in the shrimp at the beginning and after 3, 6, 12, 24, and 48 hours unchallenged (T1) and challenged with *Chromolaena odorata* leaf flour components (CO) (T2).

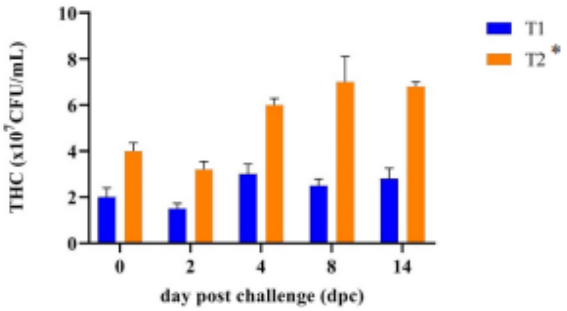

Fig. 4. Mean ± SD total hemocyte counts (THC) (n = 3) of whiteleg shrimp before, 2nd, 4th, 8th, and 14th day post-challenge. T1, control diet; T2, CO-supplemented diet; *, a significant difference ($p < 0.05$).

that after a feeding trial for 45 days of farmed rearing, whiteleg shrimp fed a CO supplementation diet (T2) significantly increased their THC value (4×10^7 CFU/mL) compared to that (2×10^7 CFU/mL) of shrimp fed a control diet (T1). The result of the THC observation indicated that a higher THC in shrimp fed the CO-supplemented diet (T2) had been shown before the challenge test compared with the control shrimp (T1). THC levels were greatly reduced in shrimp fed the control diet may be because there had been an infection caused by pathogenic bacteria or viruses since being farmed.

Differential hemocyte counts (DHC)

After whiteleg shrimp were fed a CO-supplemented diet (T2) and control diet (T1) and challenged with *V. parahaemolyticus*, the proportions of the three hemocyte types changed (Table 5 and Fig. 5), and the percentage of GC increased dramatically. The pattern of changes in GC values in whiteleg shrimp in both treatments had the same tendency; however, GC values in shrimp fed diet T1 were always lower than GC values in shrimp fed diet T2. On the 2nd dpc, the granular cell number decreased in the T2 treatment and increased in the T1 treatment. Furthermore, on the 4th, 8th, and 14th dpc, the granular cell number decreased to the GC values lower than the baseline values in both shrimp treatments. The hyaline in the T1 treatment decreased on the 2nd dpc, then increased on the 4th, 8th, and 14th dpc. Unlike hyaline in the shrimp group (T1), the shrimp group (T2) increased on the 2nd dpc, then decreased on the 4th, 8th, and 14th dpc. Since the 2nd dpc, semi-granular in the T1 treatment decreased; conversely, semi-granular in the T2 treatment

Table 5. DHC (n = 3) of whiteleg shrimp before, 2nd, 4th, 8th, and 14th day post-challenge

Treatment	Parameter	Initial	2nd dpc	4th dpc	8th dpc	14th dpc
Diet T1	Hyaline (%)	31.00	28.00	33.00	35.00	50.00
	Granular (%)	45.00	49.00	45.00	43.84	31.00
	Semi-granular (%)	24.00	23.00	22.00	21.16	19.00
Diet T2	Hyaline (%)	29.00	30.00	27.00	26.00	25.00
	Granular (%)	61.00	56.90	49.67	48.30	48.00
	Semi-granular (%)	10.00	13.10	23.33	25.70	27.00

T1, control diet; T2, CO supplementation diet.

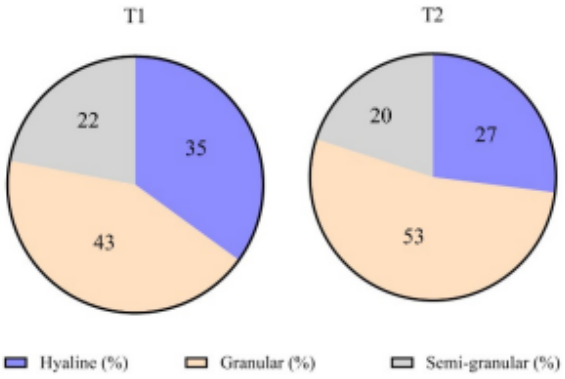

Fig. 5. The average differential hemocyte counts (DHC) (n = 3) of whiteleg shrimp before, 2nd, 4th, 8th, and 14th day post-challenge. T1, control diet; T2, CO supplementation diet.

increased. The proportion change of the three hemocyte types was summarized in Table 5.

Prophenoloxidase (proPO)

The current study found that the average proPO activity in shrimp fed the CO-supplemented diet (0.068) (T2) was higher and significantly different ($p < 0.05$) than that in shrimp fed the control diet (0.017) (T1). The proPO activity in the treatment shrimp (T2) decreased on the 2nd dpc, but their proPO activity was still higher than that in the control shrimp (T1) and then dramatically increased on the 8th dpc and remained relatively stable until the 14th dpc. In contrast, the proPO activity in control shrimp remained stable and lower than that of treatment shrimp during the challenge test (Fig. 6).

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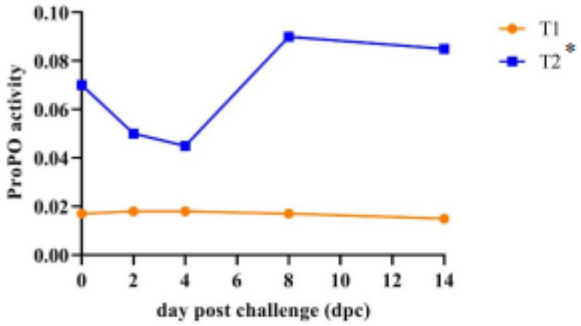

Fig. 6. proPO activity (n = 3) of whiteleg shrimp before, 2nd, 4th, 8th, and 14th dpc. T1, control diet; T2, CO supplementation diet; *, a significant difference ($p < 0.05$).

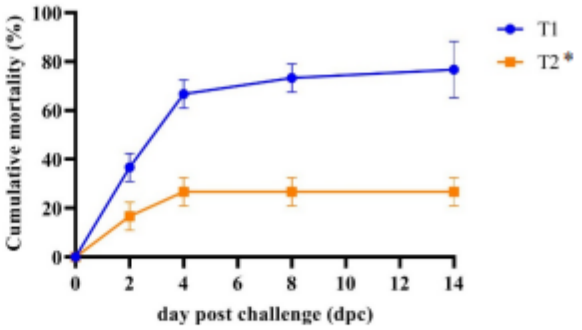

Fig. 7. Mean ± SD (n = 3) cumulative mortality of whiteleg shrimp before, 2nd, 4th, 8th, and 14th after *Vibrio parahaemolyticus* injection. T1, control diet; T2, CO-supplemented diet; *, a significant difference ($p < 0.05$).

Survival rate (SR)

The mortality rate of shrimp receiving the control diet (T1) exhibited a sharp increase on the 2nd day post-challenge (dpc) and persisted until the end of the study, as illustrated in Fig. 7. Conversely, a consistently high survival trend was observed in shrimp fed the CO-supplemented diet (T2) from the 2nd dpc onward. By the 14th dpc, nearly all shrimp fed the control diet (T1) had succumbed. Between the 4th and 14th dpc, shrimp provided with the CO-supplemented diet (T2) demonstrated significantly greater resistance ($p < 0.05$) to *V. parahaemolyticus* infection (73.33 ± 5.77% SR) compared to those fed the control diet (T1) (23.33 ± 5.77% SR). This data indicated the increased survival of shrimp fed the CO-supplemented diet by 50%, which may be the effect of *C. odorata* leaf bioactive compounds.

Discussion

The study found that the MeOH extract of *C. odorata* leaves contains natural ingredients such as flavonoids, alkaloids, triterpenoids, and steroids. The specific chemical composition of *C. odorata* may vary depending on the species, season, and plant location. The presence of phytochemical compounds such as alkaloids, flavonoids, terpenoids, and steroids in the leaves of *C. odorata* was suspected to increase the immune parameters of crustaceans such as shrimp.

In this study, *C. odorata* leaf meal showed its efficacy in reducing *V. parahaemolyticus* population at 3 hours to 48 hours after the challenge test. The population of *V. parahaemolyticus* in the shrimp decreased by 45.58% or 3.90×10^2 CFU/mL after 48 hours of challenge testing (Fig. 3). Flavonoids modulate the immune system to enhance its function, whereas alkaloids serve as both immunostimulants and antibacterial agents, as documented by Idris Affandi et al. (2019). A similar result was also obtained by Behl et al. (2021) that the content of phytochemicals consisting of alkaloids, tannins and phenols has activity as an immunostimulant that can improve the quality of host immune cells. Flavonoids, alkaloids, and saponins have been shown to have pharmacological activity that can help protect whiteleg shrimp from bacterial diseases caused by *Vibrio* sp., called vibriosis (Nur et al., 2020; Santiago et al., 2021). Alkaloids, for example, boost shrimp's immune system by enhancing the activity of macrophage cells, which can engulf and destroy pathogens.

After 45 days of rearing shrimp was farmed, the whiteleg shrimp fed with a CO-supplemented diet (T2) had a higher ABW, body weight gain, and SGR than those in the shrimp fed a control diet (T1). The shrimp increased its growth parameter because the CO-supplemented diet contained nutrients such as carbohydrates, protein, lipids, minerals, and bioactive compounds (Table 3) that promote growth (Aziz et al., 2020). *C. odorata* also contains bioactive compounds that function as antiseptic substances that can inhibit bacterial growth (Rasyid et al., 2020). The presence of flavonoids and alkaloids in the leaves of *C. odorata*, which have antioxidant and antibacterial properties, could help protect aquatic animals from oxidative stress and bacterial infection caused by environmental factors (Eze & Jayeoye, 2021). *C. odorata* leaf flour can be used as a food supplement for Bali cattle, as it contains nutrients that improve their consumption, digestibility, pH, N-ammonia, volatile

fatty acids (VFA), and growth. Grape extract has been found to improve white shrimp's antioxidant activity and growth performance, specifically *L. vannamei* (Chien et al., 2023a). The shrimp fed the CO-supplemented diet showed a weight after 45 days of rearing in the pond of 7.71 ± 0.24 grams (ABW). This ABW was higher than that (around 5.00 g) reported by Purnamasari et al. (2017). The daily growth rate (SGR) in this study reached 13.23 ± 0.14%/day, which was higher than the growth of whiteleg shrimp after being fed dry grape extract of 2.76 ± 0.06%/day (Chien et al., 2023b) and Seaweed Polysaccharide (7.59 ± 0.33) (Abbas et al., 2023). Several plant extract applications, such as *Boesenbergia pandurata*, *Solanum ferox* extract (Hardi et al., 2022), and *Phyllanthus amarus* extract, have been reported to enhance the growth of shrimp (Ngo et al., 2020). The increased growth rate of whiteleg shrimp obtained after 45 days of rearing could also come from pond water quality under normal conditions, as noted in Table 4.

Bioactive compounds in *C. odorata* increased immune responses (Harlina et al., 2019). The increased formation of phagocytic cells is essential in controlling the attack of microorganisms. Elevated THC levels are associated with increased cellular and humoral immune responses (Braak, 2002; Mulyadi & Iba, 2020). In this study, THC shrimp fed the CO-supplemented diet before facing the challenge test showed an increase in hemocyte counts compared to shrimp that were only given a control diet. It indicates a proliferation of hemocytes, hinting that the shrimp's immune system has improved, making it better prepared to deal with pathogenic infections.

The average value of the THC post-challenge test of whiteleg shrimp fed the CO-supplemented diet (T2) (5.40×10^7 cells/mL) was significantly higher ($p < 0.05$) than whiteleg shrimp fed the control diet (T1) (2.36×10^7 cells/mL). This study showed that CO may have a better effect on whiteleg shrimp blood parameters after being challenged with *V. parahaemolyticus* than without CO. Decreased and increased THC levels can occur rapidly after a bacterial infection due to the body's defence response. THC levels decreased on the 2nd day after a bacterial infection was detected and then increased again on the 4th day as part of the shrimp's natural immune system recovery process as described in the study of Risjani et al. (2021). If the number of shrimps hemocytes increases, the body's defences also increase to avoid infection. Conversely, hemocyte numbers decrease because many die after destroying foreign macromolecules when the infection is overcome and resistance to foreign bodies is achieved.

The high amount of shrimp THC after being challenged with *V. parahaemolyticus* showed that the *C. odorata* bioactive compound could increase the immune response by stimulating the production of immune cells and molecules, such as cytokines and antibodies (Vijayaram et al., 2022) and growth promotion. Shrimp are not equipped with adaptive immune systems, so they must rely on the innate immune system (Kumar et al., 2022). The high THC value found in the shrimp group (T2) could improve the disease resistance of shrimp to reduce the risk of infection, which has an impact on a higher survival (Fig. 7) in shrimp group T2 after being challenged with *V. parahaemolyticus* compared to the shrimp group T1. In the earlier study of different species, tiger shrimp given a diet supplemented with *C. odorata* leaf flour had a higher THC value than the shrimp group fed without *C. odorata* leaf flour (Harlina et al., 2019). Like the whiteleg shrimp in the current study, the THC value obtained was much higher, possibly due to the shrimp being fed a diet supplemented with CO for a longer time in the pond or different shrimp responses due to pathogens entering. THC values in shrimp before the challenge test with *V. parahaemolyticus* had shown differences. In a more general sphere, it is thought that environmental conditions play an important role in influencing crustacean immune responses and resilience, especially in shrimp farms where environmental factors such as laboratory environments are often difficult to control. Many reports have noted the use of plants to enhance non-specific immune parameters in crustacea (Hardi et al., 2022; Ngo et al., 2020).

DHC refers to the proportion of different types of hemocytes (HC, GC, and SGC) in the hemolymph of shrimp. These three hemocyte cell types played different roles in the immune system. Hyaline cells are responsible for executing the phagocytic role within the shrimp immune system, while the collaborative efforts of granular and semi-granular cells contribute to the synthesis and subsequent release of antimicrobial peptides. According to Xian et al. (2017), the presence of an immune response is indicated by changes in hemocytes. In this study, before the challenge test, shrimp fed the CO-supplemented diet (T2) during the 45-day rearing period showed lower values of semi-granular cells and hyaline cells than these values in shrimp fed without CO-supplemented diet (T1) (Table 5 and Fig. 5). The lower semi-granular cells obtained could indicate the possibility that the bioactive compounds of *C. odorata* leaf triggered the maturation of hemocyte cells more quickly than in control shrimp during the grow-out in the pond. The change in the

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number of hyaline cells is closely related to the semi-granular cell number derived from late-stage hyaline cell development. Thus, a reduction in the quantity of semi-granular cells is observed, attributed to the incapacity of hyaline cells to undergo differentiation into semi-granular cells (Risjani et al., 2021).

The number of differential hemocyte cells during the challenge test changed over time, and it can be found that hyaline and granular cells may play a key role in immune system function. The smallest cell type is hyaline cells, with a high ratio between nucleus and cytoplasm and relatively few cytoplasmic granules. These cells play a crucial role in the body's immune defense system, with the quantity of hyaline cells typically rising in correlation with heightened phagocytic activity. In instances of hyaline cell infection, a notable increase is commonly observed as an initial response in the body's defense mechanisms (Risjani et al., 2021). In this study, shrimp hyaline cells in T1 treatment groups decreased on the 2nd dpc, then increased on the 4th dpc, and continued to increase until the 14th, indicating an infection in hyaline cells.

In contrast, hyaline cells in the T2 groups increased on the 2nd dpc and then decreased on the 4th, 8th, and 14th dpc; instead, semi-granular cells continued increasing, indicating shrimp recovery from the infection. The granular and semi-granular cells are involved in crustaceans' recognition and defence system. Bioactive compounds of *C. odorata* leaf flour had increased shrimp protection from *V. parahaemolyticus* infection by increasing semi-granular cells associated with continuously increasing semi-granular cells since the 2nd dpc. Similar results have been reported in the use of *Xylocarpus granatum* leaf extract, which increased the number of semi-granular cells in tiger shrimp tested with *V. harveyi* by as much as 1.55 times higher than semi-granular cells in control shrimp (Saptiani et al., 2020).

The proportion of granular cells predominates among the three hemocyte cells (Fig. 2). The shrimp fed a CO-supplemented diet had granular cells of 53%, higher than the granular cells in the shrimp fed without a CO-supplemented diet (43%). The highest percentage of granular cells, also known as neutrophils, serve as pioneers in immune defence against Gram-negative bacteria; granular cells in this study indicated that bioactive compounds of *C. odorata* leaf could increase the srimps'immune system by inducing hemocyte improvement (Xian et al., 2017). They have several mechanisms to enhance the immune response, including phagocytosis, degranulation, and NETosis. Neutrophils can engulf and kill pathogenic bacteria through multiple bactericidal pathways. In addition, they have granules and enzyme pathways that help to eliminate pathogenic microbes. These granules can release cytokines that activate antigen-presenting cells and amplify the immune response.

Furthermore, neutrophils can produce lysozyme, an antimicrobial enzyme that can kill certain bacteria independently of peptidoglycan hydrolytic activity and modulate the host's immune response to infection. Dietary supplementation with methionine (Met) can improve growth performance and increase the activities of antioxidant enzymes in shrimp (Huang et al., 2020). Hyaline and granular cells were the two dominant cell types that were always observed to be higher than semi-granular cells after the 4th, 8th, and 14th dpc. The same pattern on both cell types was observed using seaweed extract *Sargassum* sp. to improve immune parameters (THC and DHC) in white shrimp *L. vannamei* juveniles challenged with *V. algynolyticus* (Mulyadi & Iba, 2020).

The proPO system's activity is a key element in the immune response associated with the defence against pathogens and their removal (González-Rete et al., 2019). The proPO cascade is triggered by a small number of microbe-derived molecules such as lipopolysaccharides, β-1, 3-glucans, or peptidoglycan, which are recognized by pattern-recognition proteins. ProPO is identified as a critical element within the immune system of shrimp, serving a pivotal role in safeguarding against microbial infections. PO, a crucial enzyme of the proPO system in invertebrates, promotes humoral defence mechanisms and subsequently eliminates pathogens. The proPO activity in the shrimp fed a CO-supplemented diet during the 45-day culture period in the pond was much higher than in the shrimp fed the control diet. This result is like previous studies that obtained higher proPO activity in shrimp who received diet supplementation with herbs material/extract (Ngo et al., 2020). The proPO activity enhancement in treated shrimp hemolymph (T2) indicates an increased immune response to the pathogen, which may impact shrimp SR of 73.33%.

On the other hand, the low SR observed in the control group (23.33%) (T1) may be due to the low activity of proPO. The decreased proPO activity can cause failed phagocytosis and tissue damage. A similar finding of the proPO activity in white-leg shrimp was obtained in tiger shrimp when challenged with *V. harveyi*. The *C. odorata* leaf flour-supplemented diet significantly increased the proPO activity in the treatment tiger shrimp group compared with the control tiger shrimp group (without *C. odorata* leaf flour supplementation) (Harlina et al., 2019).

Conclusion

The leaves of *C. odorata* were confirmed to contain bioactive components such as alkaloids, flavonoids, triterpenoids, and steroids, which are suspected to improve shrimp health. *L. vannamei* that received the CO at 1.5 g/kg of diet showed improved growth parameters and enhanced resistance to *V. parahaemolyticus* infection. The heightened THC, along with increased levels of granular and semi-granular cells and proPOpost the feeding trial, are associated with the supplementation of CO in the diet. The findings of this investigation propose that these dietary components have the capacity to serve as immunostimulants in shrimp, consequently enhancing growth and bolstering the immune response, thereby augmenting the resistance of whiteleg shrimp against bacterial infection. These results provide useful information for sustainable shrimp culture and the potential use of CO as a shrimp diet additive to improve growth, survival, immune responses, and resistance to bacterial infection. To explore the future perspectives of how the specific phytochemicals in CO stimulate the immune system, further research and investigations involving the study of the mechanisms of action of these phytochemicals, their interactions with the immune system components, and their effects on immune-related gene expression or signaling pathways in shrimp are needed.

Competing interests

No potential conflict of interest relevant to this article was reported.

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Availability of data and materials

Upon reasonable request, the datasets of this study can be available from the corresponding author.

Ethics approval and consent to participate

This research has been approved by the UMI Health Research Ethics Commission Number: 237/A.1/KEPK-UMI/VI/2022 (June 12, 2022).

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