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Research Article

Vibrio Dynamics and Health Status of Pacific White Shrimp Fed with Cinnamaldehyde-Containing Feed

Dian Eka Ramadhani¹, Andri Hendriana¹, Dinamella Wahjuningrum^{2*} , and Muhammad Arif Mulya¹

¹Study Program Production Technology and Management of Aquaculture, College of Vocational Studies, IPB University, Bogor, West Java, 16680. Indonesia

²Departement of Aquaculture, Faculty of Fisheries and Marine Science, IPB University, Bogor, West Java, 16680. Indonesia



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*) Corresponding author:

E-mail: dinamellawa@apps.ipb.ac.id

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Abstract

Disease can cause shrimp mortality and high economic losses caused by Vibriosis group. One alternative is using cinnamaldehyde which function as an anti-microbial and inhibit the production of toxins released by microorganisms. This study aimed to evaluate the administration of cinnamaldehyde on the dynamics of Vibrio bacteria and the health status of Pacific white shrimp fed with cinnamaldehyde-containing feed. This study used a completely randomized design (CRD) consisted of 4 treatments and 3 replications consisting of treatment K, A, B, and C which mean feeding without cinnamaldehyde, feeding containing cinnamaldehyde dose of 0.05%, 0.1%, and 0.2% respectively. Parameters included total bacteria (green and yellow Vibrio) in water, intestine, and hepatopancreas, as well as immune responses parameters, including total hemocyte count (THC), respiratory burst (RB), and phenol-oxidase (PO). All data were tabulated in Microsoft Excel 2016 and all statistical analysis was conducted in the SPSS v.22.0 software with one-way ANOVA followed by Duncan Multiple Range Test (DMRT ($P=0.05$)). The results showed that the administration of cinnamaldehyde influences the dynamics of Vibrio and health status of shrimp. At the beginning and at the end of cinnamaldehyde administration, green and yellow Vibrio bacteria are mostly found in water, intestine, and hepatopancreas. The administration of cinnamaldehyde to Pacific white shrimp can reduce the number of green Vibrio bacteria both in the intestine and hepatopancreas as shown in treatments C and B. Cinnamaldehyde also affected the higher percentage of yellow Vibrio bacteria in treatments C and B compared to control (K). The highest immune responses of THC and RB were produced in treatments A and C compared to K. The decrease of green Vibrio bacteria and increased colonization of yellow Vibrio bacteria were correlated with immune responses. The best treatment in this study is treatment C.

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1. Introduction

Pacific white shrimp (*Litopenaeus vannamei*) is one of many important aquaculture commodities and the main export commodity by Indonesian Ministry of Maritime Affairs and Fisheries (MMAF). Based on a magazine from Global Seafood Alliance (GOAL), world shrimp production has increased since 2007 to 2017 (Anderson et al., 2019). Indonesia ranked fourth in shrimp production in Asia after China, Thailand, and Vietnam (Anderson et al., 2019). Data from the Central Statistics Agency (CSA) recorded shrimp as a famous marine and fishery commodity for exports in January to April 2021 (CSA, 2021). The growth of shrimp consumption showed a positive trend during the Covid-19 pandemic. Not only domestically, but there are also increases in export. The value of Indonesia shrimp exports in 2021 reached US\$2.23 billion which amounted to around 39% of the total export value of fishery products. This value has increased 9.3% compared to 2020 (CSA, 2021). The main export destination of Indonesian shrimp is The United States with US\$1.6 billion (71.6% of total shrimp exports), followed by Japan, the European Union (27 countries) and China. In 2022, Ministry of Marine Affairs and Fisheries (MMAF) has set a target for Indonesian shrimp production to grow 20-30%.

Disease is a major threat to shrimp farming because it can cause mass death of shrimp and substantial economic losses (Anderson et al., 2019). One of the common diseases that attack shrimp, among others, is from the Vibriosis group. This disease can cause shrimp mortality and high economic losses. Various species of *Vibrio* have been reported to cause disease in Penaeid shrimp, namely *Vibrio harveyi*, *V. parahaemolyticus*, *V. vulnificus*, *V. damsela*, *V. campbellii*, and *V. splendidus* (Vandenbergh et al., 1998). Bacteria *V. parahaemolyticus* is the cause of Acute Hepatopancreatic Necrosis Disease (AHPND). The incidence of this disease was first reported in China and Vietnam in 2010, Malaysia in 2011, and Thailand in 2012. This disease is characterized by pale atrophic hepatopancreas, soft shells skin, intermittent bowel contents or no intestinal contents, black spots on the hepatopancreas and a spongy hepatopancreas consistency. Clinical symptoms and mortality began to appear 10 days after stocking in the pond, then the dying shrimp gathered at the bottom of the pond (NACA, 2014).

Several studies reported that cinnamaldehyde functions as an anti-microbial (Vasconcelos, 2018). In addition, cinnamaldehyde has also been shown to effectively inhibit the growth of various types of microorganisms such as bacteria, fungi, and several studies

have also reported that it can inhibit the production of toxins released by microorganisms (Friedman, 2017; Firmino, 2018). Trans-cinnamaldehyde and its derivatives have anti-inflammatory roles. Nuclear factor- κ B is a transcription factor that regulates the expression of inflammatory and immune genes. Cinnamaldehyde and its derivatives also act as antifungals. Several studies have shown that cinnamaldehyde has antifungal activity against *Aspergillus flavus* (Sun et al., 2016; Li et al., 2015), *A. fumigatus*, and *Trichophyton rubrum* (Khan and Ahmad, 2011). In addition, trans-cinnamaldehyde also has antibacterial properties against both Gram-positive and Gram-negative bacteria, including *Escherichia coli*, *Bacillus subtilis* (Calo et al., 2015), *Vibrio* spp., and *Streptococcus pyogenes* (Nazzaro et al., 2013). Another study also reported that the inhibition of bacteria by cinnamaldehyde could be seen in the morphology of the bacterial biofilm using confocal laser scanning microscopy (CLSM) and scanning electron microscope (SEM). Both techniques were used by Li et al. (2018) to observe the effect of adding cinnamaldehyde on the formation of *Pseudomonas fluorescens* biofilms. While given cinnamaldehyde, there was a decrease in the biofilm which was characterized by bacterial aggregation and bacterial morphology damage. Both Gram positive and negative bacteria can perform quorum sensing (QS) such as bioluminescence, enzyme secretion production of virulence factors and biofilms. Brackman et al. (2008) also reported that cinnamaldehyde and its derivatives can inhibit the bioluminescence of *Vibrio harveyi* BB170 for around 62-65%.

Some groups of green *Vibrio* bacteria are mostly pathogenic for some shrimp farming, while a few yellow *Vibrio* bacteria become beneficial bacteria in the water and the digestive tract of shrimp. Therefore, it is very important to evaluate the use of cinnamaldehyde on the dynamics of *Vibrio* bacteria and the health status of shrimp during rearing. However, all previous studies were focused on the cinnamaldehyde as antibacterial, anti-inflammatory and focused more on the incidence of the disease. Meanwhile, nowadays there has been no research studying the effect of using cinnamaldehyde given through feed for Pacific white shrimp on the dynamic of *Vibrio* green and yellow bacteria and their correlation with health status of Pacific white shrimp. The objectives of the present study are to evaluate the effect of administration of cinnamaldehyde through feed on the dynamics of *Vibrio* bacteria and the health status of Pacific white shrimp.

2. Materials and Methods

2.1 Material

The materials used include cinnamaldehyde (C₉H₈O) GRM3277-Himedia 500ML, medium thiosulphate citrate bile-salt sucrose (TCBS, Himedia M961-500G), distilled water, incubator, shaker, microbiology equipments (spiritus, alcohol 70%, alcohol 96%, bunsen burner), disposable petri dish, medium bacto agar, and autoclave. The cinnamaldehyde was purchased from PT. Intralab Ekatama (Bogor, West Java, Indonesia). The cinnamaldehyde supplied by the company is a clear colourless to yellow liquid having strong odor of cinnamon with density 1.045-1.055 g/mL and 98% purity.

2.1.1 Ethical approval

All animal experimental and rearing procedures were handled complied with the animal welfare under the national accreditation no. SNI 7311:2009 of Republic of Indonesia.

2.2 Method

This research was conducted in June-December 2021 at the Field Laboratory, College of Vocational Studies, IPB. The status of Pacific white shrimp health analysis was carried out at the Fish Health Laboratory, Department of Aquaculture, IPB. Analysis of *Vibrio* bacteria and water quality was carried out on the Field Laboratory, Fisheries BAK, College of Vocational Studies, IPB.

2.2.1 Preparation of containers, maintenance media and materials

Preparation of the container includes cleaning the aquarium, drying, filling with water, setting aeration, and checking water quality before use. The aquariums used were 60 x 40 x 40 cm³ as many as 15 units equipped with a heater and 96 L of water volume each. The maintenance medium used seawater with a salinity of about 28-31 ppt and was given chlorine 30 ppm for the disinfection process. The shrimp used is Pacific white shrimp (*L. vannamei*) from PT. SyAqua Indonesia, Anyer, Banten and reared from post-larvae size (PL) 12 to ± 2 g/shrimp. The number of shrimps reared in each aquarium is 30 shrimps. The materials used were cinnamaldehyde. For purpose of bacteria determination (*Vibrio* green and yellow bacteria), TCBS medium) (89.08 g in 1 L sterile distilled water) was used.

2.2.2 Rearing of shrimp

The maintenance of Pacific white shrimp was carried out for 30 days. Pacific white shrimp were given pellet feed according to the treatment with the fre-

quency of feeding 4 times a day, at 07.00 am, 11.00 am, 03.00 pm, and 07.00 pm (GMT+7). Water exchange during maintenance was done every 2-3 days as much as 5-10%. The maintenance of water quality was maintained according to the standard of SNI-7311-2009, including temperature 29-30°C, pH 7.0-8.3, salinity 28-31 ppt, and TAN <0.05 ppm.

2.2.3 Experimental design

This study used completely randomized design (CRD) with 4 treatments and 3 replications, namely K (feeding without cinnamaldehyde), A (feeding containing cinnamaldehyde dose of 0.05%), B (feeding containing cinnamaldehyde dose of 0.1%), and C (feeding containing cinnamaldehyde dose 0.2%). The cinnamaldehyde was dissolved in distilled water and tween 80, according to the treatment dose i.e 0.05% is 0.05 mL cinnamaldehyde in 100 g feed, then mixed in the feed.

2.2.4 Biochemical test

The samples used were Pacific white shrimp intestine and hepatopancreas as much as 1-2 g in each and 200 mL of shrimp rearing water. Parameter measurements were carried out at the beginning of maintenance and at the end of maintenance. Parameters measured included total bacteria (green and yellow *Vibrio*) in the water, intestine, and hepatopancreas using spread plate method. Each organ of the intestine and hepatopancreas were crushed and homogenized in 9 mL sterile PBS (phosphate buffer saline; 0.8 g NaCl, 0.02 g KH₂PO₄, 0.15 g Na₂HPO₄, 0.02 g KCL, 100 mL distilled water). The serial dilution was then made (1:10). The suspension 50 µL was spread onto TCBS medium and incubated in incubator at 29°C for 18-24 hours. The immune responses were measured at the end of treatment. The observed parameter included total haemocyte count (THC), phenoloxidase activity (PO), and respiratory burst activity (RB). The procedure for determination of THC followed the method described by [Nurhayati et al. \(2015\)](#), while the procedure for PO and RB assay followed the method described by [Huynh et al. \(2011\)](#), respectively.

2.3 Analysis Data

All data was tabulated in Microsoft Excel 2016 and all statistical analysis was conducted in the SPSS v.22.0 software (SPSS Inc., USA) by one-way ANOVA followed by the Duncan Multiple Range Test (DMRT) (P=0.05).

3. Results and Discussion

3.1 Results

The following are the results of measurements of total bacteria consisting of total green *Vibrio* and yellow *Vibrio* bacteria both in water, intestine, and hepatopancreas of Pacific white shrimp (Table 1). Green *Vibrio* bacteria at the beginning of maintenance were observed in water, intestine and hepatopancreas. The highest CFU of green *Vibrio* bacteria in the water at the beginning of maintenance was produced in treatment K 0.026×10^5 CFU/mL and the lowest in treatment B 0.021×10^5 CFU/mL, but significantly different ($P>0.05$) with other treatments. The highest CFU of green *Vibrio* bacteria in the intestine at the beginning of maintenance was produced in treatment K 64.4×10^5 CFU/g and the lowest in treatment A 64.10×10^5 CFU/g, but not significantly different ($P>0.05$) with other treatments. The highest CFU of green *Vibrio* bacteria in hepatopancreas at the

beginning of maintenance was produced in treatment A 4.77×10^5 CFU/g and the lowest in treatment K 4.27×10^5 CFU/g, but not significantly different ($P>0.05$) with other treatments. Generally, green *Vibrio* bacteria at the beginning of maintenance are mostly found in the intestine, then in hepatopancreas, and then lastly in the water. Moreover, treatment K at the beginning of maintenance produces more green *Vibrio* bacteria compared to other treatments, but not significantly different ($P>0.05$) with other treatments.

Yellow *Vibrio* bacteria at the beginning of maintenance were observed in water, intestine and hepatopancreas. The highest CFU of yellow *Vibrio* bacteria in the water at the beginning of maintenance was produced in treatment K 0.075×10^5 CFU/mL and the lowest in treatment A 0.058×10^5 CFU/mL, but not significantly different ($P>0.05$) with other treatments. The highest CFU

Table 1. Total green and yellow *Vibrio* bacteria in water, intestine, and hepatopancreas at the beginning of Pacific white shrimp rearing

Treatments	Green <i>Vibrio</i>			Yellow <i>Vibrio</i>		
	Water (10^5 CFU/mL)	Intestine (10^5 CFU/g)	Hepatopancreas (10^5 CFU/g)	Water (10^5 CFU/mL)	Intestine (10^5 CFU/g)	Hepatopancreas (10^5 CFU/g)
K	0.026±0.04 ^a	64.400±0.40 ^a	4.270±0.46 ^a	0.075±1.20 ^a	1.380±0.18 ^a	0.034±0.01 ^a
A	0.024±1.43 ^a	64.100±0.10 ^a	4.770±0.77 ^a	0.058±0.57 ^a	0.975±0.14 ^a	0.033±0.01 ^a
B	0.021±1.16 ^a	63.400±0.30 ^a	4.400±0.40 ^a	0.067±1.82 ^a	1.213±0.45 ^a	0.038±0.01 ^a
C	0.023±0.10 ^a	64.200±0.20 ^a	4.700±0.10 ^a	0.066±1.39 ^a	1.150±0.23 ^a	0.041±0.01 ^a

Description: K (feeding without cinnamaldehyde), A (feeding containing cinnamaldehyde dose of 0.05%), B (feeding containing cinnamaldehyde dose of 0.1%), and C (feeding containing cinnamaldehyde dose 0.2%). Different superscript letters show the significance between treatments.

Table 2. Total green and yellow *Vibrio* bacteria in water, intestine, and hepatopancreas at the end of Pacific white shrimp rearing

Treatments	Green <i>Vibrio</i>			Yellow <i>Vibrio</i>		
	Water (10^5 CFU/mL)	Intestine (10^5 CFU/g)	Hepatopancreas (10^5 CFU/g)	Water (10^5 CFU/mL)	Intestine (10^5 CFU/g)	Hepatopancreas (10^4 CFU/g)
K	0.022±0.80 ^a	5.87±0.41 ^a	0.138±1.20 ^a	0.061±1.20 ^a	2.49±0.40 ^a	0.235±0.94 ^c
A	0.030±1.43 ^a	5.03±2.07 ^a	0.023±3.55 ^a	0.057±0.54 ^a	2.18±0.00 ^a	0.164±0.19 ^b
B	0.027±1.16 ^a	2.13±0.93 ^a	0.022±0.23 ^a	0.056±0.00 ^a	2.58±0.00 ^a	0.468±0.00 ^d
C	0.022±0.10 ^a	2.67±0.04 ^a	0.004±0.57 ^a	0.068±1.34 ^a	7.97±0.01 ^b	0.012±0.19 ^a

Description: K (feeding without cinnamaldehyde), A (feeding containing cinnamaldehyde dose of 0.05%), B (feeding containing cinnamaldehyde dose of 0.1%), and C (feeding containing cinnamaldehyde dose 0.2%). Different superscript letters show the significance between treatments.

of yellow Vibrio bacteria in the intestine at the beginning of maintenance was produced in treatment K 1.38×10^5 CFU/g and the lowest in treatment A 0.975×10^5 CFU/g, but not significantly different ($P>0.05$) with other treatments. The highest CFU of yellow bacteria in hepatopancreas at the beginning of maintenance was produced in treatment C 0.04×10^5 CFU/g and the lowest in treatment A 0.033×10^5 CFU/g, but not significantly different ($P>0.05$) with other treatments. Generally, yellow Vibrio bacteria at the beginning of maintenance are mostly found in the intestine, then in hepatopancreas, and in water. Moreover, treatment K produces the highest of yellow Vibrio bacteria in intestine and water, as well as treatment C produces more yellow Vibrio bacteria in hepatopancreas than other treatments. At the beginning of maintenance, generally treatment K produces the highest number of green and yellow Vibrio bacteria both in intestine and water. Meanwhile, treatment A produces the highest of green Vibrio bacteria and treatment C produces the highest of yellow Vibrio bacteria in hepatopancreas. Furthermore, the following are the results of measurements of total green and yellow Vibrio bacteria in the intestine and hepatopancreas at the end of treatments (Table 2).

Green Vibrio bacteria at the end of treatment were observed in water, intestine, and hepatopancreas.

The highest CFU of green Vibrio bacteria in the water at the end of treatment was produced in treatment A 0.03×10^5 CFU/mL and the lowest in treatment K and C which is equal to 0.022×10^5 CFU/mL, but not significantly different ($P>0.05$) with other treatments. The highest CFU of green Vibrio bacteria in the intestine at the end of treatment was produced in treatment K 5.87×10^5 CFU/g and the lowest in treatment B 2.13×10^5 CFU/g, but not significantly different ($P>0.05$) with other treatments. The highest CFU of green Vibrio bacteria in hepatopancreas at the end of treatment was produced in treatment K 0.138×10^5 CFU/g and the lowest in treatment C 0.004×10^5 CFU/g, but not significantly different ($P>0.05$) with other treatments. Generally, green Vibrio bacteria at the end of treatment decrease in the amount in the water, intestine, and hepatopancreas. Green Vibrio bacteria at the end of maintenance are mostly found in the intestine, then in hepatopancreas, and then lastly in the water. Moreover, treatment K at the end of treatment produces more green Vibrio bacteria in the intestine and hepatopancreas and treatment A produce greener Vibrio bacteria in water.

Yellow Vibrio bacteria at the end of treatment were observed in water, intestine, and hepatopancreas. The highest CFU of yellow Vibrio bacteria in the water at the end of treatment was produced in treatment

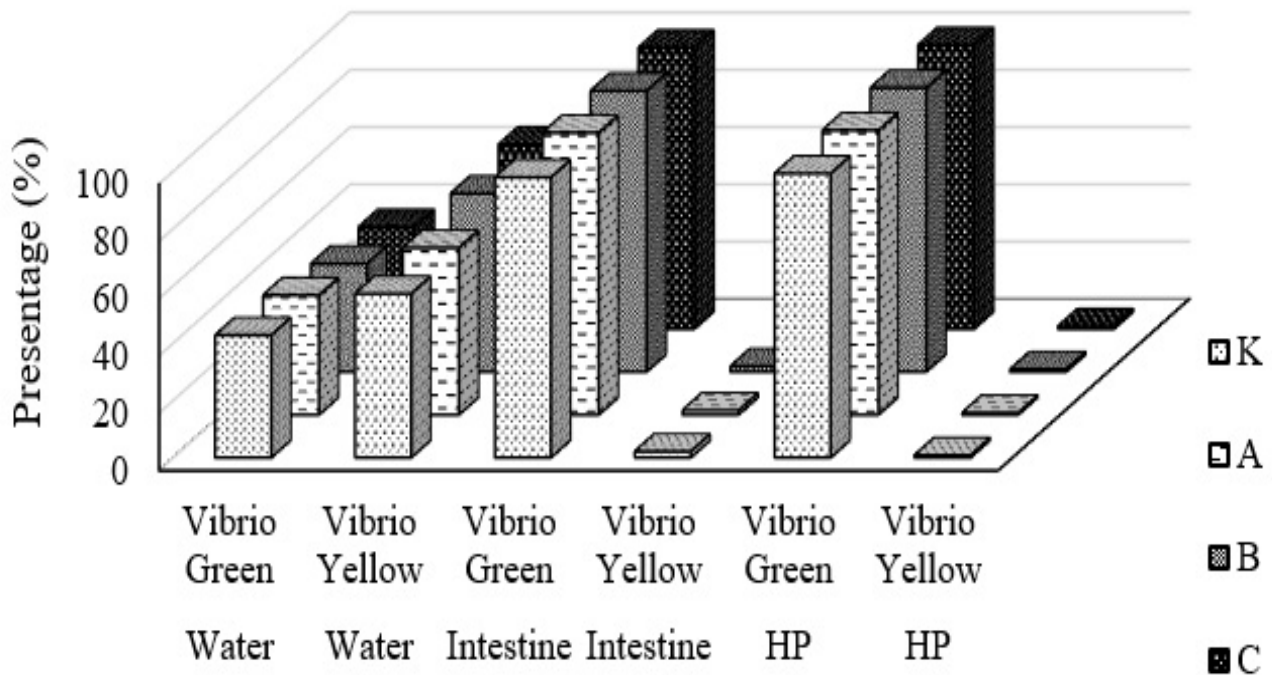


Figure 1. Percentage of green and yellow Vibrio bacteria in water, intestine, and hepatopancreas (HP) at the beginning of maintenance.

Description: K (feeding without cinnamaldehyde), A (feeding containing cinnamaldehyde dose of 0.05%), B (feeding containing cinnamaldehyde dose of 0.1%), and C (feeding containing cinnamaldehyde dose 0.2%).

C 0.068×10^5 CFU/mL and the lowest in treatment B 0.056×10^5 CFU/mL, but not significantly different ($P>0.05$) with other treatments. The highest CFU of yellow Vibrio bacteria in the intestine at the end of treatment was produced in treatment C 7.97×10^5 CFU/g and significantly different ($P<0.05$) with other treatments, while the lowest in treatment A 2.18×10^5 CFU/g. Inter-treatment K, A, and B is not significantly different ($P>0.05$). The highest CFU of yellow Vibrio bacteria in the hepatopancreas at the end of treatment was produced in treatment B 0.468×10^5 CFU/g and the lowest in treatment C 0.012×10^5 CFU/g, as well as significantly different ($P<0.05$) with other treatments. Generally, yellow Vibrio bacteria at the end of treatment decrease in the amount in water, while in the intestine and hepatopancreas increase after the administration of cinnamaldehyde. Yellow Vibrio bacteria at the end of treatment are mostly found in the intestine, then in hepatopancreas, and then lastly in the water. Moreover, treatment C produces more yellow Vibrio bacteria in the intestine and water, as well as treatment B produces more yellow Vibrio bacteria in hepatopancreas. The following is a graph of the percentage of green and yellow Vibrio bacteria in water, intestine, and hepatopancreas at the beginning of maintenance (Figure 1).

At the beginning of maintenance, the composition between green and yellow Vibrio bacteria in the

water was as following: the highest percentage of green Vibrio bacteria was produced in treatment K 42.78% and the lowest was in treatment C 35.61%, but not significantly different ($P>0.05$) to other treatments. Meanwhile, the highest percentage of yellow Vibrio bacteria was produced in treatment K 64.39% and the lowest in treatment K 57.22%, meanwhile there was no significant difference ($P>0.05$) compared to other treatments. In the intestine, the highest percentage of green Vibrio bacteria was produced in treatment A 98.48% and the lowest in treatment K 97.86%, but not significantly different ($P>0.05$) to other treatments. The highest percentage of yellow bacteria in intestine was obtained in treatment K 2.14% and the lowest in treatment A 1.52%, but not significantly different ($P>0.05$) to other treatments. In hepatopancreas, the highest percentage of green Vibrio bacteria was found in treatment A 99.32% and the lowest in treatment B and C 99.13%, but not significantly different ($P>0.05$) to other treatments. Meanwhile, the highest percentage of yellow Vibrio bacteria in treatment C 0.87% and the lowest in treatment A 0.68%, but not significantly different ($P>0.05$) to other treatments. The following is graph of percentage green and yellow Vibrio bacteria in the water, intestine and hepatopancreas at the end of treatment (Figure 2).

At the end of treatment, the composition of green and yellow bacteria in water, intestine and hepatopancreas

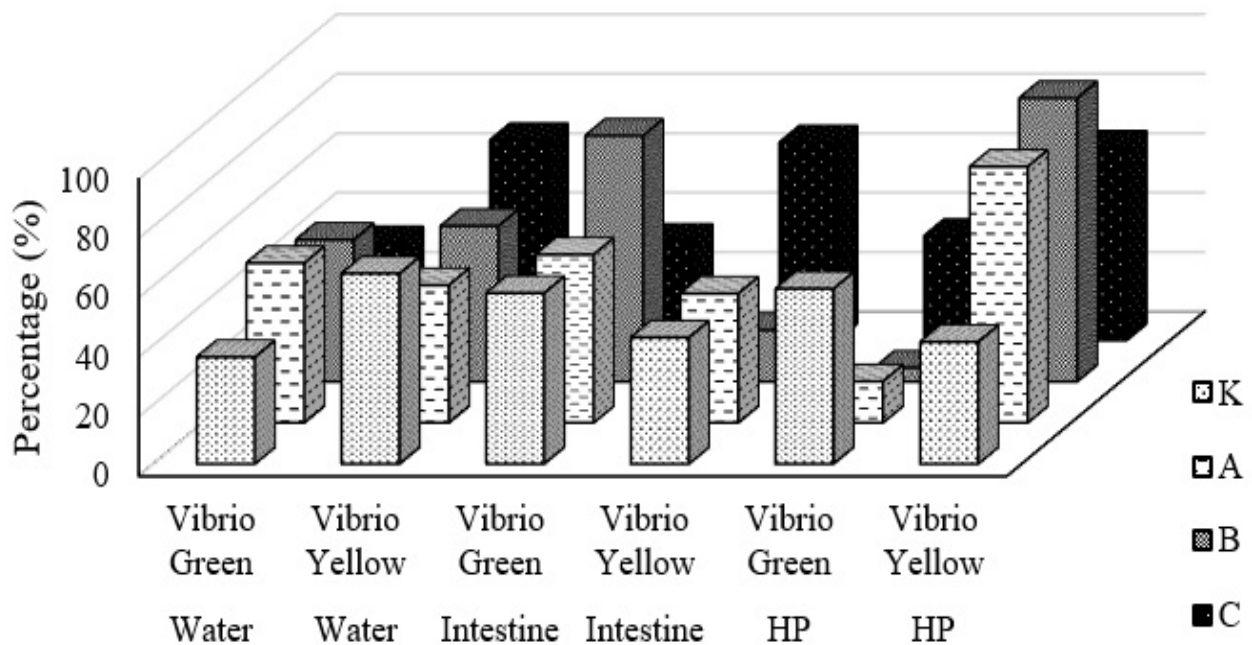


Figure 2. Percentage of green and yellow Vibrio bacteria in water, intestine, and hepatopancreas (HP) at the end of treatment.

Description: K (feeding without cinnamaldehyde), A (feeding containing cinnamaldehyde dose of 0.05%), B (feeding containing cinnamaldehyde dose of 0.1%), and C (feeding containing cinnamaldehyde dose of 0.2%).

changes. In water, the highest percentage of green *Vibrio* bacteria was found in treatment A 53.71% and significantly different ($P < 0.05$) with treatment C 32.65%. Meanwhile, treatment C was not significantly different ($P > 0.05$) with treatment K and B. Meanwhile, the highest percentage of yellow *Vibrio* bacteria was found in treatment C 67.35% and significantly different ($P < 0.05$) with treatment A 46.29%. Moreover, treatment C was not significantly different ($P > 0.05$) with treatment K and B. In intestine, the higher percentage of green *Vibrio* was obtained in treatment B 82.69% and A 64.79%, however it was not significantly different ($P > 0.05$) to treatment K. Meanwhile, the highest percentage of yellow *Vibrio* bacteria was found in treatment C 66.56% and significantly different ($P < 0.05$) to treatment K. The percentage of green and yellow *Vibrio* bacteria in hepatopancreas, the highest percentage of green *Vibrio* bacteria was found in treatment K 58.95% and the lowest in treatment B 4.66%, but not significantly different ($P > 0.05$) to other treatments. Meanwhile, the highest percentage of yellow *Vibrio* bacteria was found in treatment B 95.34% and the lowest was found in treatment K 41.05%, but not significantly different ($P > 0.05$) to other treatments

Table 3. Results of measurements of THC, RB, and PO at the end of rearing white shrimp

Treatments	THC (10^3 cells/mm ³)	RB (OD $\lambda=630$)	PO (OD $\lambda=490$)
K	2.277±2.13 ^a	0.282±0.05 ^a	0.078±0.005 ^a
A	4.587±3.84 ^a	0.206±0.03 ^a	0.067±0.011 ^a
B	2.753±1.60 ^a	0.272±0.05 ^a	0.060±0.014 ^a
C	2.397±1.22 ^a	0.310±0.18 ^a	0.069±0.015 ^a

Description: K (feeding without cinnamaldehyde), A (feeding containing cinnamaldehyde dose of 0.05%), B (feeding containing cinnamaldehyde dose of 0.1%), and C (feeding containing cinnamaldehyde dose 0.2%). Different superscript letters show the significance between treatments.

Generally, after administration of cinnamaldehyde, the percentage of green *Vibrio* bacteria in the water tends to increase in treatment A and decrease in other treatments. The percentage of yellow *Vibrio* bacteria in the water tends to increase, especially in treatment K, B, and C. The percentage of green *Vibrio* bacteria in the intestine tends to decrease in all treatments, while the percentage of yellow *Vibrio* bacteria tends to increase, especially in treatment C. The percentage of green *Vibrio* bacteria in the hepatopancreas tends to decrease in all treatments and the percentage of yellow *Vibrio* bacteria tends to increase. Generally, the administration of

cinnamaldehyde had the effect of reducing the percentage of green *Vibrio* bacteria in the hepatopancreas and intestine, as well as increasing the percentage of yellow *Vibrio* bacteria. Below is the health status of Pacific white shrimp with several parameters including total haemocyte count (THC), respiratory burst (RB) and phenol oxidase (PO) (Table 3).

The highest THC value was found in treatment A 4.58×10^3 cells/mm³ and was not significantly different ($P > 0.05$) compared to other treatments. Meanwhile, the highest RB and PO values were found in treatment C, 0.310 and 0.069; it was not significantly different ($P > 0.05$) to other treatments.

3.2 Discussion

The microbiota in the digestive tract plays an important role in the growth and development of the host body by maintaining balance and resistance to pathogens. *Vibrio* is an opportunistic pathogen and is a normal flora commonly found in the digestive organs of fish. The results showed that the addition of cinnamaldehyde in the feed affected the population dynamics of the normal flora of the digestive tract of shrimp in the water, intestine, and hepatopancreas. Several studies report that the microbiota of the digestive tract of aquatic animal is strongly influenced by the composition of the diet, the food trophic level, and the quality of the rearing water (Cornejo-Granados *et al.*, 2018; Landsman *et al.*, 2019). The results of this study showed that the administration of cinnamaldehyde influenced the dynamics of *Vibrio* in water, intestine, and hepatopancreas. At the beginning of maintenance, generally, green and yellow *Vibrio* bacteria are mostly found in the intestine, then in hepatopancreas, and lastly in the water. Moreover, treatment K produces more green and yellow *Vibrio* bacteria in intestine and water, as well as treatment C produces more yellow *Vibrio* bacteria in hepatopancreas than other treatments. At the end of treatment, the composition of green and yellow bacteria in water, intestine and hepatopancreas changes. In this study, the results showed that after the administration of cinnamaldehyde there was the effect of reducing the percentage of green *Vibrio* bacteria in hepatopancreas and intestine, as well as increasing the percentage of yellow *Vibrio* bacteria especially in treatment C (dose 0.2% of cinnamaldehyde) compared to control.

Phylum Proteobacteria, Class Gamma-proteobacteria, *Vibrio* genus is commonly found in the water column and in aquatic animals including fish and crustaceans. Some *Vibrio* species are pathogenic and infectious leading to the disease "vibriosis". Opportunistic

Vibrio species such as *V. harveyi*, *V. parahaemolyticus*, and *V. vulnificus* are now the main agents of this disease (Valente and Wan, 2021). The majority of the *Vibrio* group of bacteria are saprophytic to pathogenic under certain environmental conditions and bacterial density (Kadriah et al., 2011). Based on that research, *Vibrio* bacteria have the ability to fluoresce indicate the presence of a virulent gene that determines the pathogenic nature of these bacteria. Pang et al. (2005) and Marlina et al. (2008) reported that there is an indication of the presence of the *toxR* gene in *V. cholerae* as a positive transcriptional regulator of *ctx* gene that encodes the toxin in *V. cholerae*. In addition, this gene was also found in *V. parahaemolyticus*. The *toxR* gene plays an important role in the regulation of the *ctx* toxin gene and others to produce a toxin in the form of haemolysin. This haemolysin is the main factor for *Vibrio* bacteria in the process of pathogenicity. Haemolysin is an exotoxin that is responsible for the absorption of haemocyte membranes or the process of haemolysis of blood cells. The gene encoding haemolysin has been reported to be found in several bacteria of the *Vibrio* genus. In crustaceans, *Vibrio* species can colonize or accumulate in hepatopancreas, intestine, hemolymph, gills, somatic muscle, and so on (Bi et al., 2016). The hepatopancreas can be examined for *Vibrio* group detection and for diagnosis of AHPND, analysis of the digestive tract is recommended because there is a high density of pathogenic *Vibrio* (Soto-Rodriguez et al., 2015).

In this study, treatment with cinnamaldehyde tends to decrease the density and percentage of green *Vibrio* bacteria in hepatopancreas and intestine compared to control. Meanwhile, the density and percentage of yellow *Vibrio* bacteria increased after the administration of cinnamaldehyde especially in treatment C (dose 0.2% of cinnamaldehyde) compared to control. Laboratory isolation of *Vibrio* from hepatopancreas and intestine used TCBS agar culture medium. This medium causes sucrose-positive strains to form yellow colonies, while sucrose-negative strains form green colonies (Kaysner et al., 2019). Most pathogenic *Vibrio* found in crustaceans are non-sucrose species and from this reason, sucrose can be added to the water column to create unfavorable conditions for *Vibrio*. TCBS medium can be used to observe *Vibrio* bioluminescence such as among green *Vibrio* bacteria *V. harveyi*, *V. campbellii*, *V. cholerae*, and *V. splendidus* (Gomez-Gil and Roque, 2006).

Modified TCBS medium can also be used to improve isolation of *Vibrio* and produced higher colony plate counts. Chromogenic *Vibrio* (CV) agar can be

used, and it is a suitable medium for detection, selection, differentiation, and isolation of *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus* and *V. cholerae*. Generally, in shrimp culture green bacteria *V. parahaemolyticus* are known to be pathogens that cause vibriosis and acute hepatopancreatic necrosis disease (AHPND), as well as causing huge losses for shrimp farmers. Meanwhile, yellow *Vibrio* bacteria are known to rarely cause disease, even some of them such as *V. alginolyticus* bacteria is one of the beneficial bacteria (probiotics) that can increase the growth and immune response of shrimp (Widanarni et al., 2008a, 2008b, 2010, 2012a, 2012b; Nurhayati et al., 2015; Oktaviana et al., 2014). This is also supported by Sarida and Harpeni (2010) reported that the presence of yellow *Vibrio* bacteria (CP1) in intestine and hepatopancreas as beneficial bacteria can reduce the green *Vibrio* bacteria such as *V. harveyi* MR5339 and resulted the highest survival rate 90% in Pacific white shrimp compared to treatment with the highest amount of *V. harveyi* MR5339. The previous research supports the results obtained in this study where cinnamaldehyde with dose 0.2% can reduce the number of green *Vibrio* bacteria detected, especially in the intestine and hepatopancreas.

Cinnamaldehyde is a bioactive compound that acts as the main component of cinnamon bark extract. This compound has been proven to be anti-bacterial, anti-fungal, and anti-fungi activity through usage of bark, oils, and pure cinnamaldehyde (Suresh et al., 1992; Bilgrami et al., 1992; Pacheco et al., 1993; Doyle and Stephens, 2019). Trans-cinnamaldehyde and its derivatives also have anti-inflammatory roles. Nuclear factor- κ B is a transcription factor that regulates the expression of inflammatory and immune genes. Cinnamaldehyde and its derivatives also act as anti-fungal. Several studies have shown that cinnamaldehyde has anti-fungal activity against *Aspergillus flavus* (Sun et al., 2016; Li et al., 2015), *A. fumigatus* and *Trichophyton rubrum* (Khan and Ahmad, 2011). In addition, trans-cinnamaldehyde also has anti-bacterial properties against both Gram-positive and Gram-negative bacteria including *E. coli*, *B. subtilis* (Calo et al., 2015), *Vibrio* spp. and *S. pneumoniae* (Nazaro et al., 2013). The green and yellow *Vibrio* bacteria were mostly found in the intestine, because intestine has role of bacterial colonization. Meanwhile, in this study, the administration of cinnamaldehyde decreased the number of green *Vibrio* bacteria. The present study found that there is a possibility of cinnamaldehyde as an anti-bacterial especially for green *Vibrio* bacteria.

The administration of cinnamaldehyde does not only influence the dynamics of *Vibrio*, but also the

health status of Pacific white shrimp. Although there was no significant difference ($P > 0.05$) of Pacific white shrimp health status, the treatment with cinnamaldehyde resulted in higher immune responses compared to control. Shrimp has an open circulation where it could distribute haemocytes in both vascular system and tissues (Maftuch *et al.*, 2013). Humoral defense mechanisms in shrimp body can be measured with total haemocyte count (THC), phenol-oxidase (PO), and respiratory burst (RB) activity has been proposed as a procedure to evaluate the immune responses of shrimp (Shahverdi *et al.*, 2007; Inouye *et al.*, 2001). The activation of pro-PO system by microbial cell wall components such as lipopolysaccharides (LPS) from Gram-negative bacteria has been demonstrated in several crustacean species (Lee *et al.*, 2001). Phenol-oxidase (PO) activity demonstrated the enhanced capability of the shrimp in distinguishing foreign particles, meanwhile RB activity defined the release of foreign particles by phagocytes involving degradative enzyme released to phagosome (oxygen-dependent killing) (Wongsasak *et al.*, 2014). This study showed that the administration of cinnamaldehyde could increase immune responses (THC, DO, and RB activity) better than control. This result is similar to Lai *et al.* (2005) where the addition of leaf extract of *Cinnamomum kanehirae* can increase the activity of the pro-PO system of penaeid shrimp. In addition, Yeh *et al.* (2009) reported that *Cinnamomum kanehirae* extract can enhance the immune system and disease resistance of *V. alginolyticus*. It may have a growth-inhibiting effect against specific bacteria from water, intestine, and hepatopancreas.

In this study, the administration of cinnamaldehyde can decrease the number of green *Vibrio* bacteria; there is an indication that the decrease of green *Vibrio* bacteria can improve the immune system of Pacific white shrimp. Moreover, increased colonization of yellow *Vibrio* bacteria can also indicate a trigger in the hepatopancreas and intestine to produce immune cells as a defense against pathogens both humorally and cellularly. In shrimp culture, majority *Vibrio* group with the green colony is opportunistic pathogen that can cause the vibriosis disease where it accumulates in hepatopancreas and intestine, meanwhile yellow *Vibrio* bacteria is known as beneficial bacteria. The results of this study indicate that cinnamaldehyde has an anti-bacterial role, especially against green *Vibrio* bacteria, but we found indications that yellow *Vibrio* bacteria can colonize properly in the intestine and hepatopancreas after the administration of cinnamaldehyde. This is shown in the treatment C of 0.2% cinnamaldehyde. We examined that it is a possibility that yellow *Vibrio* bacteria can utilize

compound from cinnamaldehyde for its growth. This is supported by Zhu *et al.* (2022) who stated that dietary compounds in the digestive tract interact directly with the intestinal epithelium and the commensal gut microbiota. It is possible to induce either direct immunomodulatory effects on mucosal immune cells or indirect modulate immune responses due to independent prebiotic modification of the gut microbiota and production of microbial-derived metabolites. Zhu *et al.* (2022) also reported that cinnamaldehyde intake altered the composition of the gut microbiota thus raising the question as to whether a prebiotic effect from cinnamaldehyde may be responsible for the immunomodulatory activity. Several research also reported that the prebiotic honey has a similar mode of action with cinnamaldehyde as anti-bacterial for some bacteria. The administration of prebiotic honey can fight against *V. parahaemolyticus* infection in shrimp (Fuandila *et al.*, 2019) and it can improve the number of probiotic bacteria as well as alter the microbiota composition in the digestive tract of Nile tilapia. We examined the cinnamaldehyde with the content of 98% pure cinnamaldehyde with dose 0.2% which resulted the best treatment in this study.

4. Conclusion

The administration of cinnamaldehyde to Pacific white shrimp in this study can reduce the number of green *Vibrio* bacteria both in the intestine and hepatopancreas as shown in treatments C (dose 0.2% of cinnamaldehyde) and B (dose 0.1% of cinnamaldehyde). This decrease was correlated with higher yellow *Vibrio* bacteria colonization in treatments C and B. In addition, the administration of cinnamaldehyde also affected the higher percentage of yellow *Vibrio* bacteria in treatment C and B compared to control (K). The highest immune responses of THC and RB were produced in treatment A and C compared to control (K). The decrease in the number of green *Vibrio* bacteria and the increased colonization of yellow *Vibrio* bacteria in this study was correlated with immune responses. The best treatment in this study is treatment C.

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Authors' Contributions

All authors have contributed to final manuscript. The contribution of each author as follow, Dian (DER); collected the data, drafted the manuscript and designed the table as well as graph. Andri (AHA) and Arif (MAM); devised the main conceptual ideas and searching the related journals, Dinamella (DMW); critical revision of the article. All authors discussed the results and contributed to the final manuscript.

Conflict of Interest

The authors declare that they have no competing interests.

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