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EFFECTIVENESS OF BIOFLOC, PROBIOTICS AND THE COMBINATIONS ON GROWTH, IMMUNE RESPONSES AND RESISTANCE OF VANNAMEI SHRIMP INFECTED WITH *Vibrio parahaemolyticus*

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ABSTRACT

Vibrio parahaemolyticus strain that produces PirA and PirB toxins is the main causative agent of Acute Hepatopancreatic Necrosis Disease (AHPND) in vannamei shrimp. This study aimed to evaluate the effect of biofloc application, probiotic Pseudoalteromonas piscicida 1Ub*, and their combination on growth, immune response and resistance of vannamei shrimp infected with V. parahaemolyticus strain. This study used a completely randomized design consisting of biofloc-based system treatment with or without probiotic* 1Ub *and normal seawater as control. All treatment groups were challenged with V. parahaemolyticus AHPND strain at a cell density of 10^6 CFU mL^{-1} through immersion, while the negative control was reared without being pathogenic challenged. The shrimp used were in averaged body weight of 1.3 ± 0.002 g, reared for 21 days and fed five times a day at 06:00, 10:00, 14:00, 18:00, and 22:00 WIB. The results showed that the B+Pro combination challenge test treatment resulted the best growth performance (specific growth rate, absolute length gain and feed conversion ratio) ($P<0.05$) compared to other challenge test treatments. Shrimp treated with B+Pro also showed a lower intestinal cell population of V. parahaemolyticus* Rf^R*, and significantly higher immune response values ($P<0.05$) than those of other challenge test treatments and K+. Furthermore, those parameters supported positive impact on final shrimp survival rates in the experiment. This study shows that the application of combination of biofloc and* 1Ub *probiotic bacteria can significantly protect and increase the resistance of vannamei shrimp to V. parahaemolyticus AHPND infection.*

KEYWORDS: *Vannamei shrimp; AHPND; Vibrio parahaemolyticus; biofloc; probiotic* 1Ub

INTRODUCTION

Indonesia is one of the largest shrimp producing countries in the world with an export value of USD 2.2 billion (KKP, 2022), one of the leading commodities is vannamei shrimp (*Litopeneaus vannamei*). Globally, the production value of vannamei shrimp represents 51.7% of the total production of the crustacean group, and Indonesia is in the fourth position with production from the crustacean group reaching 892 thousand tons (FAO, 2022). The high production is partly generated through the application of intensive and super-intensive farming systems. Increasing stocking density and artificial feeding will increase the production of waste derived from inedible feed, feces and metabolic waste of shrimp. This leads to several problems such as reducing the quality of the aquaculture environment due to high concentrations of nitrogenous waste, as well as increasing the population of pathogenic bacteria, thereby increasing the risk of disease in shrimp (Watts *et al.,* 2017; Liu *et al.,* 2017; Khoa *et al.,* 2020).

Vibriosis disease is one type of disease that attacks vannamei shrimp, the pathogen that causes this disease is bacteria from the *Vibrio* genus including *Vibrio parahaemolyticus*. These bacteria can cause necrosis of the gills, anorexia, slow growth and mass mortality in a short time, especially at early period of cultivation (Aguirre-Guzman *et al.,* 2010; Kumar *et al.,* 2014; Raja *et al.,* 2017). In addition to being one of the causes of vibriosis, certain strains of *V. parahaemolyticus* that produce PirA and PirB toxins are known to be the main cause of AHPND (*Acute Hepatopancreatic Necrosis Disease*) (Tran *et al.,* 2013;

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Yuhana & Afiff, 2023), with symptoms of sudden death, shrunken hepatopancreas and pale color, empty intestines and stomach, and when histological observation of tubular epithelial cells in the hepatopancreas is necrosis (Lai *et al.,* 2015).

Control of bacterial diseases in shrimp can be done by improving environmental conditions and increasing shrimp immunity (Wicaksono *et al.,* 2020). As is known, the use of antibiotics to suppress bacterial populations in aquaculture has been limited because it can cause the emergence of resistant properties of bacteria and the content of antibiotic residues in shrimp after harvest (PERMEN-KP, 2019). An alternative solution to reduce the use of antibiotics in overcoming bacterial disease problems is to apply biofloc technology and probiotics. In aquaculture, bioflocs maximizing the nitrogen assimilation from the utilization of feed waste and metabolic waste of cultured organisms by heterotrophic bacteria through modification of the C/N ratio in water and forming bacterial biomass and other organisms (Avnimelech, 2009). The results of previous studies showed that the application of biofloc systems can increase the immune response of vannamei shrimp (Kumar *et al.,* 2018), improved the growth performance of vannamei shrimp (Ekasari *et al.,* 2014), and water quality (Zhang *et al.,* 2020). In addition, biofloc application has been reported to provide protection, and to reduce the virulence of *V. parahaemolyticus* bacteria (Gustilatov *et al.,* 2022).

Probiotics if supplemented in optimal amounts can benefit the health of the host; either in the stage of living bacteria (Wang *et al.,* 2019); or in the form of inactive bacteria which so-called as paraprobiotic (Yuhana *et al*., 2024). *Pseudoalteromonas piscicida* 1Ub bacteria is one type of probiotic bacteria that is reported to be able to increase the immune response in vannamei shrimp. *P. piscicida* 1Ub belongs to one of Gram-negative bacteria, is motile, isolated from seawater-origin that have tolerance to a low pH and have antimicrobial properties (Richards *et al.,* 2017). The results of research by Widanarni *et al.* (2009), stated that *P. piscicida* 1Ub bacteria have antagonistic properties against *Vibrio harveyi* bacteria and increase the survival rate of tiger shrimp larvae, even though exposed in disease outbreaks and fluctuated environmental condition in the field (Ermawati *et al.,* 2023). Different study conducted by Hamsah *et al.* (2019), reported that *P. piscicida* 1Ub applied to vannamei shrimp larvae through bioencapsulated *Artemia* sp. were able to increase the immune response, and shrimp resistance when tested with *V. harveyi.*

Vannamei shrimp have a non-specific immune system in overcoming pathogen attacks in the body. Activation of the shrimp immune response begins with foreign body recognition and is mediated by pattern recognition receptors (PRRs) which are considered the first step in recognizing non-self-recognition material that enters the body (Ji *et al.,* 2009; Kulkarni *et al.,* 2021). PRRs can recognize microorganism cell material such as lipopolysaccharides, peptidoglycans and β-glucans in the biofloc constituent material and 1Ub probiotics known as pathogen-associated molecular patterns (PAMPs). Furthermore, PRRs will bind to PAMPs and will induce a series of cellular and humoral immune responses through the activation of hemocytes in the shrimp body (Kim *et al.,* 2014).

Several other studies have shown that the addition of probiotics to biofloc systems can improve shrimp health status and reduce pathogenic bacteria populations (He *et al.,* 2023; Gustilatov *et al.,* 2023). However, evaluations on the application of bioflocs, probiotic *P. piscicida* 1Ub, and the combination of both have not been conducted. Therefore, this study aimed to evaluate the effect of biofloc application, probiotic *P. piscicida* 1Ub, and their combination on growth performance, immune response and resistance of vannamei shrimp infected with *V. parahaemolyticus* bacteria.

MATERIALS AND METHODS

Research Design

This research was conducted from September to December 2023 at the Aquatic Organism Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University, Indonesia. The research design used was a completely randomized design (CRD) with eight treatments and three replicates including K- (without biofloc and probiotics and not challenged with *V. parahaemolyticus* Rf^R), K+ (without biofloc and probiotics but challenged with *V. parahaemolyticus* Rf^R), treatment without challenge test *V. parahaemolyticus* Rf^R, namely biofloc control (KB), probiotic control (KPro), biofloc + probiotic control (KB+Pro), and treatment with challenge test *V. parahaemolyticus* Rf^R, namely biofloc (B), probiotics (Pro), biofloc + probiotics (B+Pro).

Cultivation of the Shrimp

The tested animals were *L. vannamei* shrimp certified *Specific-Pathogen Free* (SPF), with an average size of 1.3±0.002 g. The rearing container used was an aquarium measuring 60×30×30 cm^3 and covered

with black plastic, filled with 28 ppt salinity seawater with a water volume of 30 L and installed water heater and aeration. Vannamei shrimp were stocked in the treatment container at a density of 30 shrimps/ aquarium. Commercial feed (39-40% protein, 7% lipid, 3% fiber, 13% ash, and 10% moisture content) was given five times a day at 06:00, 10:00, 14:00, 18:00, and 22:00 WIB, with a feeding rate of 8% of shrimp biomass weight and maintained for 21 days. Floc volume in the biofloc and biofloc + probiotic application treatments during rearing was maintained in the range of 15-20 mL L^{-1}. Water quality checks during the rearing of vannamei shrimp were carried out periodically including temperature (28.0-29.4°C), pH (7.51-8.46), *dissolved oxygen* (6.2-7.5 mg L^{-1}), salinity (28-29 g L^{-1}) (BSN 2014).

Biofloc Provision

The source of bioflocs suspension used as an inoculum for shrimp culture in this study was obtained from another vannamei shrimp culture in a container (working volume 200 L) with a biofloc system that used molasses as a source of organic C (carbon). The addition of initial bioflocs to the aquarium was carried out the day before treatment (the ratio of biofloc stock volume : seawater = 2 : 3) (Gustilatov *et al.,* 2022). Molasses, as a carbon source, was added daily to the culture medium once a day, 2 hours after morning feeding (at 08:00 WIB), with an estimated C: N ratio of 10 (Ekasari *et al.,* 2014). The amount of carbon added to the rearing medium was calculated based on the equation of De Schryver *et al.* (2008) with the assumption of 40% feed protein; 16% of the amount of nitrogen (N) of feed protein; 85% of the amount of feed wasted as waste; C:N ratio of 10; 43.31% carbon content of molasses, so the addition of molasses was about 12.56 g per 10 g of feed.

Bacteria Preparation and Challenge Test

The bacteria used in this study were *V. parahaemolyticus* bacteria as pathogenic bacteria in the challenge test, and *P. piscicida* 1Ub bacteria used as probiotic bacteria, which came from the Aquatic Organism Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University, Indonesia. Both bacteria were made resistant to the antibiotic rifampicin 50 μg mL^{-1} (1 g rifampicin, 95 mL absolute ethanol and 5 mL distilled water) as a marker on agar media and coded Rf^R. *V. parahaemolyticus* bacteria were cultured on Thiosulfate Citrate Bile-Salt Sucrose (TCBS) agar media, while *P. piscicida* 1Ub bacteria were cultured on Sea Water Complete (SWC) agar media (Widanarni *et al.,* 2009). Administration of probiotic bacteria *P. piscicida* 1Ub with cells density of 10^6 CFU mL^{-1} to probiotic treatments with or without challenge test, and biofloc + probiotic combination treatments with or without challenge test was included one day before being given *V. parahaemolyticus* Rf^R. The challenge test using *V. parahaemolyticus* Rf^R bacteria began simultaneously from the beginning of the treatment through immersion with a bacterial density of 10^5 CFU mL^{-1} referring to the LC50 results (the concentration of *V. parahaemolyticus* that causes 50% shrimp mortality) using the Reed & Muench method (1938).

Observed Parameters

Parameters observed in this study were growth performance parameters including specific growth rate (SGR), absolute length gain (ALG), and feed conversion ratio (FCR). The SGR was calculated as a percentage using the formula: SGR (%) = [(ln final weight (g) – ln initial weight (g)) / rearing period (days)] × 100 (Zokaeifar *et al.,* 2012), then ALG was determined by subtracting the initial average length from the final average length, using the formula: ALG (cm) = final average length (cm) – initial average length (cm) (Widanarni *et al.,* 2009), and for FCR was calculated by dividing the total amount of feed given (g) by the biomass gain, using the formula: FCR = amount of feed given (g) / (final biomass (g) – initial biomass (g)) (Yang *et al.,* 2015).

Bacterial abundance parameters in the rearing medium and shrimp body include total *V. parahaemolyticus* Rf^R count (VPC) in the hepatopancreas, while total *P. piscicida* 1Ub and total *Vibrio* sp. count (TVC) in the intestine, calculated using the Total Plate Count scatter plate method (Madigan *et al.,* 2012). Thiosulfate Citrate Bile-Salt Sucrose (TCBS) selective media with the addition of the antibiotic rifampicin (50 μg mL^{-1}) was used to measure *V. parahaemolyticus* Rf^R, while TCBS media without the addition of rifampicin was used to measure total *Vibrio* sp. count (Liu *et al.,* 2010). Sea water complete (SWC) media with the addition of the antibiotic rifampicin (50 μg mL^{-1}) was used to measure total *P. piscicida* 1Ub. Samples of rearing media, hepatopancreas and shrimp intestine were crushed then serially diluted using sterile phosphate-buffered saline (PBS) and spread as much as 50 μL on each of these media. TCBS media was incubated for 24 hours, while SWC media containing culture was incubated for 48 hours at 37-40°C. Sampling of bacterial abundance was carried out on days 0, 1, 11 and 21.

Health status parameters include total haemocyte count (THC) referring to the method of Hamsah *et*

al. (2019), which is by inserting shrimp samples into a mortar containing anticoagulant (Na-citrate 3.8%) in a ratio of 1: 3 (shrimp weight: anticoagulant). The shrimp were then crushed, then the body fluid was taken with a micropipette, then dripped onto a hemacytometer, then observed and counted under a microscope at 100x magnification. Phagocytosis activity (PA) was measured by mixing a 100 µl sample of shrimp hemolymph with a suspension of *Staphylococcus aureus* (10^7 CFU mL^{-1}) 25 µl and then incubated for 20 minutes, then made a review preparation, then dried, fixed with methanol for five minutes and dried again, then stained with giemsa dye for 20 minutes and observed using a 400x magnification microscope (Anderson & Siwicki, 1993). Respiratory burst activity (RB) was performed according to the working method of Cheng *et al.* (2004), RB was expressed as NBT reduction per 10 µl hemolymph with optical density (DO) 630 nm. Phenoloxidase (PO) activity was measured by dopachrome formation produced by L-DOPA, DO measurement using a spectrophotometer with a wavelength of 492 nm (Liu & Chen, 2004). Sampling of shrimp immune response was conducted on days 1, 11 and 21.

Histopathological parameters on hepatopancreas organs in all treatments were performed to determine the level of tissue damage due to the challenge test with *V. parahaemolyticus* Rf^R bacteria and the effect of bioflocs, probiotics and their combination. Hepatopancreas tissues were fixed with Davidson's solution for 24 hours. Hepatopancreas tissue was cut 3-5 mm thick measuring 0.5 × 0.5 cm, then dehydrated, cleared, embedded, paraffin blocked, sectioned, stained, and examined microscopically at 100x magnification (Munaeni *et al.,* 2020). Classification of the degree of tissue damage is done by scoring histology results referring to Wolf *et al.* (2015), namely P < 20% (score 0, normal), 20% ≤ P < 40% (score 1, mildly damaged), 40% ≤ P < 60% (score 2, moderately damaged), 60% ≤ P < 80% (score 3, severely damaged), P ≥ 80% (score 4, severely damaged). Water quality parameters (TAN, Nitrite, Nitrate and TSS) were observed on days 0, 11 and 21.

The shrimp survival rate (SR) was calculated as the percentage of shrimp that survived until the end of the treatment period, using the formula: SR (%) = (number of shrimp at the end of the treatment / number of shrimp at the beginning of the treatment) × 100 (Widanarni *et al.,* 2009).

Data Analysis

The data obtained were tabulated using Microsoft Excel 2021. Data analysis of specific growth rate (SGR), absolute length gain (ALG), feed conversion ratio (FCR), total *V. parahaemolyticus* Rf^R count (VPC), total *P. piscicida* 1Ub, total *Vibrio* sp. count (TVC), total haemocyte count (THC), phagocytosis activity (PA), respiratory burst activity (RB), phenoloxidase activity (PO), water quality (TAN, Nitrite, Nitrate and TSS) and shrimp survival rate was tested using analysis of variance (ANOVA) using the SPSS version 26 application. Treatments that are significantly different will be further tested using the Duncan test with a 95% confidence interval. Histopathological data were analyzed by scoring.

RESULTS AND DISCUSSION

Growth Performance

The growth performance of vannamei shrimp with the application of biofloc, probiotic *P. piscicida* 1Ub, and the combination of both for 21 days of rearing can be seen in Table 1. The initial average weight was not significantly different (P > 0.05) in all treatments. After 21 days of rearing, the KB + Pro treatment

Table 1. Growth performance of vannamei shrimp reared in biofloc system, probiotics and their combination for 21 days of rearing

Treatments	Parameters				
	W0 (g)	W21 (g)	SGR (%)	ALG (cm)	FCR
K-	1.31 ± 0.005	2.48 ± 0.01^{b}	3.03 ± 0.02^{b}	1.16 ± 0.08^{b}	1.68 ± 0.05^{cd}
K+	1.31 ± 0.003	2.22 ± 0.08^{a}	2.51 ± 0.18^{a}	0.66 ± 0.10^{a}	2.19 ± 0.19^{e}
KB	1.31 ± 0.002	2.86 ± 0.06^{d}	3.71 ± 0.11^{d}	1.65 ± 0.04^{d}	1.38 ± 0.08^{b}
B	1.31 ± 0.004	2.47 ± 0.00^{b}	3.02 ± 0.01^{b}	1.20 ± 0.10^{bc}	1.84 ± 0.11^{d}
KPro	1.31 ± 0.002	2.65 ± 0.11^{c}	3.36 ± 0.22^{c}	1.32 ± 0.03^{c}	1.57 ± 0.13^{bc}
Pro	1.31 ± 0.002	2.44 ± 0.13^{ab}	2.97 ± 0.25^{b}	1.19 ± 0.12^{bc}	1.88 ± 0.13^{d}
KB + Pro	1.31 ± 0.001	3.27 ± 0.05^{e}	4.35 ± 0.09^{e}	1.95 ± 0.03^{e}	1.11 ± 0.02^{a}
B + Pro	1.31 ± 0.000	2.77 ± 0.03^{d}	3.56 ± 0.07^{cd}	1.57 ± 0.06^{d}	1.43 ± 0.17^{b}

Notes: Different superscript letters in the same column indicate significantly different results (Duncan P < 0.05). W0: average initial weight, W21: average final weight, SGR: specific growth rate, ALG: absolute length gain, FCR: feed conversion ratio.

showed significantly different effects (P<0.05) on the final weight, SGR, ALG and FCR of all treatments. The B+Pro treatment, which was challenged with *V. parahaemolyticus* through immersion, had the best effect on final weight, SGR, ALG and FCR and was significantly different (P<0.05) from other challenge treatments and the positive control.

The results obtained in this study indicate that the application of a combination of biofloc and probiotic *P. piscicida* 1Ub can improve better growth performance in the control treatment (KB+Pro) and the B+Pro challenge test treatment with *V. parahaemolyticus* Rf^R compared to other treatments (Table 1) and was able to provide the same results as control treatment KB and KPro. This higher growth is because biofloc has a composition of nutrients such as protein, fatty acids, minerals, and vitamins that can be utilized as natural food that is always present in the culture medium (Toledo *et al.,* 2016), and contains many bioactive compounds such as carotenoids, chlorophyll, phytosterols, bromophenols, and amino acids that act as growth promoters (Kumar *et al.,* 2018). Meanwhile, probiotic 1Ub is known to be able to produce protease, lipase and amylase enzymes that help the digestive system of vannamei shrimp, making it easier to absorb nutrients for shrimp growth (Ramadhani *et al.,* 2019). The research by Nababan *et al.* (2022) showed that the supplementation of probiotic 1Ub into shrimp dietary can enhance vannamei growth rate. Similarly, the study by Hamsah *et al.* (2017) also found that the synbiotic between probiotic 1Ub and *Artemia* sp. was able to enhance the growth rate of vannamei shrimp. The effect of the combination is indicated by higher final weight value (W21), specific growth rate (SGR), absolute length gain (ALG), and reducing the feed conversion ratio (FCR).

Total *V. parahaemolyticus* Count

The observation results of the total *V. parahaemolyticus* Rf^R count (VPC) value in the rearing medium and hepatopancreas of vannamei shrimp are presented in Figure 1. VPC values (Figure 1) on day 0 before the challenge test and day 1 according to the treatment tested. On the 11th day of observation, VPC values in the rearing medium (Figure 1a) of treatment B and in the hepatopancreas of vannamei shrimp (Figure 1b) of treatments B and B+Pro were significantly lower (P<0.05) than the positive control. Furthermore, the VPC value after 21 days of challenge test in the rearing medium (Figure 1a) of B and B+Pro treatments, and in the hepatopancreas of vannamei shrimp (Figure 1b) B+Pro treatment was significantly lower (P<0.05) than the positive control.

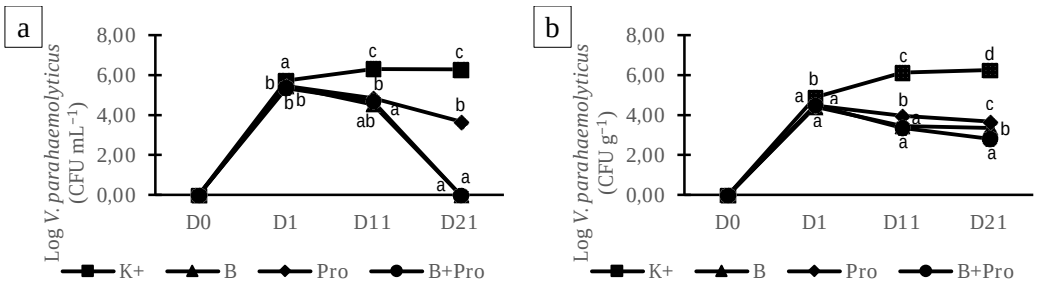

Figure 1. *V. parahaemolyticus* Rf^R bacteria in rearing medium (a) and hepatopancreas of vannamei shrimp (b) after challenge test with *V. parahaemolyticus* Rf^R by immersion. Different letters above the graph at the same observation period indicate significantly different (Duncan P<0.05).

V. parahaemolyticus bacteria have a quorum sensing (QS) mechanism as a regulator of their pathogenic activity, which allows these bacteria to communicate through signaling molecules and express certain genes simultaneously including in terms of pathogenicity and virulence (Raja *et al.,* 2017). The effect of biofloc application and its combination with probiotic bacteria 1Ub was able to significantly reduce the density of *V. parahaemolyticus* Rf^R bacteria in the rearing medium and in the hepatopancreas of shrimp (Figures 1a and 1b) compared to the positive control. The population of *V. parahaemolyticus* Rf^R bacteria in the rearing medium (Figure 1a) of treatment B and B + Pro was not observed on day 21. In line with the results of Gustilatov *et al.* (2022), the density of *V. parahaemolyticus* bacteria tested by immersion in shrimp rearing media with biofloc applications was not observed on day 9. This is because biofloc is a consisted of microalgae, bacteria, protozoa, fungi and other organic matter, which is capable of producing hydrolytic degradative enzymes as well as polymeric extracellular substances, and several antimicrobial compounds such as bromophenols, carotenoids and poly-betahydroxybutirate (Fatimah *et al.*, 2019; Kumar *et al.,* 2020), and *P. piscicida* 1Ub bacteria are reported to produce antimicrobial metabolites such as alkaloids, polyketides and peptides (Offret *et al.,* 2016) that can inhibit bacterial growth and can interfere with the QS activity of *V. parahaemolyticus.* Gustilatov *et al.* (2023) reported

that the probiotic 1Ub in the biofloc system were capable of competing with and reducing the population density of *V. parahaemolyticus*, highlighting it's potential role in pathogen control within aquaculture environments. In addition, bioflocs can act as biocontrol agents of pathogenic bacteria because they can produce extracellular compounds that interfere with bacterial intercellular communication activities, thereby reducing the virulence factor of these bacteria (Aguilera-Rivera *et al.,* 2019).

Total *P. piscicida* 1Ub Count

The results of the observation of the total value of probiotic bacteria *P. piscicida* 1Ub in the rearing medium and the intestine of vannamei shrimp are presented in Figure 2. Total bacteria *P. piscicida* 1Ub in the rearing medium (Figure 2a) was highest and significantly different ($P < 0.05$) in the KPro treatment on days 1, 11 and 21 compared to other treatments. While in the treatments tested for challenge, the value of *P. piscicida* 1Ub bacteria on day 11 in the B + Pro treatment and day 21 in the Pro treatment was significantly higher ($P < 0.05$) than the other challenge test treatments. The observation of *P. piscicida* 1Ub bacteria in the intestines of vannamei shrimp (Figure 2b) showed significant differences ($P < 0.05$), the highest value on day 1 in the KB + Pro treatment, on day 11 in the KPro and KB + Pro treatments, then on day 21 in the KPro treatment, while in the challenge test treatment there was no significant difference in each observation.

P. piscicida 1Ub bacteria are Gram-negative bacteria that are reported to produce bioactive compounds with antibacterial and antibiofilm activities that are able to damage and reduce protein material from pathogenic bacteria (Wang *et al.,* 2018). *P. piscicida* 1Ub bacteria are able to surround and adhere to *V. parahaemolyticus* bacteria, then transfer *lytic vesicles* to the bacterial cell wall which produces several holes that damage the bacterial cell, thus killing the bacteria (Richards *et al.,* 2017). This causes the addition of 1Ub bacteria to the biofloc system to be able to compete and reduce the population of *V. parahaemolyticus* Rf^R bacteria, as evidenced by the population density of *P. piscicida* 1Ub bacteria in the challenge test treatment (Figures 2a and 2b) which is higher than the density of *V. parahaemolyticus* Rf^R bacteria in the rearing media and hepatopancreas of vannamei shrimp (Figures 1a and 1b). A similar finding was also reported by Hamsah *et al.* (2019), in which the probiotic 1Ub were able to reduce the population of *V. harveyi* in the body of vannamei shrimp.

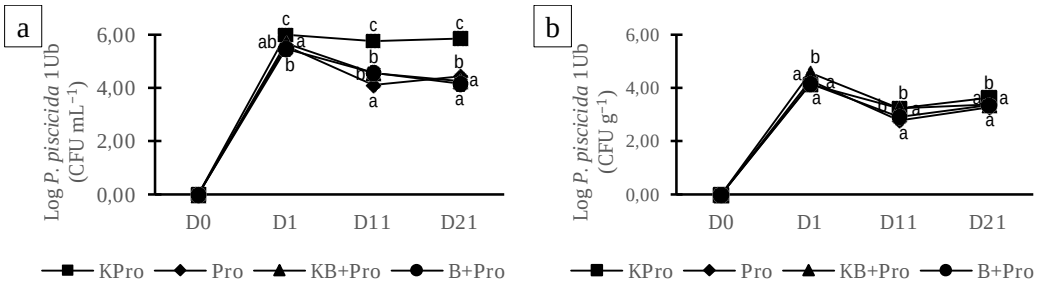

Figure 2. *P. piscicida* 1Ub bacteria in rearing medium (a) and intestine of vannamei shrimp (b) reared in control treatment (KPro, KB + Pro) and challenge treatment (Pro, B + Pro) with *V. parahaemolyticus* by immersion. Different letters above the graph at the same observation period indicate significantly different (Duncan $P < 0.05$).

Total *Vibrio* sp. Count

The observation of the total *Vibrio* sp. count (TVC) value showed an increase in TVC values starting from day 1 and 11 compared to day 0 in all control and challenge treatments in the rearing medium and vannamei shrimp intestine (Table 2). Whereas, on day 21, TVC values decreased compared to day 11, especially in the rearing medium of treatment B, which was tested for *V. parahaemolyticus* challenge through immersion, and the intestines of vannamei shrimp treated with KB + Pro compared to other treatments ($P < 0.05$).

Vibrio sp. bacteria are bacteria that exist in the marine environment naturally and are a type of bacteria that have opportunistic properties, which can become pathogenic if environmental and host conditions deteriorate (Wicaksono *et al*., 2020). The competitive effect of *P. piscicida* 1Ub and biofloc bacteria against *V. parahaemolyticus* Rf^R with extracellular products that are antagonistic to these bacteria also has an impact on reducing the value of TVC in the rearing media and the body of vannamei shrimp. The results of the study by Gustilatov *et al*. (2022), where the application of a biofloc system in vannamei shrimp culture not only led to a reduction in the density of *V. parahaemolyticus* but also resulted in a significant decrease in the overall TVC.

Health Status

Observations of the health status of vannamei shrimp tested with *V. parahaemolyticus* for 21 days, showed that treatments B, Pro and B+Pro were able to provide higher THC, PA, RB and PO values and significantly different (P<0.05) compared to the positive control (Figure 3). THC values (Figure 3a) on days 1 and 11 of treatments B, Pro and B+Pro were significantly different (P<0.05) compared to the positive control. PA values (Figure 3b) on day 1 of B and B+Pro treatments, then day 11 and 21 of B+Pro treatment were significantly different (P<0.05) compared to the positive control. RB values (Figure 3c) on day 1 of B, Pro and B+Pro treatment, day 11 of B treatment and day 21 of Pro and B+Pro treatment were significantly different (P<0.05) compared to the positive control. PO values (Figure 3d) on day 1 of Pro treatment, day 11 of Pro and B+Pro treatment and day 21 of Pro treatment were significantly different (P<0.05) compared to the positive control.

Table 2. Total *Vibrio* sp. count in rearing media and intestine of vannamei shrimp reared in biofloc system, probiotics and their combination during 21 days of rearing

Treatments	Rearing media (Log CFU mL^{-1}) on Day			
	D0	D1	D11	D21
K-	3.58 ± 0.05^{c}	3.69 ± 0.06^{a}	5.14 ± 0.05^{a}	5.90 ± 0.04^{c}
K+	3.62 ± 0.03^{c}	6.03 ± 0.03^{e}	6.47 ± 0.04^{e}	6.63 ± 0.07^{d}
KB	3.30 ± 0.04^{b}	5.22 ± 0.13^{d}	5.85 ± 0.04^{c}	5.49 ± 0.07^{b}
B	3.26 ± 0.10^{ab}	6.30 ± 0.01^{f}	6.30 ± 0.01^{d}	5.34 ± 0.08^{a}
KPro	3.77 ± 0.03^{d}	4.43 ± 0.08^{b}	6.25 ± 0.01^{d}	5.89 ± 0.02^{c}
Pro	3.66 ± 0.05^{cd}	6.36 ± 0.04^{f}	6.32 ± 0.05^{d}	5.53 ± 0.12^{b}
KB+Pro	3.15 ± 0.13^{a}	4.66 ± 0.06^{c}	5.51 ± 0.08^{b}	5.47 ± 0.08^{b}
B+Pro	3.19 ± 0.11^{ab}	6.28 ± 0.03^{f}	6.28 ± 0.03^{d}	5.50 ± 0.04^{b}
Treatments	Shrimp intestinal (Log CFU g^{-1}) on Day			
	D0	D1	D11	D21
K-	4.48 ± 0.09^{b}	4.77 ± 0.07^{a}	5.28 ± 0.04^{a}	5.90 ± 0.07^{e}
K+	4.49 ± 0.06^{b}	5.95 ± 0.02^{c}	6.15 ± 0.14^{e}	6.66 ± 0.02^{f}
KB	4.64 ± 0.06^{cd}	5.49 ± 0.07^{b}	5.85 ± 0.04^{d}	5.63 ± 0.05^{d}
B	4.26 ± 0.10^{a}	6.14 ± 0.03^{d}	6.04 ± 0.08^{e}	5.47 ± 0.08^{c}
KPro	4.65 ± 0.07^{cd}	5.89 ± 0.08^{c}	5.89 ± 0.02^{d}	5.73 ± 0.07^{d}
Pro	4.66 ± 0.05^{cd}	6.38 ± 0.02^{e}	5.73 ± 0.08^{c}	5.89 ± 0.05^{e}
KB+Pro	4.53 ± 0.05^{bc}	5.88 ± 0.06^{c}	5.47 ± 0.08^{b}	4.77 ± 0.09^{a}
B+Pro	4.45 ± 0.12^{b}	7.23 ± 0.03^{f}	5.62 ± 0.04^{c}	5.35 ± 0.02^{b}

Notes: Different *superscript* letters in the same column indicate significantly different results (Duncan P<0.05).

The observation of total haemocyte count (THC) (Figure 3a) of vannamei shrimp reared in biofloc system, probiotics and their combination showed significant differences. Especially in the challenge test treatment with *V. parahaemolyticus* bacteria, the THC values of treatments B, Pro and B + Pro were higher than the positive control on days 1 and 11. Foreign particles that enter the shrimp body will be recognized by hemocytes, which are the cellular defense system in shrimp and responded through several mechanisms such as intracellular, phagocytosis, encapsulation, and nodular aggregation (Hamsah *et al.,* 2019). Elevated hemocyte levels may enhance the shrimp's ability to overcome pathogen infection through phagocytosis activity (PA), which is the primary defense system against pathogens in shrimp bodies (Gustilatov *et al.,* 2022). Phagocytosis is the process of swallowing foreign particles carried out by hemocyte cells to protect the host from pathogens (Suleman *et al.,* 2019). The PA value (Figure 3b) of the KB+Pro treatment shrimp and the B+Pro challenge test treatment on days 11 and 21 produced the highest value and was significant compared to the positive control.

Bioflocs are mostly composed of bacteria containing lipopolysaccharides and peptidoglycans, fungi containing â-glucans, and other microorganisms, and *P. piscicida* 1Ub bacteria are Gram-negative bacteria whose cell membranes contain lipopolysaccharides. All components within the biofloc and probiotic 1Ub are identified as pathogen-associated molecular patterns (PAMPs) (Kim *et al*., 2014), which are then recognized by pattern recognition receptors (PRRs) as a receptors to further stimulate an increase in the number of hemocytes along with a non-specific immune

response that will activate the regulation of cytokines and antimicrobial molecules in the shrimp body to provide defense against incoming foreign bodies from spreading throughout the shrimp body (Habib *et al.,* 2021; Gustilatov *et al.,* 2023). One type of PRRs found in shrimp is the Lipopolysaccharide and β-1,3-glucan Binding Protein (LGBP), with an estimated molecular weight of 30–45 kDa. LGBP expression is inducible upon exposure to Gram-negative bacteria, suggesting its role in early immune recognition. Upon ligand binding, LGBP activates the prophenoloxidase (proPO) cascade, contributing to the non-self-recognition mechanism in the innate immune system (Yang *et al.,* 2015).

Humoral immune responses in shrimp are shown through respiratory burst (RB) activity. RB is an advanced process of phagocytosis and produces reactive oxygen intermediates (ROI: hydrogen peroxide H_2O_2, singlet oxygen $1O_2$, hydroxyl radical OH, and other reactive compounds) that possess microbicidal and radical properties to destroy foreign particles by involving the release of degradative enzymes into phagosomes (Rodriguez & Mullac, 2000; Tassanakajon *et al.*, 2013). The RB measurement results (Figure 3c) on day 21 in the KB + Pro treatment and the Pro and B + pro challenge test treatments had significantly higher RB values compared to the positive control. The higher the RB value indicates a good immune system (Pardede *et al.,* 2023). The next humoral immune response in shrimp is through phenoloxidase (PO) activity. PO is a precursor to melanin formation that inactivates and prevents the spread of pathogens in the shrimp body by coating pathogens using melanized particles (Kulkarni *et al.,* 2021). Overall, the observation of PO values (Figure 3d) in the Pro and B + Pro challenge test treatments resulted in the highest values compared to other treatments. According to Gustilatov *et al.* (2023), RB and PO activities in biofloc applications and their combination with 1Ub probiotics are able to protect shrimp from *V. parahaemolyticus* bacterial infection and neutralize radical compounds due to the elimination process of these bacteria.

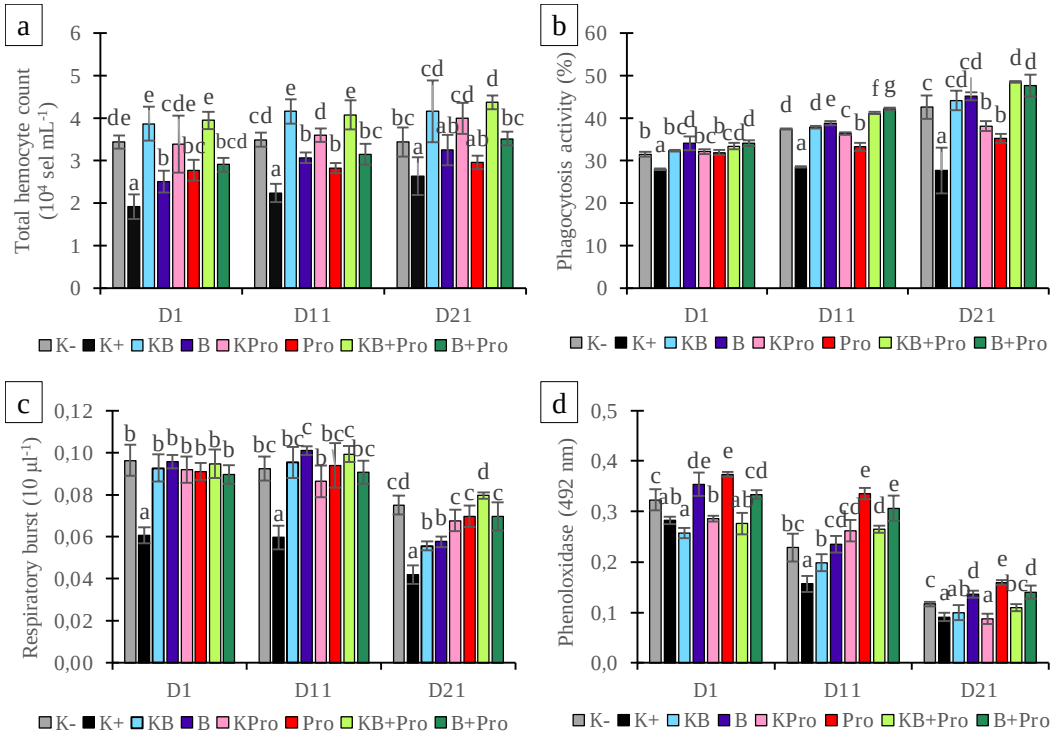

Figure 3. Total haemocyte count (a), phagocytosis activity (b), respiratory burst (c), and phenoloxidase (d) of vannamei shrimp reared in biofloc system, probiotics and their combination. Different letters above the bar at the same observation period indicate significantly different (Duncan $P < 0.05$).

Histopathology of Hepatopancreas

Histopathological observations on the hepatopancreas of vannamei shrimp reared for 21 days (Figure 4) showed that the level of hepatopancreas damage in the treatment tested with *V. parahaemolyticus* Rf^R was less severe than the positive control treatment. Hepatopancreas damage in the positive control treatment was indicated by tubule necrosis, vacuolization, B cell necrosis, and enlarged lumen due to *V. parahaemolyticus* Rf^R infection.

The percentage of necrosis that occurs in the hepatopancreas organs in all treatments can be seen

in Table 3. After observation and then classified showed that all control treatments without challenge test had normal damage. Treatment B and B + Pro tested challenged with *V. parahaemolyticus* Rf^R had mild damage, then Pro treatment had moderate damage, while in the treatment K + shrimp hepatopancreas had severe damage.

Shrimp hepatopancreas is the main target organ of *V. parahaemolyticus* bacteria (Wang *et al.,* 2020). Reduced damage to the hepatopancreas organs in treatments B and B + Pro compared to the positive control treatment was due to the application of bioflocs and their combination with probiotic 1Ub was able to reduce the population of *V. parahaemolyticus* and improve the health status of shrimp so as to minimize damage to the hepatopancreas of shrimp. This is consistent with the findings reported by Aguilera-Rivera *et al*. (2014), which showed that the application of probiotics in a biofloc system prevents pathogenic bacteria from disrupt-

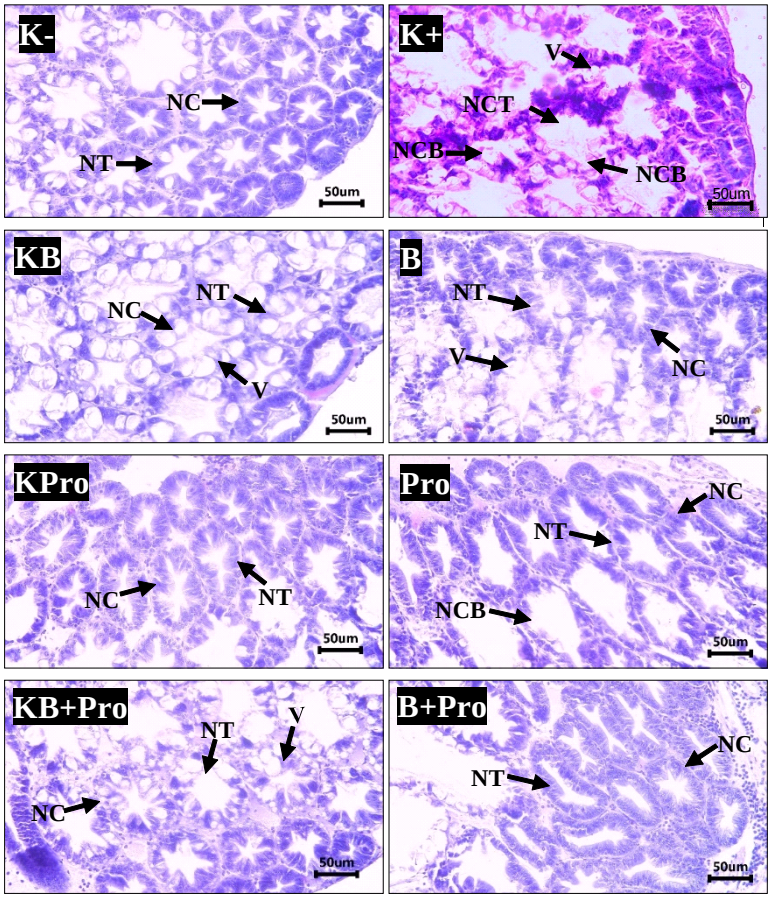

Figure 4. Histopathology of hepatopancreas organs of vannamei shrimp treated without challenge (K-, KB, KPro, KB+Pro) and treated with challenge (K+, B, Pro, B+Pro) with *V. parahaemolyticus* Rf^R through immersion for 21 days. Arrows indicate normal tubules (NT), necrotizing B cells (NCB), vacuoles (V), normal cells (NC), and necrotizing tubules (NCT).

Table 3. Scoring values of hepatopancreas organ damage in vannamei shrimp reared for 21 days

Treatments	Necrosis	Score	Level of damage
K-	11.86%	0	Normal
K+	73.81%	3	Severely damaged
KB	9.02%	0	Normal
B	28.01%	1	Lightly damaged
KPro	10.85%	0	Normal
Pro	51.36%	2	Moderately damaged
KB+Pro	13.94%	0	Normal
B+Pro	25.98%	1	Lightly damaged

Description: $P < 20\%$ (score 0, normal), $20\% \leq P < 40\%$ (score 1, lightly damaged), $40\% \leq P < 60\%$ (score 2, moderately damaged), $60\% \leq P < 80\%$ (score 3, severely damaged), $P \geq 80\%$ (score 4, severely damaged).

ing the balance of the microbial community, thereby failing to massively infect the shrimp and cause severe damage to the hepatopancreas tissue as one of the key organs involved in the immune response of vannamei shrimp.

Water Quality

The results of water quality measurements are presented in Table 4. The parameters TAN, nitrite (NO_2^-) on day 0, 11 and 21 observations and nitrate (NO_3^-) on day 21 observations in the biofloc system treatment showed significantly lower values ($P<0.05$) than the control and probiotic treatments. While TSS values in all biofloc treatments were significantly higher ($P<0.05$) than the control and probiotic treatments.

The addition of a carbon source with an appropriate C:N ratio to the biofloc system can increase the population of heterotrophic bacteria that have the ability to convert and reduce toxic nitrogen concentrations in the aquaculture environment into microbial biomass (Kumar *et al.,* 2018). This can be seen from the results of water quality observations (Table 4) which showed nitrogen parameters (TAN, nitrite, nitrate) were generally lower in the biofloc treatment compared to the control and probiotic treatments. Wei *et al.* (2016), stated that the reduction of nitrogen in the biofloc system was caused by nitrogen uptake by phototrophic organisms and was subsequently dominated by the assimilation process carried out by heterotrophic bacteria to form flocs.

Table 4. Chemical parameters of water quality of vannamei shrimp rearing media in biofloc system, probiotics and their combination during 21 days of rearing

Observation days	Treatments	TAN (mg L^{-1})	NO_2^- (mg L^{-1})	NO_3^- (mg L^{-1})	TSS (mg L^{-1})
Day-0	K-	0.007 ± 0.003^{a}	0.132 ± 0.012^{b}	0.098 ± 0.005	40.0 ± 8.00^{a}
	K+	0.009 ± 0.002^{a}	0.154 ± 0.033^{b}	0.098 ± 0.014	44.0 ± 4.00^{a}
	KB	0.040 ± 0.018^{b}	0.507 ± 0.029^{c}	0.122 ± 0.012	106.7 ± 8.33^{b}
	B	0.040 ± 0.009^{b}	0.464 ± 0.018^{c}	0.113 ± 0.007	112.0 ± 14.42^{bc}
	KPro	0.010 ± 0.003^{a}	0.059 ± 0.040^{a}	0.094 ± 0.024	38.7 ± 8.33^{a}
	Pro	0.012 ± 0.003^{a}	0.091 ± 0.009^{a}	0.108 ± 0.017	39.0 ± 2.65^{a}
	KB+Pro	0.050 ± 0.008^{b}	0.472 ± 0.012^{c}	0.111 ± 0.017	122.7 ± 8.33^{cd}
	B+Pro	0.040 ± 0.009^{b}	0.496 ± 0.009^{c}	0.113 ± 0.011	130.7 ± 6.11^{d}
Day-11	K-	0.385 ± 0.015^{b}	0.537 ± 0.046^{b}	0.871 ± 0.045	89.3 ± 8.33^{a}
	K+	0.392 ± 0.014^{b}	0.499 ± 0.012^{b}	0.907 ± 0.009	90.7 ± 6.11^{a}
	KB	0.238 ± 0.021^{a}	0.094 ± 0.035^{a}	0.864 ± 0.046	324.0 ± 8.00^{c}
	B	0.248 ± 0.020^{a}	0.074 ± 0.049^{a}	0.907 ± 0.066	302.7 ± 8.33^{b}
	KPro	0.399 ± 0.011^{b}	0.463 ± 0.055^{b}	0.898 ± 0.096	85.3 ± 8.33^{a}
	Pro	0.395 ± 0.026^{b}	0.528 ± 0.057^{b}	0.862 ± 0.070	88.0 ± 8.00^{a}
	KB+Pro	0.226 ± 0.012^{a}	0.098 ± 0.034^{a}	0.897 ± 0.061	321.3 ± 10.07^{c}
	B+Pro	0.244 ± 0.021^{a}	0.091 ± 0.033^{a}	0.883 ± 0.010	324.0 ± 8.00^{c}
Day-21	K-	0.552 ± 0.028^{b}	0.564 ± 0.051^{b}	3.025 ± 0.154^{b}	148.0 ± 4.00^{a}
	K+	0.559 ± 0.037^{b}	0.552 ± 0.015^{b}	2.981 ± 0.123^{b}	143.3 ± 21.20^{a}
	KB	0.221 ± 0.025^{a}	0.138 ± 0.057^{a}	1.439 ± 0.042^{a}	437.3 ± 10.07^{b}
	B	0.182 ± 0.015^{a}	0.144 ± 0.057^{a}	1.451 ± 0.221^{a}	454.7 ± 12.22^{bc}
	KPro	0.513 ± 0.021^{b}	0.525 ± 0.025^{b}	2.901 ± 0.101^{b}	144.0 ± 10.58^{a}
	Pro	0.540 ± 0.043^{b}	0.554 ± 0.035^{b}	2.895 ± 0.242^{b}	148.0 ± 4.00^{a}
	KB+Pro	0.193 ± 0.042^{a}	0.133 ± 0.021^{a}	1.443 ± 0.256^{a}	469.3 ± 10.07^{c}
	B+Pro	0.205 ± 0.014^{a}	0.126 ± 0.042^{a}	1.362 ± 0.089^{a}	456.0 ± 8.00^{bc}

Shrimp Survival

The results of observations of the survival rate of vannamei shrimp after the challenge test with *V. parahaemolyticus* Rf^R after 21 days are presented in Figure 5. Vannamei shrimp reared in the KB and KB+Pro treatments gave a significantly different effect ($P<0.05$) compared to other treatments. While in the *V. parahaemolyticus* challenge test treatment through immersion, the B+Pro treatment showed higher survival rates and significantly different ($P<0.05$) compared to other challenge test treatments and positive controls (Figure 5).

The positive role of the combination of biofloc and probiotics 1Ub is able to improve the growth performance of vannamei shrimp, can suppress the growth of bacteria *V. parahaemolyticus* Rf^R and increase the immune response so that it has an impact on the survival rate of shrimp (Figure 5) tested challenged with *V. parahaemolyticus* Rf^R. This is evidenced by the survival rate of vannamei shrimp in the B + Pro combination treatment which is higher than the other challenge test treatments and the positive control. This effect may also be attributed to the ability of *P. piscicida* 1Ub to persist in the culture environment and shrimp intestine in the challenged treatment. The study conducted by He *et al*. (2023), reported that the addition of probiotics to the biofloc system can enhance the survival rate of vannamei shrimp. Furthermore, Gustilatov *et al*. (2023), also demonstrated that the combination of probiotic 1Ub and the biofloc system can further improve the survival rate of vannamei shrimp when challenged with *V. parahaemolyticus*.

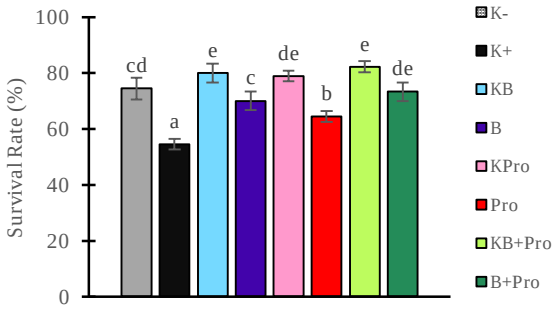

Figure 5. Survival of vannamei shrimp reared in biofloc system, probiotics and their combination with treatment without challenge test (K-, KB, KPro, KB+Pro) and treatment with challenge test (K+, B, Pro, B+Pro) with *V. parahaemolyticus* through immersion for 21 days. Different letters above the bar indicate significantly different (Duncan $P<0.05$).

CONCLUSION

The combination of biofloc system and probiotic bacteria *P. piscicida* 1Ub can provide the best growth performance, reduce the population of *V. parahaemolyticus* Rf^R in rearing media and shrimp hepatopancreas, optimize immune response and increase survival rate of vannamei shrimp when infected with *V. parahaemolyticus* Rf^R bacteria compared to only biofloc or probiotics treatment.

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