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GROWTH, IMMUNITY, AND RESISTANCE OF CATFISH (*Clarias* sp.) REARED IN BIOFLOC SYSTEM SUPPLEMENTED WITH *Bacillus* NP5 AGAINST *Aeromonas hydrophila* INFECTION

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ABSTRACT

Catfish *Clarias sp.* is one of the leading commodities in demand and has economic value. Low survival due to cannibalism and disease is the main constrain of *Clarias sp.* hatchery. One of the way to overcome this problem by the application of biofloc supplemented with probiotic *Bacillus* NP5. This study aimed in evaluating the appropriate dose of *Bacillus* NP5 probiotic supplemented to the biofloc system to improve the health status, growth performances, water quality, and resistance to *A. hydrophila*. The catfish fry (*Clarias sp.*) with an average weight of 1.79 ± 0.05 g were reared in tanks with a dimension of 60 × 30 × 35 cm^3. The experiment applied a completely randomized design (CRD) consisted of five treatments with three replicates which were negative control (KN), positive control (KP), biofloc without probiotic bacteria (BFT), biofloc supplemented with *Bacillus* NP5 10^4 CFU mL^{-1}(BFT4), biofloc supplemented with *Bacillus* NP5 10^6 CFU mL^{-1}(BFT6). Treatments tested were challenged with *A. hydrophila* at density 10^4 CFU mL^{-1} by immersion, except the negative control. After 40 days of rearing, biofloc supplemented with *Bacillus* NP5 treatments had a significant effect ($p < 0.05$) on growth performance, immune response, water quality, total bacteria in water and the gut compared to the positive control and negative control ($p < 0.05$). In addition, total *A. hydrophila* in liver, kidney and water were lower ($p < 0.05$) in BFT4 and BFT6 treatments than the controls. As the conclusion, the bioflocs supplemented with *Bacillus* NP5 improved the growth performance, immune response and resistance of catfish to *A. hydrophila* infection.

KEYWORDS: *Aeromonas hydrophila*; *Bacillus* NP5; Biofloc; *Clarias* sp.

INTRODUCTION

African catfish (*Clarias* sp.) is one of the important aquaculture commodities in Indonesia, with a production of 1.04 million tons in 2021 and an increase of 32% in 2022 (Indonesian Ministry of Marine Affairs and Fisheries, 2022). The production target for catfish in 2024 is 1.65 million tons. This can be achieved through an intensification system, which requires high fry quality and quantity. However, intensification activities have an impact on increasing organic matter, metabolic products (feces and ammonia), and uneaten feed (Khanjani *et al*., 2023), this affects high nitrogen concentrations, reduces environmental quality, and stresses the fish, which negatively affect growth performance, immunity, and disease, leading to low survival.

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One of the diseases identified to infect African catfish is *Motile Aeromonas Septicaemia* (MAS) disease, caused by *Aeromonas hydrophila* (Sharma *et al*., 2021). The *A. hydrophila* is an opportunistic bacterium, motile, facultatively anaerobic, and found in aquatic environments (Rai *et al*., 2023). Infection of this bacteria causes economic losses to fish farmers due to the high mortality up to 80% within 1-2 weeks (Abdelrahman *et al*., 2023). The use of antibiotics to control bacterial infections in aquaculture makes the fish resistant, produces residual effects, and disrupts the balance of bacteria in aquaculture environment (Chen *et al*., 2021). Therefore, environmentally intensive aquaculture technology is needed, one of which is by the use of biofloc and probiotics to support high growth and productivity and to fulfill the targets of fish farmers.

Bioflocs Technology (BFT) is an alternative solution in aquaculture to enhance water quality, growth, health status, and disease resistance (Azimi *et al*., 2022; Hassan *et al*., 2022). This technology has the

principle of converting nitrogenous wastes from uneaten feed and metabolic by-products into microbial biomass by maintaining a certain C/N ratio and adding carbon sources to the water or modified the feed formulation (Ekasari *et al*., 2023). Bioflocs consist of an aggregation of living and dead particles, including bacteria, microalgae, fungi, zooplankton, and other organic matter that can be utilized by cultured organisms (Ogello *et al*., 2021). Several studies have shown that the positive effects of biofloc application can increase fish biomass, survival, immune response, reduce cannibalism even at high densities, and reduce pathogen populations, especially *A. hydrophila* in water and fish gut which has further effects on increasing productivity (Kim *et al*., 2014; Dauda *et al*., 2018; Pérez-Fuentes *et al*., 2018).

Probiotics and bioflocs as a combination treatment has also been reported to improve feed utilization, the immune system, bacterial diversity in maintaining water stability, degrading nitrogen components in water, and resistance to bacterial infections (Amjad *et al*., 2022; Haraz *et al*., 2023). *Bacillus* NP5 can act as probiotic (Putra & Widanarni, 2014). This bacteria is a gram-positive, rod-shaped bacterium that has been tested against *Streptococcus agalactiae, Vibrio parahaemolyticus*, and *Vibrio harveyi (*Agung *et al*., 2015; Muharrama *et al., 2022*). The application of *Bacillus* NP5 and prebiotic mannan oligosaccharides can increase immune response and resistance to *A. hydrophila* infection (Tamamdusturi *et al*., 2016). In addition, the combination of *Bacillus* NP5 and *Artemia* sp. can reduce the coefficient diversity of fish length and suppress cannibalism in *Pangasianodon hypophthalmus* (Widanarni *et al*., 2022). Research on the addition of *Bacillus* NP5 to BFT system needs to be conducted to evaluate its effect on growth performances, immune response, and catfish resistance to *A. hydrophila*.

MATERIALS AND METHODS

Experimental Diets and Design

Bacillus NP5 Rf^R and *Aeromonas hydrophila* Rf^R that used in this study were resistant to the antibiotic rifampicin (Rf^R) at dose of 50 µg mL^{-1}. Both types of bacteria were collected from the Aquatic Organism Health Laboratory, Department of Aquaculture, Faculty of Fisheries and Marine Science, Bogor Agricultural University. The experiment was designed into a completely randomized design (CRD) consisting of five treatments with three replicates. The treatments were negative control (KN), positive control (KP), biofloc application without *Bacillus* NP5 addition (BFT), biofloc-based with cells addition of *Bacillus* NP5 10^4 CFU mL^{-1} (BFT4), biofloc-based with cells addition of *Bacillus* NP5 10^6 CFU mL^{-1} (BFT6). All treatments were challenged with *A. hydrophila* at a density of 10^4 CFU mL^{-1} (based on LC $test_{50}$) by immersion, except the negative control.

Experimental Fish and Feeding Management

The test fish were catfish fry (*Clarias* sp.) obtained from fish farmers in Bogor. The average length and weight of the fish were 4.54 ± 0.11 cm and 1.79 ± 0.05 g, respectively. The fish were reared in aquarium with the dimension of 60 × 30 × 35 cm^3 as many as 15 aquariums, with a rearing density of 50 fish per aquarium. Before being used, the aquarium was throughly washed and disinfected using 30 ppm chlorine. The aquarium was then filled with 45 L of water. The fish were reared for 40 days and fed with the commercial feed (Hi Pro Vite 781) twice a day (07.00 and 16.00 WIB) to satiation.

Bacterial and Biofloc Preparation

Before being used, *Bacillus* NP5 and *A. hydrophila* bacteria were made resistant to rifampicin antibiotics (Rf^R) as the antibiotic resistance markers to monitor the bacterial population in water and gut of catfish fry. Both bacteria were then cultured into *t*ryptic soy agar (TSA) media which were added with rifampicin antibiotic at dose of 50 µg mL^{-1} at 29R"C for 18 hours. The growing colonies were then inoculated into 25 mL of tryptic soy broth (TSB) media and incubated in a shaker at a speed of 160 rpm for 24 hours at 29R"C. Following this, *Bacillus* NP5 was added according to the target density applied in each treatment. *Bacillus* NP5 was supplemented into the rearing medium once a week. In term of biofloc preparation, flocs were first grown in separated fiber tanks for seven days with the addition of organic carbon source which was molasses with an estimated C/N ratio of 15 (Ekasari *et al*., 2014). After the floc is formed, then floc is added to the aquarium as much as 10% of the volume of the rearing aquarium. The amount of molasses added to the rearing aquarium was calculated based on the equation of De Schryver *et al*. (2008). The C/N ratio was calculated based on the following assumptions: feed protein is 31%; 16% of protein is nitrogen; approximately 75% of ingested N is excreted by the fish; and 38% of molasses is carbon. Then, the amount of molasses added to the aquarium was 147.05 g.

Sample Collection

Water quality parameters including dissolved oxygen (DO), pH, temperature,volume floc were measured daily using a DO meter, while total ammonia nitrogen (TAN), nitrite, and nitrate were measured every 20 days. Sampling of weight, blood, gut, liver

and kidney were taken every 10 days for observation of immune response and bacterial population. Histopathology of the kidney and liver organs were sampled at the end of the study. The observations of these parameters were performed at the Aquatic Organism Health Laboratory, Department of Aquaculture, Bogor Agricultural University.

Data Calculation

Growth Performances

Growth performances were calculated using the following formula: Survival rate (SR) (%) = (final fish number/initial fish number) × 100; Specific Growth Rate (SGR) (%) = [(ln final fish weight (g) - ln initial fish weight (g))/duration of the experiment (days)] × 100; Absolute length gain (cm) = final fish length – initial fish length (Effendi, 1997). Coefficient of variation (%) = standard deviation/sample mean × 100 (Steel & Torrie, 1993). Feed Conversion Ratio (FCR) = feed intake/(final weight (g) - initial weight (g)) (Zokaeifar *et al*., 2012).

Hematological and Immune Response

The total erythrocytes and leukocytes were calculated using the Blaxhall & Daisley method (1973). Concentration of hemoglobin were measured according to the Wedemenyer & Yasutake method (1977). Hematocrit, phagocytic activity, and respiratory burst were calculated according to Anderson & Siwicki (1995) procedures.

Total Bacterial Count and Histopathology

Calculation of total bacterial cells, *Bacillus* NP5, and *A. hydrophila* in organs and rearing media following the Zhao *et al*. (2021) method. Gut, liver, and kidney organs were pulverized approximately 0.1 g and dissolved in 1 mL of sterile PBS. The rearing media was taken approximately 1 mL for bacterial analysis. TSA medium was used for counting total bacteria, TSA medium+Rif and Rimmler-Shotts (RS) medium+Rif for counting of *Bacillus* NP5 and *A. hydrophila,* respectively. Method performed for histopathology followed Hossain *et al*. (2007), by taking kidney and liver organs from each treatment. The organs were fixation with BNF solution for 24 hours. The organs that has been fixed was cut into 1×1 cm thick pieces of 3-5 mm and put into a histology tissue cassette. Dehydrate the sample with graded alcohol gradually. After that, the sample was cleared by soaking it in xylol solution in stages, xylol I, xylol II, xylol III each for 30 minutes. The samples were immersed in liquid paraffin in stages, each for 45 minutes. Samples were removed and put into molds that had contained liquid paraffin and frozen. Tissue blocks were cut using a microtome with a thickness of 35 mm. The cut results are placed in water to be attached to a glass object and dried. Staining was done by immersing the glass object using xylol I and xylol II in stages, each for 5 minutes. Next, the samples were immersed using graded alcohol in stages, 100% I, 100% II, 95%, 90%, 80%, and 70%, each for 3 minutes, then immersed with hematoxylin and eosin, each for 5 minutes and then rinsed with running water. Next, it was soaked again with graded alcohol for 3 minutes and xylol solution for 5 minutes. Then cover the preparation with a cover glass by dripping with entellan rapid mounting medium and dried for 24 hours. Observations were made using a microscope.

Data Analysis

Data on growth performances, hematological and immune response, and total bacterial count were analyzed using one-way ANOVA using SPSS software version 22. If the data were significantly different ($p < 0.05$), continued with the Tuckey test at a 95% confidence interval. Data on histopathology were descriptively analyzed.

RESULTS AND DISCUSSIONS

Growth Performances

The growth performance of catfish observed during 40 days of rearing are presented in Table 1. The absolute length gain and SGR values were significantly higher ($p < 0.05$) in the BFT, BFT4 and BFT6 treatments compared to the controls. The addition of probiotic *Bacillus* NP5 showed a lower coefficient of variation ($p < 0.05$) compared to the controls. Furthermore, fish reared in the biofloc system also showed significant differences in FCR values ($p < 0.05$) with the controls. The lowest FCR was found in the BFT4 treatment (0.92 ± 0.04) presumably due to the abundance of floc particles in the media as an additional feed that can be utilized by fish to support the growth.

The ability of *Bacillus* to colonize in the gut of fish will increase the production of organic acids, trigger digestive enzymes, and further have a positive effect on the absorption of nutrients in the feed (Adeoye *et al*., 2016). The presence of this enzyme in the gut will hydrolyze protein into amino acids, carbohydrates into glucose, fat into fatty acids resulting an increase in growth rate and feed efficiency. Based on research by Mirzakhani *et al*. (2019), bioflocs and probiotics can increase the number of goblet cells and the length of microvilli so that the absorbed nutrients can be improved.

Table 1. Growth performances of *Clarias* sp. reared in control, biofloc without probiotic bacteria, and biofloc supplemented with *Bacillus* NP5 system at 40 days of the experimental period.

Treatments	SGR (%)	Absolute Length Gain (cm)	FCR	SR (%)	Coefficient of Variation (%)
KN	2.72 ± 0.09^{b}	2.98 ± 0.04^{a}	1.22 ± 0.01^{a}	70 ± 1.41^{b}	2.58 ± 0.012^{b}
KP	2.55 ± 0.06^{a}	2.97 ± 0.06^{a}	1.27 ± 0.03^{a}	55 ± 3.00^{a}	3.17 ± 0.023^{a}
BFT	3.37 ± 0.09^{c}	4.30 ± 0.02^{b}	1.04 ± 0.03^{b}	84 ± 2.45^{c}	2.48 ± 0.003^{c}
BFT4	4.27 ± 0.03^{e}	4.31 ± 0.03^{b}	0.92 ± 0.02^{c}	90 ± 1.41^{d}	1.86 ± 0.005^{e}
BFT6	4.00 ± 0.05^{d}	4.17 ± 0.04^{b}	0.94 ± 0.03^{c}	87 ± 1.00^{cd}	2.14 ± 0.008^{d}

Description: Different superscript letters following the mean (± SD) on the same column indicate significant differences ($P < 0.05$). specific growth rate (SGR), feed conversion ratio (FCR), survival rate (SR).

Biofloc and *Bacillus* NP5 treatments increased fish survival ranging from 84 - 90% and showed significant differences with the controls ($p < 0.05$) (Table 1). The lowest survival was obtained in the KP treatment. In line with the research of He *et al*. (2023) that the provision of *Bacillus* into biofloc resulted in a survival rate of up to 93%. The low survival rate in the positive control was indicated by the cannibalism factor in catfish. This is due to the infection of pathogenic bacteria to the KP treatment fish causing stress. This condition can decrease fish appetite and growth of more diverse fish. The larger fish will survive and eat the smaller fish. This is also supported by the data on the coefficient of variation of fish length in the KP treatment which is higher than the BFT, BFT4, and BFT6 treatments. High coefficient of variation of fish length indicates high size variation in catfish which can cause cannibalism. This cannibalized condition of fish has an negative impact on the survival rate in aquaculture tanks (Onwuteaka & Prince 2015). In addition, the low survival rate in the positive control was sucpected to be due to pathogen infection which resulted in a weakened immune system that can be seen from symptoms such as body discoloration, fin demage, hemorrhage in the abdomen, ulcers on the head. According to Ahmad *et al*. (2016), fish that consume a collection of microbial flocs containing *pathogen associated molecular patterns* (PAMPs) such as α-1, 3 glucans, peptidoglycans and lipopolysaccharides cause activation of the non-specific immune system and reduce susceptibility to *A. hydrophila* infection.

Hematological and Immune Response

Observations on the hematology and immune response of *Clarias* sp. treated with biofloc and probiotic *Bacillus* NP5 and challenged with *A. hydrophila* showed mixed results (Figure 1&2). The addition of *Bacillus* NP5 probiotic showed better values than the controls (KN and KP).

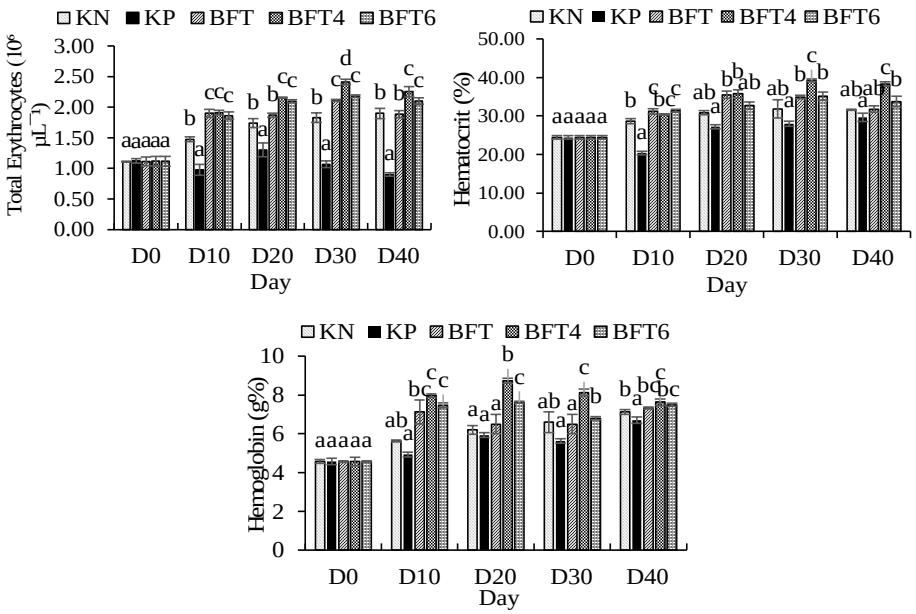

Figure 1. Hematological of *Clarias* sp reared in control, biofloc and *Bacillus* NP5 system, challenged with *A. hydrophilla.* Different letters above the bars indicate significant differences ($p < 0.05$).

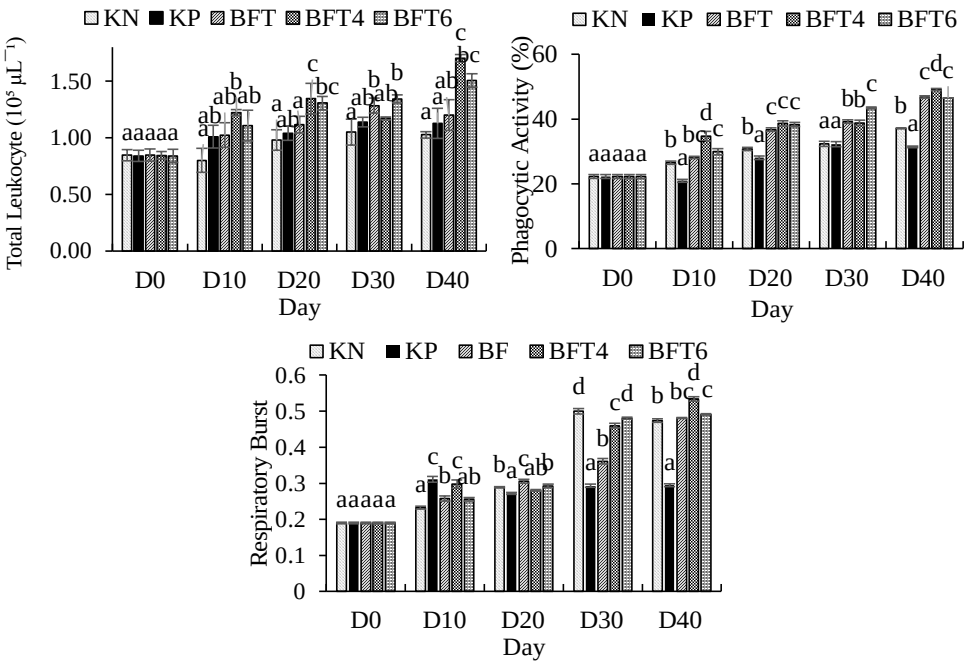

Figure 2. Immune response of *Clarias* sp. reared in control, biofloc and *Bacillus* NP5 system, challenged with *A. hydrophilla.* Different letters above the bars indicate significant differences ($p < 0.05$).

After 10 days of infection, a significant decrease ($p < 0.05$) in erythrocytes was observed in the KP treatment. On the other hand, erythrocyte values of biofloc and *Bacillus* NP5 treatments on days 10 to 30 increased and were significantly different ($p < 0.05$) from the two controls (KN and KP). A similar trend was observed in the leukocyte values of BFT4 and BFT6 treatments until the end of the study and were significantly higher ($P < 0.05$) than the controls. During fish rearing, the hemoglobin and hematocrit value of catfish treated with biofloc and probiotic *Bacillus* NP5 showed significant differences ($P < 0.05$) with the controls. BFT4 treatment has a higher value than others.

Innate immunity becomes the first defense system against pathogenic infections and leukocytes play an important role in this mechanism that will localize and eliminate pathogens (Buchmann, 2022). Bioflocs produce several components, including carotenoids, bromophenols, chlorophyll, polysaccharides, phytosterols, and taurine, which could enhance the hematology, and immune response in farmed species, stress tolerance according to Saha *et al.* (2022). A low erythrocyte count is an indicator of anemia and a decreased fish immune system. Hematocrit and hemoglobin are also strongly related to erythrocytes. Hematocrit is used to measure the percentage of the number of erythrocytes to the volume of blood in the fish body. Thus, the hematocrit value is influenced by the number of erythrocytes. Hemoglobin is an erythrocyte pigment consisting of proteins that bind and distribute oxygen throughout the body. The low number of erythrocytes is followed by a decrease in hematocrit and hemoglobin values.

In this study, immune response parameters such as phagocytic activity (AF) and respiratory burst (RB) showed significant differences ($p < 0.05$) among treatments. Probiotics work by releasing antimicrobial substances, inhibiting colonization and attachment of pathogens so that it will stimulate immune function more effectively (Fijan, 2023). Bioflocs contain microbial-associated molecular patterns (MAMPs), such as peptidoglycans and α -glucans that can stimulate immune parameters (Gustilatov *et al.*, 2022). Bioflocs added with probiotics have the potential to increase disease resistance to pathogens by stimulating cellular and humoral immune functions (Chen *et al.*, 2018).

Total Bacterial Count

The high immune response treated with biofloc and *Bacillus* NP5 apparently was influenced by the number of the various bacterial cells presented in the water and gut. The total number of bacteria in water and gut was higher ($p < 0.05$) in the biofloc and probiotic treatments compared to the two controls (KN and KP) (Table 2). Meanwhile, the abundance of *Bacillus* NP5 bacteria in water and gut at initial and final rearing period ranged from 10^4-10^6 CFU mL^{-1}. Microbial communities in floc contains algae, plankton, bacteria and other beneficial microorganisms, which can promote the self protection of gut barrier, eliminate pathogens through competitive binding of

substrates, while antagonizing the adhesion and colonization of pathogenic bacteria, which reduce the activity of pathogen bacteria and thus able to increase the host immune system (Crab *et al*. 2010; Yu *et al.* 2020).

After 40 days of fish rearing, the population of pathogenic *A. hydrophila* in the biofloc and probiotic treatments decreased in water, kidney and liver from 10^4 CFU mL^{-1} at initial rearing time to 10^3 CFU mL^{-1} (Figure 3). Futhermore, the population of *A. hydrophila* in the positive control fluctuated, and significantly increased on day 5 in the liver and kidney organs. At day 10, *A.hydrophila* in the positive control increased in the rearing medium. Populations of *A. hydrophila* in the liver, kidney and rearing medium showed that biofloc and *Bacillus* NP5 treatment were significantly different ($p < 0.05$) from the positive control.

A study showed that bioflocs can reduce the population of *A. hydrophila* in the kidney and liver of tilapia (Menaga *et al*., 2019). This bacteria also produce virulence factors such as adhesins, cytotoxins, aerolysins, elastase, lipase and then form biofilms and express them through bacterial intercellular communication (quorum sensing) (Ivey *et al*., 2016). From the results of this study, it is believed that BFT, BFT4, and BFT6 treatments are able to suppress bacterial growth, disrupt intercellular communication of *A. hydrophila* bacteria and reduce the virulence of these bacteria. Kumar *et al*. (2020) reported that bioflocs are able to change the phenotypic characteristics of pathogenic bacteria to become non-pathogenic so that the level of virulence decreases.

Table 2. Total bacterial and *Bacillus* NP5 count in water and gut of the *Clarias* sp.

Parameter	Day	Treatments				
		KN	KP	BFT	BFT4	BFT6
Total Bacterial Count in Water (Log CFU mL^{-1})	10	5.81 ± 0.01^a	5.94 ± 0.01^b	6.05 ± 0.02^c	6.66 ± 0.00^d	7.68 ± 0.00^e
	20	5.68 ±0.02^a	6.05 ± 0.03^b	7.62 ± 0.02^c	7.62 ± 0.01^c	7.98 ± 0.00^d
	30	6.05 ± 0.04^a	5.88 ± 0.09^a	6.60 ± 0.02^b	7.15 ± 0.04^c	7.69 ± 0.00^d
	40	6.35 ± 0.00^a	6.67 ± 0.00^b	7.15 ± 0.03^c	7.70 ± 0.00^d	8.65 ± 0.01^e
Total Bacterial Count in Gut (Log CFU mL^{-1})	10	7.25 ± 0.01^b	6.76 ± 0.05^a	8.31 ± 0.02^c	8.40 ± 0.02^c	8.38 ± 0.01^c
	20	7.67 ±0.00^b	7.01 ± 0.01^a	8.34 ± 0.01^d	8.03 ± 0.02^c	8.63 ± 0.00^e
	30	8.02 ± 0.00^b	7.05 ± 0.00^a	8.03 ± 0.01^b	8.34 ± 0.00^c	9.06 ± 0.02^d
	40	7.00 ± 0.00^a	7.22 ± 0.01^b	8.17 ± 0.03^c	8.37 ± 0.00^d	9.17 ± 0.01^e
Total *Bacillus* NP5 in Water (Log CFU mL^{-1})	10	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	5.72 ± 0.03^b	6.97 ± 0.01^c
	20	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	4.95 ± 0.03^b	7.47 ± 0.01^c
	30	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	4.13 ± 0.01^b	6.22 ± 0.01^c
	40	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	4.37 ± 0.00^b	6.59 ± 0.02^c
Total *Bacillus* NP5 in Gut (Log CFU mL^{-1})	10	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	5.69 ± 0.01^b	6.98 ± 0.00^c
	20	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	5.76 ± 0.03^b	7.33 ± 0.02^c
	30	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	5.76 ± 0.06^b	7.13 ± 0.05^c
	40	0.00 ± 0.00^a	0.00 ± 0.00^a	0.00 ± 0.00^a	4.87 ± 0.02^b	6.58 ± 0.06^c

Meanwhile, probiotics also produce antibacterial molecules such as bacteriocins that directly inhibit other bacteria, actively fight infection, inhibit the movement of other bacteria towards the gut wall, repairs the mucosa by increasing the production of non-specific immune responses (Cerezuela *et al*., 2013). Probiotics can produce extracellular polymeric substances (EPS) that act as bioflocculants (Hashim *et al*., 2019). The composition of EPS includes proteins, exopolysaccharides, polysaccharides, glycoproteins, nucleic acids, and cellulose (Feng & Xu, 2008; Kumar *et al*., 2004). These biopolymers are capable of flocculating residual particles, dead cells, and colloidal solids (Zaki *et al*., 2011). Probiotics and microbes in flocs are able to interact with each other (quorum sensing) for synergistic activities. Probiotics such as *Bacillus* sp. and *Pseudoalteromonas* sp. are reported to have high flocculation activity of more than 90% and are able to produce polysaccharide bioflocculants (DeSchryver *et al*., 2012). Research conducted by Gustilatov *et al*. (2023) proved that supplementation of the probiotic *P. piscicida* 1Ub in the biofloc system could significantly protect and increase the resistance of shrimp to *V. parahaemolyticus* infection.

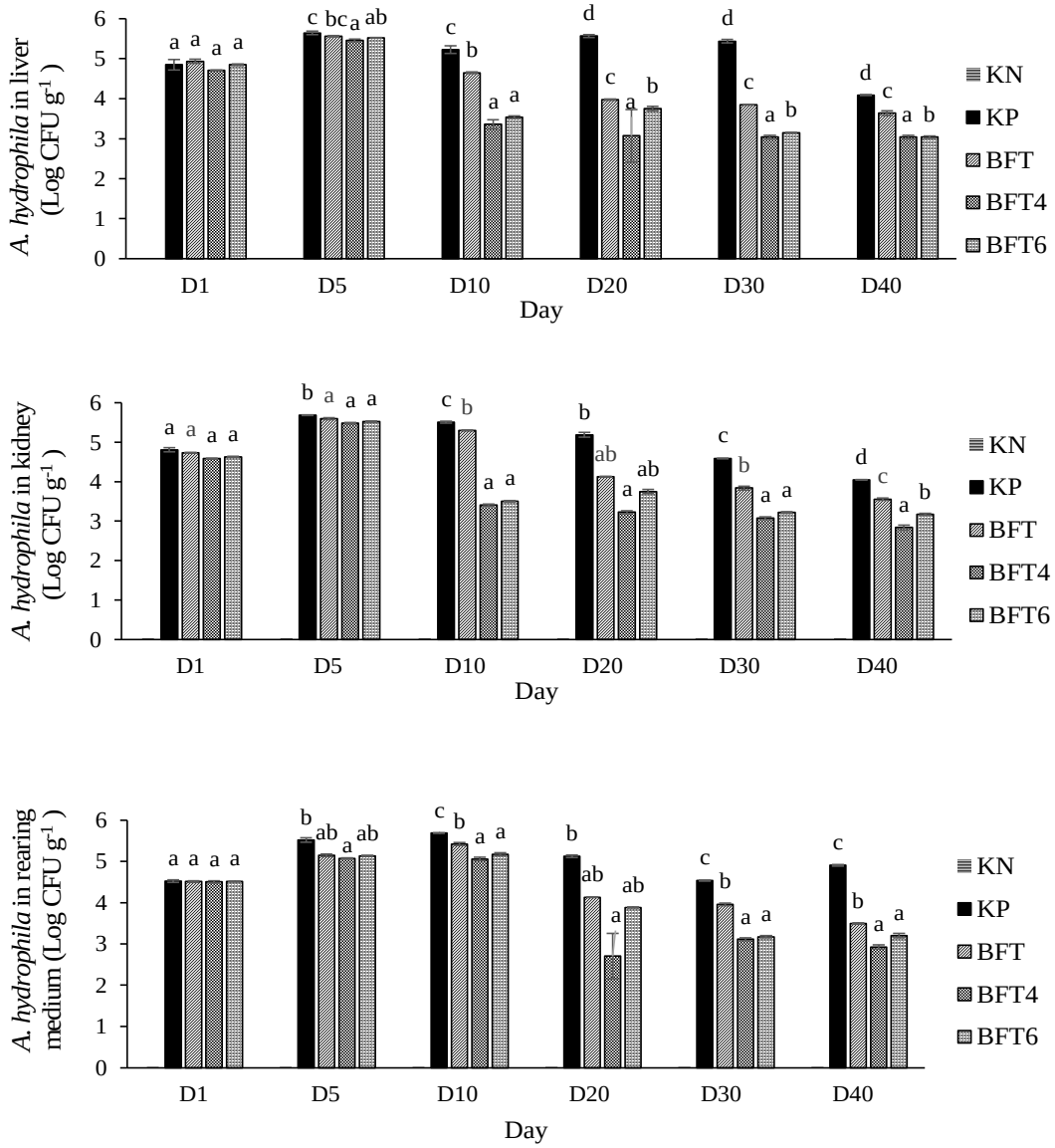

Figure 3. The density of *A. hydrophila* in liver, kidney, and rearing medium of *Clarias* sp. with a biofloc and probiotic system and addition of *A. hydrophila* at 10^4 CFU mL^{-1}.

Histopathology

Histopathological observations of the liver showed differences between the positive control with BFT, BFT4 and BFT6. There was a remarkable tissue damages in the liver detected in the positive control characterized by necrosis, pycnosis and vacuoles (Figure 4). Likewise in the kidney, there was necrosis, vacuoles, and damage to the hematopoetic tissue (Figure 5) while biofloc and probiotic treatments showed much less tissue damage.

Histopathological observations in biofloc and *Bacillus* NP5 treatments showed a less damage to the liver and kidney organs than to the positive control. The reduction of *A. hydrophila* population in water, liver and kidney treated with bioflocs and probiotics also reduced damage to the liver cells (Figure 4) and kidney (Figure 5).

Severe damage to the positive control after *A. hydrophila* infection showed vacuoles, pycnosis, and necrosis in the liver, while damage to the kidney organs including necrosis in the distal tubules, vacuoles, and hematopoetic tissue had degenerative cells characterized by dilated organelles and necrosis. In line with the results of research reported by Dauda *et al*. (2018); Menaga *et al*. (2019), which showed that the application of biofloc systems can disrupt the balance of pathogenic bacteria in infecting the host and reduce damage to liver and kidney tissue.

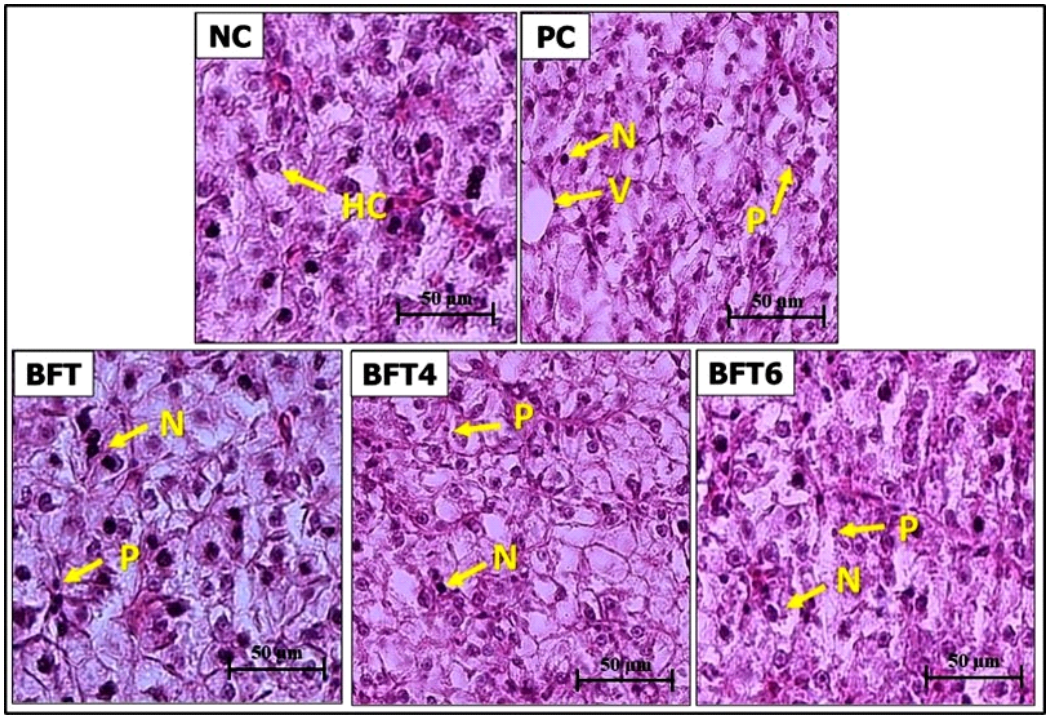

Figure 4. *Clarias* sp liver histopathology post challenge test with *A. hydrophila*. The scale bar (—) 50 µm; hepatocyte cell (HC); necrosis (N), pyknosis (P), loss of nucleus (LN) in the liver tissue. negative control (NC), positive control (PC).

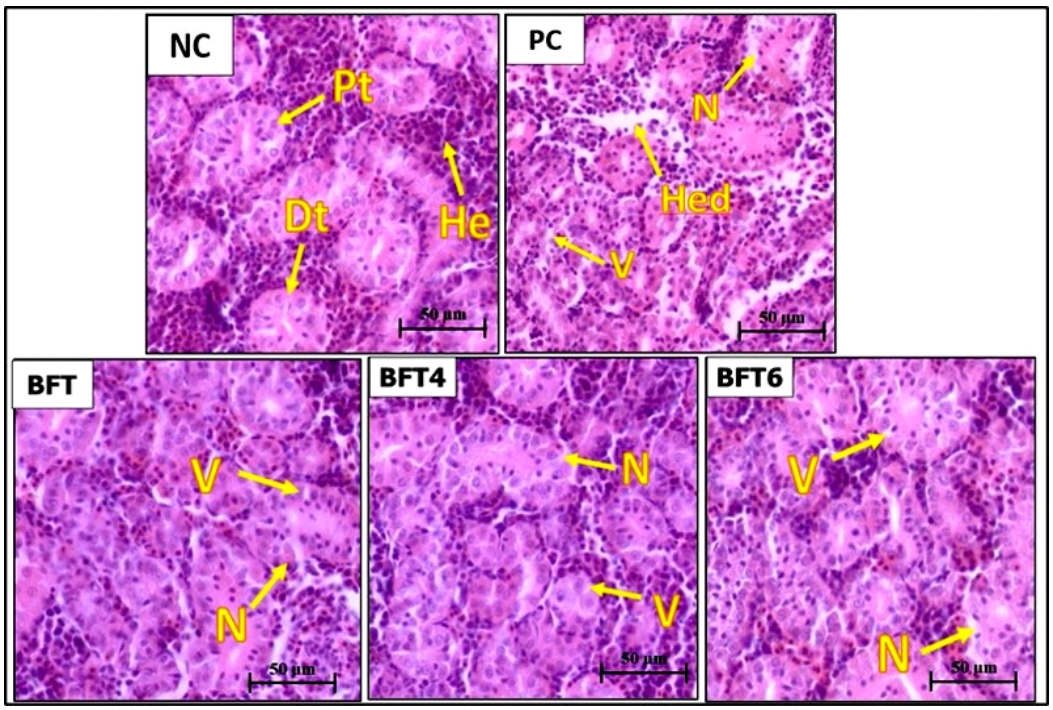

Figure 5. *Clarias* sp kidney histopathology post challenge test with *A. hydrophila*. The scale bar (—) 50 µm; necrosis (N), proximal tubules (Pt), distal tubules (Dt), vacuole (v), normal hematopoetic tissue (He), damaged hematopoetic tissue (Hed) in the kidney tissue. negative control (NC), positive control (PC).

Water Quality

Overall water quality including the pH, temperature, and DO values were at optimum conditions for rearing catfish (Table 3).

The biofloc and probiotic treatments had lower TAN-NO_2 values than the controls. Meanwhile, higher values of NO_3 were observed in the BFT, BFT4 and BFT6 treatments than the controls. The results of

Table 3. Water quality in the *Clarias* sp. rearing medium given biofloc and *Bacillus* NP5, challenged with *A. hydrophila.*

Parameter	Treatment				
	KN	KP	BFT	BFT4	BFT6
DO (mg L^{-1})	5.95 ± 0.01^{b}	5.36 ± 0.12^{a}	6.40 ± 0.03^{c}	6.30 ± 0.02^{c}	6.31 ± 0.03^{c}
pH	6.90 ± 0.01^{a}	6.88 ± 0.02^{a}	7.03 ± 0.01^{b}	7.05 ± 0.04^{b}	7.08 ± 0.01^{b}
Temperature (°C)	28.83 ± 0.19^{a}	28.68 ± 0.21^{a}	29.07 ± 0.03^{a}	28.96 ± 0.14^{a}	28.46 ± 0.01^{a}
TAN (mg L^{-1})	1.229 ± 0.10^{b}	1.252 ± 0.02^{b}	0.396 ± 0.30^{a}	0.284 ± 0.18^{a}	0.400 ± 0.27^{a}
$N0_2$ (mg L^{-1})	0.469 ± 0.17^{b}	1.277 ± 0.20^{b}	0.715 ± 0.37^{a}	0.517 ± 0.19^{a}	0.542 ± 0.22^{a}
$N0_3$ (mg L^{-1})	0.104 ± 0.05^{a}	0.140 ± 0.04^{a}	0.895 ± 0.31^{ab}	1.055 ± 0.37^{b}	1.055 ± 0.37^{b}
Volume floc (mg L^{-1})	14.65 ± 0.31^{b}	10.56 ± 1.27^{a}	20.75 ± 1.14^{c}	40.06 ± 2.58^{d}	50.95 ± 0.83^{e}

Description: dissolved oxygen (DO), total ammoniacal nitrogen (TAN), nitrite ($N0_2$), nitrate ($N0_3$).

this study also reported that the addition of probiotics to the biofloc system had an impact on the nitrification and denitrification processes. Ammonia and nitrite levels in the treatment groups were lower than the controls. Water quality conditions in the biofloc and *Bacillus* NP5 can support the high survival of catfish. Research conducted by He xi *et al*., (2023) proved that the addition of *Bacillus subtilis* to biofloc can optimize total ammoniacal nitrogen, nitrite, and nitrate so that the growth and immune system of cultured organisms increases.

CONCLUSION

The addition of *Bacillus* NP5 to the biofloc system improve hematological parameters and immune response, growth performance of *Clarias* sp. infected with *A. hydrophila* and reduce ammonia and nitrite levels of the culture media. Supplementation of *Bacillus* NP5 10^4 CFU mL^{-1} in the biofloc system is the appropriate dose for culture of catfish fry (*Clarias* sp).

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