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Inhibition of *Vibrio parahaemolyticus* infection in whiteleg shrimp reared in a biofloc system with different volumes

Penghambatan infeksi bakteri *Vibrio parahaemolyticus* pada udang vaname yang dipelihara pada sistem bioflok dengan volume berbeda

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ABSTRACT

This study aimed to evaluate the inhibition of *V. parahaemolyticus* infection in the whiteleg shrimp (*Penaeus vannamei*) reared in different volumes of biofloc system. Post-larval shrimp with an average body weight of 0.28 ± 0.01 g were reared in 15 aquariums with working volume of 33.3 L and stocking density of 3 shrimp per liter. The shrimp were reared for 21 days in a biofloc system (C:N ratio of 10) with biofloc volume of 5, 10, and 15 mL/L, and challenged with *V. parahaemolyticus* at a density of 10^3 CFU/mL initially. The positive control treatment involved shrimp reared without biofloc and challenged, while the negative control treatment involved shrimp reared without biofloc and without challenged. The shrimp was fed with commercial feed while the protein content of 39-40% four times a day. The results showed that the presumptive *Vibrio* count (PVC) and the population of *V. parahaemolyticus* in the biofloc treatments were significantly lower than the positive control ($p<0.05$). Furthermore, the biofloc treatment with a volume of 15 mL/L demonstrated the best results compared to other treatments in decreasing PVC and *V. parahaemolyticus* population as evidenced by the immune response, survival rate, and growth performance of whiteleg shrimp.

Keywords: biofloc, immune response, *Penaeus vannamei*, *Vibrio parahaemolyticus*

ABSTRAK

Penelitian ini bertujuan untuk mengevaluasi penghambatan infeksi *V. parahaemolyticus* pada udang vaname (*Penaeus vannamei*) yang dipelihara dalam sistem bioflok dengan volume yang berbeda. *Post-larvae* udang dengan bobot tubuh rata-rata 0,28 ± 0,01 g dipelihara dalam 15 akuarium dengan volume kerja 33.3 L dan padat tebar 3 udang per liter. Udang dipelihara selama 21 hari dalam sistem bioflok (rasio C:N 10) dengan volume bioflok 5, 10, dan 15 mL/L, dan diuji tantang dengan *V. parahaemolyticus* pada kepadatan awal 10^3 CFU/mL. Perlakuan kontrol positif melibatkan udang yang dipelihara tanpa bioflok dan diuji tantang, sementara perlakuan kontrol negatif melibatkan udang yang dipelihara tanpa bioflok dan tanpa diuji tantang. Udang diberi pakan komersial dengan kandungan protein 39-40% empat kali sehari. Hasilnya menunjukkan bahwa *presumptive Vibrio count* (PVC) dan populasi *V. parahaemolyticus* dalam perlakuan bioflok signifikan lebih rendah daripada kontrol positif ($p<0,05$). Selain itu, perlakuan bioflok dengan volume 15 mL/L menunjukkan hasil terbaik dibandingkan dengan perlakuan lain dalam menurunkan PVC dan populasi *V. parahaemolyticus* sebagaimana dibuktikan oleh respons imun, tingkat kelangsungan hidup, dan kinerja pertumbuhan udang vaname.

Kata kunci: bioflok, *Penaeus vanname*, respons imun, *Vibrio parahaemolyticus*

INTRODUCTION

Penaeus vannamei, commonly known as Pacific white shrimp, represents a key commodity in aquaculture, with global production reaching 5.81 million tons in 2020 (FAO, 2022). Production is projected to increase in 2022, with Indonesia emerging as a major producer (FAO, 2023). Data from the Ministry of Marine Affairs and Fisheries (KKP, 2020) indicates that Indonesia's shrimp production was valued at 860,000 tons, contributing approximately 2 billion USD to global exports in 2019 (FAO, 2022). This represents the highest value in Indonesia's aquaculture sector, followed by species such as Nile tilapia and catfish. The expansion of *P. vannamei* farming remains promising due to the extensive target markets including Japan, the United States, the United Kingdom, and other shrimp-importing countries (FAO, 2020).

Despite the generally increasing production trend in recent years, white shrimp farming is still constrained by disease outbreaks, particularly bacterial diseases caused by the genus *Vibrio*, commonly known as vibriosis. *Vibrio parahaemolyticus* is a significant pathogen affecting white shrimp, causing various abnormalities such as gill necrosis, lethargy, loss of appetite, and in acute cases, up to 100% mortality (Abdel-Latif *et al*., 2022). Recent years have seen particular global attention on *V. parahaemolyticus* strains with PirA and PirB toxins, responsible for acute hepatopancreatic necrosis disease (AHPND). The first AHPND outbreak occurred in China (2009), with rapid spread to countries including Vietnam (2010), Malaysia (2011), Thailand (2012), Mexico (2013), the Philippines (2015), and the United States (2016) (Kumar *et al*., 2021). In Indonesia, *V. parahaemolyticus* with AHPND (VpAHPND) has been identified in export samples and various regions including Serang, Banten, and Bangkalan, Madura (Han *et al*., 2020; Saputra *et al*., 2023; Suryana *et al*., 2023).

The pathogenicity of *V. parahaemolyticus* is regulated by quorum sensing (QS) mechanisms (Gode-Potratz & McCarter, 2011), which enable bacteria to communicate via signaling molecules (autoinducers) and synchronize gene expression, including those for virulence factors (Lu *et al*., 2019; Lin *et al*., 2022). Through QS, *V. parahaemolyticus* can produce cytotoxic compounds and molecules facilitating infection, influenced by population density in aquatic environments. One approach to mitigate *V. parahaemolyticus* infections is by enhancing shrimp immunity and controlling bacterial populations to inhibit QS (Gustilatov *et al*., 2023). This can be achieved through the application of biofloc technology in shrimp culture.

Biofloc technology utilizes and converts nitrogen from feed waste and metabolic byproducts into heterotrophic microorganism biomass by adding organic carbon sources. This process forms flocs of bacterial biomass and other microorganisms, known as biofloc (Khanjani *et al*., 2022). Besides improving environmental quality and growth parameters (Kumar *et al*., 2018), biofloc enhances the immune response of white shrimp (Ekasari *et al*., 2014; Panigrahi *et al*., 2018) and serves as a biocontrol for pathogenic bacteria by reducing QS activity and virulence factors (Panigrahi *et al*., 2018; Aguilera-Rivera *et al*., 2019; Widanarni *et al*., 2024). Aguilera-Rivera *et al*. (2014) reported that biofloc application can prevent outbreaks of opportunistic *Vibrio* bacteria in aquatic environments. Implementation of biofloc in shrimp farming has shown positive results, including higher survival rates when challenged with *V. parahaemolyticus* causing AHPND, compared to controls (Hostins *et al*., 2019; Kumar *et al*., 2020).

The findings of these studies suggest that biofloc technology can serve as an effective biocontrol agent and enhance shrimp immunity, positioning it as a viable alternative for managing *V. parahaemolyticus* infections in white shrimp culture. The volume of biofloc, influenced by different C ratios, exhibits varying characteristics and functions (Panigrahi *et al*., 2018), yet detailed information on the impact of different biofloc volumes on bacterial inhibition and pathogenicity remains scarce. Understanding the optimal protective effects of biofloc at specific volumes can help maintain microbial populations during shrimp farming. This study aims to evaluate the inhibition of *V. parahaemolyticus* infections in *P. vannamei* reared in biofloc systems with varying volumes.

MATERIALS AND METHODS

Time and place of study

This study was conducted from June to August 2022 at the Aquatic Organism Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, IPB University.

Experimental design

This research used a completely randomized design (CRD) consisting of five treatments and three replications (Table 1).

Study procedure

Preparation of containers and rearing media

The containers used for the adaptation process and initial maintenance of white shrimp seedlings are 200×100×40 cm^3 fiber tanks. Subsequently, the containers used for maintenance during the treatment period are 60×30×30 cm^3 glass aquariums, with a total of 15 units. The aquariums are thoroughly cleaned and filled with freshwater, then disinfected using chlorine at a dose of 30 µl/L for 24 hours. The aquariums are then rinsed with freshwater and dried. After that, the aquariums are filled with seawater with a salinity of 25 g/L up to a water volume of 33.3 L, and aeration is installed.

Biofloc preparation

The preparation of biofloc suspension for white shrimp maintenance comes from biofloc culture conducted in white shrimp cultivation tanks using molasses as the organic carbon source. During maintenance, molasses is directly added to the shrimp maintenance aquariums once a day, two hours after the morning feeding, with an estimated C/N ratio of 10 (Ekasari *et al*., 2010). The amount of carbon added to support the floc formation process by heterotrophic bacteria in each treatment follows the carbon requirement calculation scheme by De Schryver *et al*. (2008).

Preparation of test bacteria

V. parahaemolyticus used as the challenge test bacteria in this study were made resistant to the antibiotic rifampicin at 50 µg/mL (1 g rifampicin, 95 mL absolute ethanol, and 5 mL distilled water) as a marker on agar media.

Table 1. Experimental design for white shrimp maintenance in a biofloc system with different volumes for controlling *V. parahaemolyticus* infection.

Treatment	Annotation
K- (Negative control)	White shrimp reared without biofloc treatment and without challenge with *V. parahaemolyticus*
K+ (Positive control)	White shrimp reared without biofloc treatment and challenged with *V. parahaemolyticus*
B5	White shrimp reared in the biofloc system with volume of 5 mL/L dan challenged with *V. parahaemolyticus*
B10	White shrimp reared in the biofloc system with volume of 10 mL/L dan challenged with *V. parahaemolyticus*
B15	White shrimp reared in the biofloc system with volume of 15 mL/L dan challenged with *V. parahaemolyticus*

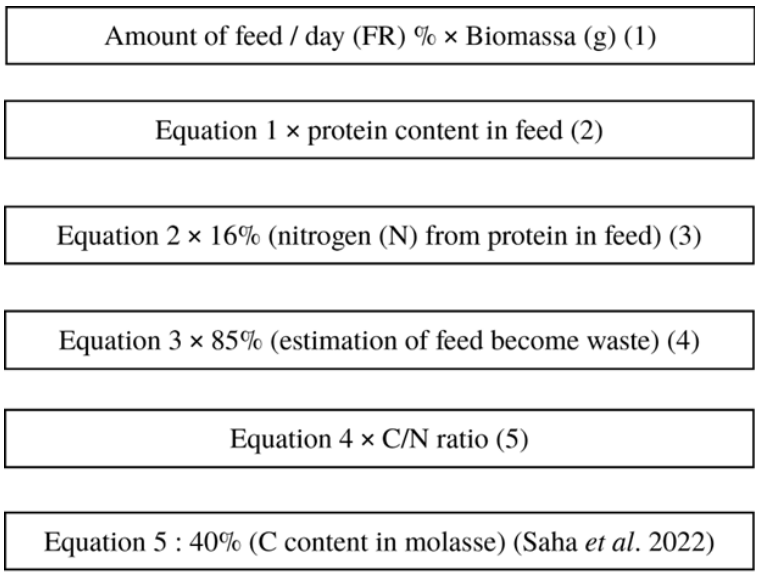

Figure 1. Organic carbon requirement calculation scheme (De Schryver *et al*., 2008).

The bacteria were then cultured on Sea Water Complete (SWC) or Thiosulfate-Citrate-Bile-Salt Sucrose (TCBS) agar. Colonies that had grown on the agar media after 24 hours were then taken using an inoculation loop and inoculated into 15 mL of liquid SWC media. The culture was then incubated using a shaker at 160 rpm for 18 hours at 28°C until a bacterial density of 10^8 CFU/mL was obtained. The bacterial density was then diluted to a concentration of 10^3 CFU/mL in the shrimp maintenance media.

White shrimp maintenance

White shrimp were stocked into the aquariums at a density of 3 individuals per liter (100 individuals per aquarium) and maintained for 21 days. During the maintenance period, the shrimp were fed commercial feed with a protein content of 39-40%, four times a day (07:00, 12:00, 17:00, and 22:00 WIB) at a feeding rate of 10%. The challenge test using *V. parahaemolyticus* was conducted using an immersion method at the start of the treatment. The challenge test used a *V. parahaemolyticus* concentration of 10^3 CFU/mL, obtained from the preliminary LC_{50} (lethal concentration 50%) test results (Torpee *et al*., 2021), as shown in Table 1.

Weekly counts of the bacterial populations of *Vibrio* sp. and *V. parahaemolyticus* Rf^R in the rearing water and shrimp gut were performed. Water quality parameters, including dissolved oxygen (DO), pH, and salinity, were measured daily, while total ammonia nitrogen (TAN), nitrite, and nitrate were measured weekly at the Aquaculture Environment Laboratory, Department of Aquaculture, IPB University.

Floc volume observation

Floc volume was measured after settling in a cone, and floc particle density was observed using the method of Sumitro *et al*. (2022). Approximately 1000 mL water sample was settled for 60 minutes in a 1000 mL capacity cone tube. If there was a significant increase in floc volume in the treatment using biofloc, dilution was performed by replacing 50% of the maintenance water to keep the floc volume stable.

Observation parameter

Total Vibrio count and total *V. parahaemolyticus* in water and shrimp bodies

On the 7th, 14th, and 21st days of maintenance, total *Vibrio* count and *V. parahaemolyticus* Rf^R were measured in the maintenance media and the bodies of white shrimp following the method of Madigan *et al*. (2003). Total bacteria were counted using the total plate count method on TCBS media, while total *V. parahaemolyticus* was counted on TCBS media supplemented with 50 µg/mL rifampicin. The maintenance water and homogenized shrimp bodies were serially diluted and 50 µL was spread on each type of media. The media with bacterial cultures were then incubated for 24 hours at 28°C-29°C. The bacteria that grew were then counted to determine the total bacterial count.

Immune response

The measurement of shrimp immune response refers to Hamsah *et al*. (2019) and Widanarni *et al*. (2020). Total hemocyte count (THC) was conducted by placing shrimp samples in a mortar containing an anticoagulant (3.8% Na-citrate) with a ratio of 1:3 (shrimp weight: anticoagulant). The shrimp were then ground, and the body fluid was extracted with a micropipette, dropped onto a hemocytometer, and observed under a microscope at 100× magnification. Phenoloxidase (PO) activity was measured by the formation of dopachrome produced by L-DOPA, with optical density (OD) measured using a spectrophotometer at a wavelength of 492 nm (Sutthangkul *et al*., 2017). Respiratory burst (RB) activity was measured according to the method of Hampton *et al*. (2020), with RB expressed as NBT reduction per 10 µl hemolymph. Immune response sampling was conducted on days 7, 14, and 21.

Growth performance

The shrimp's weight was measured at the beginning and end of the rearing period. Sampling was conducted before feeding when the shrimp's intestines were empty. Specific growth rate (SGR) and feed conversion ratio (FCR) were calculated using the equation provided by Liu *et al*. (2019):

$$\text{SGR (\%/ day)} = \frac{\ln W_t - \ln W_0}{t} \times 100$$

Note:

W_t = Average shrimp weight at the end of the rearing period (g)

W_0 = Average shrimp weight at the beginning of the rearing period (g)

B_a = Final shrimp biomass (g)

B_0 = Initial shrimp biomass (g)

B_m = Biomass of dead shrimp (g)
t = Duration of rearing (days)
FC = Total feed consumption (g)

Statistical analysis

The obtained data were tabulated using Microsoft Excel 2013. Analysis of growth performance, immune response, total bacteria, and total *V. parahaemolyticus* was performed using analysis of variance (ANOVA) with SPSS version 20. If significant differences were found, further tests were conducted using Tukey's test with a 95% confidence interval.

RESULT AND DISCUSSION

Result

Floc volume range

The floc volume in each treatment was measured daily and maintained for 21 days during the maintenance period. Throughout the maintenance period, the floc volume remained within the range corresponding to each treatment. In treatment B5, the floc volume ranged from 4.6 to 4.9 mL/L; treatment B10 had a floc volume ranging from 9.4 to 9.8 mL/L; and treatment B15 had a floc volume range of 14.3 to 14.5 mL/L. The weekly range of floc volume values is presented in Table 2.

Total Vibrio count and total V. parahaemolyticus Rf^R in water and shrimp body

Total Vibrio count and *V. parahaemolyticus* were observed through the maintenance media and the shrimp bodies. The observation of total presumptive *Vibrio* count (TPC) of Vibrio sp. in the maintenance media showed that the total *Vibrio* count for treatments B5, B10, and B15 was higher on day 7 compared to treatments KN and KP. However, on days 14 and 21, the total Vibrio count tended to decrease (Table 3). In treatment B5, the count was higher on day 7 at 5.62 log CFU/mL, but decreased on days 14 and 21 to 3.92 log CFU/mL and 3.55 log CFU/mL, respectively.

Similarly, treatments B10 and B15 had the highest total *Vibrio* count on day 7 and the lowest on day 21. The total Vibrio count in the shrimp bodies showed similar values to those in the maintenance media. Treatments B5, B10, and B15 had the highest count on day 7 at 5.98 ± 0.57 log CFU/mL and the lowest count on day 21 at 3.75 ± 0.42 log CFU/mL. The complete data for total Vibrio count in the water and white shrimp bodies are presented in Table 3.

The observation of total *V. parahaemolyticus* Rf^R bacteria in the maintenance water showed a range of 3.18–4.80 log CFU/mL on day 7, while no *V. parahaemolyticus* growth was detected on days 14 and 21. However, in the positive control

Table 2. Range of floc volume in white shrimp maintenance media during the maintenance period.

Floc Volume (mL/L)	Week-1	Week-2	Week-3
B5	4.6–5.1	4.9–5.5	4.7–5.6
B10	9.5–10,1	9.4–10.3	9.8–10.7
B15	14.3–15	14.4–15.2	14.5–15.1

Note: B5 (floc volume of 5 mL/L); B10 (floc volume of 10 mL/L); B15 (floc volume of 15 mL/L).

Table 3. Total Vibrio count in water and body of white shrimp maintained in biofloc system with different volumes and challenged with *V. parahaemolyticus*.

Treatment	TPVC (*Total presumptive vibrio count*)					
	Water (Log CFU/mL)			Shrimp (Log CFU/g)		
	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
KN	4.31 ± 0.19^{a}	5.84 ± 0.12^{c}	5.66 ± 0.03^{c}	4.66 ± 0.05^{a}	5.62 ± 0.17^{bc}	5.68 ± 0.08^{c}
KP	5.47 ± 0.16^{b}	6.7 ± 0.03^{c}	6.69 ± 0.01^{d}	5.69 ± 0.23^{ab}	6.35 ± 0.37^{c}	6.21 ± 0.34^{c}
B5	5.62 ± 0.04^{b}	3.92 ± 0.10^{b}	3.55 ± 0.24^{ab}	5.88 ± 0.55^{b}	4.45 ± 0.14^{b}	4.58 ± 0.18^{b}
B10	5.47 ± 0.07^{b}	3.62 ± 0.15^{a}	3.67 ± 0.09^{b}	5.96 ± 0.71^{b}	4.19 ± 0.87^{a}	4.41 ± 0.07^{b}
B15	5.61 ± 0.18^{b}	3.47 ± 0.13^{a}	3.31 ± 0.07^{a}	5.98 ± 0.57^{b}	4.66 ± 0.45^{bc}	3.75 ± 0.42^{a}

Note: Different superscript letters in the same column indicate significantly different results (Tukey $p<0.05$).

(KP) treatment, bacteria were found on days 14 and 21 with total counts of 3.27 ± 0.20 log CFU/mL and 1.83 ± 0.42 log CFU/mL, respectively. The observation of total *V. parahaemolyticus* Rf^R bacteria in white shrimp bodies showed higher counts in the KP treatment, ranging from 2.77 to 3.78 log CFU/mL. In the biofloc treatments (B5, B10, and B15), the highest total *V. parahaemolyticus* counts were observed on day 7, with a tendency to decrease on days 14 and 21, with the lowest count in treatment B10 at 1.72 ± 0.28 log CFU/mL (Table 4).

White shrimp immune response

The results of the immune response observations in white shrimp, including total hemocyte count (THC), phagocytic activity (PA), phenoloxidase (PO) activity, and respiratory burst (RB) activity, are presented in Table 5. On day 7, THC values showed a significant difference (P<0.05) in treatments B5, B10, and B15 compared to KN and KP treatments. The highest THC value was found in treatment B15, with a value of 7.70 ± 0.35 10^6 cells/mL, while the lowest value was in KP treatment, with a value

Table 4. Total *V. parahaemolyticus* Rf^R in water and body of white shrimp maintained in biofloc system with different volumes and challenged with *V. parahaemolyticus*.

Treatment	TVC (Total Bakteri *V. parahaemolyticus*)					
	Water (Log CFU/mL)			Shrimp (Log CFU/g)		
	Day 7	Day 14	Day 21	Day 7	Day 14	Day 21
KN	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
KP	4.80 ± 0.28^{d}	3.27 ± 0.20^{b}	1.83 ± 0.09^{b}	3.78 ± 0.14^{c}	3.75 ± 0.32^{d}	2.77 ± 0.55^{c}
B5	3.64 ± 0.23^{c}	0 ± 0^{a}	0 ± 0^{a}	3.02 ± 0.35^{bc}	2.72 ± 0.14^{c}	1.94 ± 0.12^{b}
B10	3.36 ± 0.08^{c}	0 ± 0^{a}	0 ± 0^{a}	2.88 ± 0.51^{bc}	1.69 ± 0.32^{b}	1.72 ± 0.28^{b}
B15	3.18 ± 0.12^{b}	0 ± 0^{a}	0 ± 0^{a}	2.58 ± 0.76^{b}	1.89 ± 0.47^{b}	1.85 ± 0.17^{b}

Note: Different superscript letters in the same column indicate significantly different results (Tukey p<0.05).

Table 5. Immune response of white shrimp reared in biofloc systems with different volumes and challenged with *V. parahaemolyticus*

Day	Parameter	KN	KP	B5	B10	B15
0	THC (10^6 Cell/mL)	4.33 ± 0.15^{a}	4.33 ± 0.15^{a}	4.33 ± 0.15^{a}	4.33 ± 0.15^{a}	4.33 ± 0.15^{a}
	PA (%)	34.33 ± 1.15^{a}	34.33 ± 1.15^{a}	34.33 ± 1.15^{a}	34.33 ± 1.15^{a}	34.33 ± 1.15^{a}
	PO (100 µL)	0.24 ± 0.01^{a}	0.24 ± 0.01^{a}	0.24 ± 0.01^{a}	0.24 ± 0.01^{a}	0.24 ± 0.01^{a}
	RB (10 µL)	0.15 ± 0.01^{a}	0.15 ± 0.01^{a}	0.15 ± 0.01^{a}	0.15 ± 0.01^{a}	0.15 ± 0.01^{a}
7	THC (10^6 Cell/mL)	5.23 ± 0.35^{b}	3.27 ± 0.31^{a}	7.10 ± 0.20^{c}	7.33 ± 0.21^{c}	7.37 ± 0.35^{c}
	PA (%)	49.33 ± 3.21^{b}	37.00 ± 1.00^{a}	49.33 ± 0.58^{b}	46.67 ± 2.52^{b}	49.67 ± 1.53^{b}
	PO (100 µL)	0.26 ± 0.03^{b}	0.18 ± 0.04^{a}	0.27 ± 0.03^{b}	0.28 ± 0.02^{b}	0.27 ± 0.01^{b}
	RB (10 µL)	0.23 ± 0.01^{ab}	0.2 ± 0.00^{a}	0.24 ± 0.02^{ab}	0.26 ± 0.04^{b}	0.34 ± 0.01^{c}
14	THC (10^6 Cell/mL)	5.80 ± 0.10^{b}	3.50 ± 0.10^{a}	6.47 ± 0.25^{bc}	7.33 ± 0.45^{d}	7.10 ± 0.26^{cd}
	PA (%)	52.33 ± 3.21^{b}	39.00 ± 1.00^{a}	51.33 ± 3.21^{b}	48.67 ± 3.06^{b}	51.33 ± 2.08^{b}
	PO (100 µL)	0.25 ± 0.03^{b}	0.12 ± 0.02^{a}	0.26 ± 0.04^{b}	0.26 ± 0.02^{b}	0.26 ± 0.02^{b}
	RB (10 µL)	0.27 ± 0.02^{b}	0.21 ± 0.00^{a}	0.35 ± 0.03^{c}	0.36 ± 0.02^{c}	0.38 ± 0.03^{c}
21	THC (10^6 Cell/mL)	6.03 ± 0.15^{b}	3.70 ± 0.10^{a}	7.00 ± 0.10^{c}	7.60 ± 0.50^{c}	7.7 ± 0.26^{c}
	PA (%)	57.00 ± 2.65^{b}	31.67 ± 2.08^{a}	47.33 ± 6.66^{b}	50.33 ± 2.52^{b}	54.67 ± 2.08^{b}
	PO (100 µL)	0.27 ± 0.02^{b}	0.17 ± 0.05^{a}	0.28 ± 0.04^{b}	0.29 ± 0.02^{b}	0.3 ± 0.01^{b}
	RB (10 µL)	0.31 ± 0.02^{a}	0.26 ± 0.02^{a}	0.41 ± 0.03^{b}	0.47 ± 0.04^{bc}	0.5 ± 0.00^{c}

Note: Different superscript letters in the same column indicate significantly different results (Tukey p<0.05).

of 3.27 ± 0.31 10^6 cells/mL. Phagocytic activity in the treatments with increased biofloc volume (B5, B10, and B15) on days 7, 14, and 21 of rearing showed significant differences (P<0.05) compared to KP treatment but did not differ significantly from KN treatment.

The highest phagocytic activity (PA) was observed on day 21 in the biofloc treatment (B15), with a value of 54.67 ± 2.08%, while the lowest value on day 21 was in the positive control treatment (KP), with a value of 31.67 ± 2.08%. Observations of phenoloxidase (PO) activity in all treatments on day 0 showed a value of 0.24 ± 0.01 (100 µL). PO values with increased biofloc volume (B5, B10, and B15) showed significantly different results from KP treatment but did not differ significantly from KN treatment. The highest PO activity during 21 days of rearing was observed on day 21 in the B15 treatment, with a value of 0.30 ± 0.01 (100 µL), and the lowest PO activity was observed on day 14 in the KP treatment, with a PO value of 0.12 ± 0.02 (100 µL).

The observation of respiratory burst (RB) on day 0 in all treatments showed a value of 0.15 ± 0.01 (10 µL). On days 7, 14, and 21, the increased biofloc volume (B5, B10, and B15) significantly increased RB values. A significant effect was observed on day 21, where the increased biofloc volume (B15) challenged with *V. parahaemolyticus* 10^3 CFU/mL showed higher RB activity, with a value of 0.5 ± 0.00 (10 µL), and was significantly different (P<0.05) from both the positive control (KP) and negative control (KN) treatments. However, there were no significant differences between treatments B5 and B10.

White shrimp growth performance

The growth performance of white shrimp reared in a biofloc system with different volumes is presented in Table 6. Based on Table 6, it is evident that the B10 treatment provided better feed conversion ratio (FCR) values, which were significantly different from the positive control (KP), B5, and B15 treatments according to statistical analysis. Additionally, the B10 treatment yielded higher values in final weight and specific growth rate parameters, although these values were not significantly different from the B5 and B15 treatments. The highest weight obtained during the rearing period was 0.99 ± 0.08 g, the highest specific growth rate was 6.42 ± 0.28%, and the lowest feed conversion ratio was 1.45 ± 0.08.

The total feed consumption during the 21-day rearing period ranged from 83.35 to 102.65 g. The highest survival rate during the rearing period was in the range of 84.23-89.96%, which was significantly different from the positive control (KP) but not significantly different from the negative control (KN), B5, B10, and B15 treatments. The results of water quality measurements during the rearing of white shrimp are presented in Table 7. Overall, the values of

Table 6. Growth performance of white shrimp reared in biofloc systems with different volumes and challenged with *V. parahaemolyticus.*

Parameter	KN	KP	B5	B10	B15
W_0 (g)	0.30 ± 0.03^a	0.30 ± 0,03^a	0.28 ± 0.01^a	0.28 ± 0.01^a	0.28 ± 0.01^a
W_t (g)	0.81±0.01^a	0.80 ± 0.01^a	0.94 ± 0.05^b	0.99 ± 0.08^b	0.96 ± 0.07^b
FC (g)	95.67 ± 3.74^{ab}	83.35 ± 5.96^a	98.18 ± 6.21^{ab}	102.65 ± 4.93^b	101.69 ± 7.86^b
SGR (%)	4.64 ± 0.42^a	4.80 ± 0.08^a	6.04 ± 0.37^b	6.42 ± 0.28^b	6.07 ± 0.34^b
FCR	1.93 ± 0.23^{bc}	2.18 ± 0.23^c	1.56 ± 0.12^{ab}	1.45 ± 0.04^a	1.53 ± 0.10^{ab}
Survival (%)	89.96 ± 3.31^b	61.32 ± 13.59^a	84.23 ± 3.66^b	84.23 ± 3.31^b	89.90 ± 0.00^b

Note: Different superscript letters in the same row indicate significantly different results (Tukey p<0.05).

Table 7. Water quality of the maintenance media for white shrimp maintained in the biofloc system with different volumes and tested against *V. parahaemolyticus.*

Treatment	TAN (mg/)	NO_2 (mg/L)	NO_3 (mg/L)
KN	0.131-0.384	0.457-0.696	0.385-0.733
KP	0.173-0.390	0.480-0.739	0.226-0.653
B5	0.132-0.468	0.011-0.572	0.966-1.980
B10	0.078-0.326	0.013-0.786	1.124-2.052
B15	0.073-0.279	0.013-0.683	1.273-2.284

TAN (total ammonia nitrogen) and NO_2^- (Nitrite) in the biofloc treatments were lower compared to the control treatments, while the NO_3^- (Nitrate) values in the biofloc treatments were higher than those in the control treatments. The lowest DO (Dissolved Oxygen) value was 5.0 mg/L, which is within the normal range, while the highest value was 6.10 mg/L. The pH values (7.67–8.00), temperature (26.2–29.3°C), and salinity (31–34 g/L) were all within the normal range for white shrimp rearing. In general, the water quality conditions during the white shrimp rearing were optimal and in accordance with SNI 8008:2014 BSN (2014).

Discussion

Vibriosis disease, caused by bacteria from the *Vibrio* genus, particularly *Vibrio parahaemolyticus*, is one of the most serious and prevalent diseases affecting shrimp aquaculture (Valente & Wan, 2021). *V. parahaemolyticus* is an opportunistic pathogen whose high population in aquatic environments plays a role in regulating virulence factors and contributing to bacterial defense mechanisms as well as host infection activities, such as enhanced attachment and penetration, interbacterial interactions, and environmental stress responses (Wang *et al*., 2013). Typically, bacterial infections are managed using antibiotics, but their usage has been restricted. An environmentally friendly alternative to control *V. parahaemolyticus* infections involves inhibiting bacterial growth to reduce virulence and enhance shrimp immune responses, thereby increasing resistance to bacterial infections (Zhao *et al*., 2014), such as through the application of biofloc technology (Gustilatov *et al*., 2022).

This study demonstrates that the presence of biofloc at all tested volumes (5 mL/L, 10 mL/L, and 15 mL/L) reduces the density of *Vibrio* spp. and *V. parahaemolyticus*, both in the culture medium and on the shrimp bodies. According to Gustilatov *et al*. (2023), biofloc acts as a biocontrol agent and can inhibit the growth and pathogenicity of *Vibrio* bacteria due to extracellular components that disrupt bacterial QS activity. Biofloc has also been shown to produce compounds such as bromophenol, carotenoids, poly-beta-hydroxybutyrate, and various hydrolytic enzymes (Fatimah *et al*., 2019). Gustilatov *et al*. (2022) reported that biofloc can reduce *V. parahaemolyticus* density in vitro and inhibit biofilm formation.

Biofilm formation, mediated by QS (Liu *et al*., 2018), allows bacteria to efficiently utilize nutrients, increase resistance to antimicrobial agents, stress, and enhance bacterial virulence (Packiavathy *et al*., 2013). Additionally, biofloc can play an antagonistic role by competing for nutrients, energy, and sites, thereby suppressing the growth of pathogenic bacteria (Ferreira *et al*., 2020). The immune parameter profiles measured in this study align with previous findings indicating the positive effects of biofloc on aquaculture organism immunity, as reported by Tepaamorndech *et al*. (2020), Panigrahi *et al*. (2018), and Ekasari *et al*. (2014). Biofloc, consisting of bacteria with lipopolysaccharides or peptidoglycans, fungi with β-glucans, and other microbes, can stimulate hemocytes and other immune parameters through pathogen-associated molecular patterns (Kim *et al*., 2014).

In this study, the total hemocyte count (THC) and phagocytic activity (PA) of shrimp reared in biofloc systems showed significant differences compared to controls, confirming the immunostimulatory effects of biofloc and corroborating previous research by Ferreira *et al*. (2015). The best results were observed in the 15 mL/L biofloc volume treatment compared to other volumes. Hemocytes are involved in several pathogen resistance activities, including phagocytosis, encapsulation, foreign particle aggregation, and prophenoloxidase (proPO) system functions (Sahoo *et al*., 2008). The humoral immune response in shrimp, indicated by phenoloxidase (PO) activity a precursor to melanin formation that inactivates and prevents pathogen spread was enhanced in biofloc treatments (Amparyup *et al*., 2013).

A similar trend was observed in pathogen destruction activity through reactive oxygen intermediates (ROI) represented by reactive blue (RB) parameters (Duan *et al*., 2015). Consistent with the increase in hemocytes, the activities of phenoloxidase (PO) and reactive blue (RB) in the 15 mL/L biofloc treatment were also significantly higher compared to the 5 mL/L and 10 mL/L treatments. This indicates that a 15 mL/L biofloc treatment can provide better protection against *Vibrio parahaemolyticus* infections. Based on the obtained results and corroborated by several previous studies, biofloc is capable of protecting shrimp from pathogen infections, partly through the enhancement of immune responses. The reduction in bacterial virulence impacts its ability to infect shrimp.

In addition to weakening the bacteria's ability to infect shrimp, biofloc also improves the shrimp's immune response to *V. parahaemolyticus* infections, enabling the immune system to better control the bacterial infection due to the influence of biofloc (Ekasari *et al*., 2014). The positive role of biofloc is also evident in shrimp growth performance. Higher growth rates in biofloc treatments are attributed to the comprehensive nutrient composition of biofloc, including proteins, carbon, ash, fatty acids, minerals, and other nutrients, which can serve as a natural feed consistently available in the culture medium (Toledo *et al*., 2016). Additionally, biofloc can enhance digestive enzyme activity, leading to more efficient feed utilization for growth (Wang *et al*., 2015). This effect is indicated by higher length-to-weight ratios (SGR) and lower feed conversion ratios (FCR) in biofloc treatments compared to controls in this study.

Furthermore, the survival rate of shrimp challenged with *Vibrio parahaemolyticus* was higher in biofloc treatments compared to positive controls. This improvement is due to reduced *V. parahaemolyticus* virulence and enhanced immune responses in shrimp. The survival rate in the 15 mL/L biofloc treatment was 89.90 ± 0.00%, which was not significantly different from the 10 mL/L (84.23 ± 3.31%) and 5 mL/L (84.23 ± 3.66%) treatments, but significantly higher than the positive control (61.32 ± 13.59%). These findings are consistent with reports by Sajali *et al*. (2019) and Hostins *et al*. (2019), which observed that biofloc treatment enhances the survival of white shrimp challenged with *V. parahaemolyticus*. Biofloc also positively impacts water quality by reducing ammonia and nitrite concentrations, which can be toxic to shrimp, through nitrogen assimilation by heterotrophic bacteria (Robles-Porchas *et al*., 2020).

CONCLUSION

Biofloc can inhibit *Vibrio parahaemolyticus*, enhance immune responses, improve growth performance, and increase the resistance of *Penaeus vannamei*, with the best results observed at a volume application of 15 mL/L.

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