Effects of Feeding Artemia sp. and Artificial Feed Enriched with Bacillus sp. NP5 to Catfish Pangasianodon hypophthalmus on Growth Performance, Immune Responses, and Resistance to Aeromonas hydrophila Infection

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Abstract

Motile Aeromonad Septicemia (MAS) is caused by *Aeromonas hydrophila* and often attacks juvenile catfish. Probiotics could be an alternative to prevent MAS disease. This study aims to determine the dose of probiotics to improve the survival, growth performance, and immune response of juvenile catfish *Pangasianodon hypophthalmus* to the *A. hydrophila* infection. This probiotic was contained in *Artemia* sp. and artificial feed enriched with *Bacillus* sp. NP5 at concentrations of 10^6 CFU.mL^{-1}, 10^7 CFU.mL^{-1}, 10^8 CFU.mL^{-1}, and the control. Larvae with an average weight of 1.52 ± 0.06 mg and an average length of 0.46 ± 0.007 cm were reared for 28 days in aquariums filled with 10 L of water and a stocking density of 15 individuals.L^{-1}. After the rearing period, the fish was challenged by the immersion of *A. hydrophila* with a concentration of 10^7 CFU.mL^{-1}. This study has revealed that probiotics could increase the fish's survival, length growth, daily growth rate, and feed conversion ratio. The total bacterial count in larvae and probiotics in juvenile fish is higher than those in the control. After the fish had been challenged with the probiotics at a concentration of 10^8 CFU.mL^{-1}, they had a better survival rate and immune responses (including leukocyte differential, phagocytosis activity, and respiratory burst activity) than the positive control. Probiotic *Bacillus* sp. NP5 is effectively administered through bioencapsulated *Artemia* sp. and commercial feed.

Introduction

One type of fish that has been widely cultivated in various regions in Indonesia is the catfish (*Pangasianodon hypophthalmus*), and its production has increased every year [1]. Besides Indonesia, several Asian countries, such as China, Thailand, Vietnam, India, and Bangladesh produce *P. hypophthalmus* in aquaculture [2]. However, the problem of fish farming is a disease, such as *P. hypophthalmus* which frequently appears during the larval and rearing stages. Meanwhile, *P. hypophthalmus* fish is frequently attacked by Motile Aeromonad Septicemia (MAS), caused by *Aeromonas hydrophila* [3]. Since the disease causes hemorrhage, it is also known as a hemorrhagic disease [4]. Pathogenic bacteria *A. hydrophila* has caused significant losses in the *P. hypophthalmus* aquaculture industry [5]. In addition to causing disease in fish, the bacteria also cause soft tissue wound infections and diarrhea in humans [6].

Antibiotics are frequently used to treat bacterial infections [7]. However, antibiotics can cause resistance to pathogens and disrupt the micro-ecological balance [8, 9]. Probiotics can be used as a safe and environmentally friendly alternative to prevent and control fish diseases [10, 11]. Probiotics are live microbes that have beneficial effects on the host by increasing host resistance to pathogens, inhibiting the growth or reproduction of pathogenic bacteria, activating host humoral and cellular immunity, and secreting antagonist substances to inhibit pathogens [12].

Bacillus sp. NP5 has been tested to increase the immune response and resistance of tilapia to streptococcosis disease [13, 14], goldfish to *A. hydrophila* infection [15], white shrimp to Infectious Myonecrosis Virus infection [16], catfish to *A. hydrophila* infection [17], and African catfish to *A.*

hydrophila [18]. Many studies have investigated the application of *Bacillus* sp. NP5 through artificial or commercial feed on several types of fish and shrimp to conduct an enlargement stage. However, this application has never been tested on catfish *P. hypothalamus*, especially through the natural feed for larvae and artificial feed for juveniles. In addition, Silva et al. (2020) assert that the use of different doses of probiotics results in different growth performances in tilapia. The use of probiotics depends on the host species, dose, and duration of administration [19, 20]. Thus, it is necessary to gain information related to the optimal dose of probiotic *Bacillus* sp. NP5 to give juvenile catfish significant growth, immune response, and resistance to *A. hydrophila* infection.

Material And Methods

Probiotic Preparation

This study employed probiotics *Bacillus* sp. NP5 from the digestive tract of tilapia (Putra & Widanarni, 2015) and made resistance to the antibiotic rifampin as a marker. *Bacillus* sp. NP5 was cultured on Trypticase Soy Agar (TSA) media and incubated at room temperature (27–30□C) for 24 h. Afterward, the bacteria was taken and inoculated on Tryptic Soy Broth (TSB) media. The inoculants were incubated in a water bath shaker at 29□C at 140 rpm for 24 h.

Preparation of Artemia **sp.**

This study employed 2 $g.L^{-1}$ *Artemia* sp. in the form of a system that was hatched in water with 30 $g.L^{-1}$ salinity and was given strong aeration for 24 h. After that, the nauplii of *Artemia* sp. were harvested by turning off the aeration. The hatching container was covered with dark plastic, and a light source was provided at the bottom. The shell of the hatched system will be on the surface while the unhatched system will settle at the bottom. Nauplii *Artemia* sp. will approach the light source, which was then siphoned using a hose. Meanwhile, *Artemia* sp. was given when the fish larvae were 2–5 days old.

Preparation of Tubifex **sp. (Silkworm)**

Silkworms or *Tubifex* sp. were kept in a rearing container and given aeration. Worms had been washed before being placed in different containers according to the treatment used. The finely chopped and washed silkworms were given to fish larvae aged 6–13 days. At this stage, the fish was not given probiotics.

Preparation of Artificial Feed

The artificial feed was prepared in the form of commercial feed with a protein content of 40%. Feeding was carried out at satiation with a rate of 11% of fish biomass. Artificial feed was given when the seeds were 14–28 days old.

Preparation of Maintenance Media Container

This study employed 12 aquariums with a size of 25 × 20 × 30 cm^3. The aquarium was disinfected with 20 ppm potassium permanganate (PK) for 24 h. The aquarium was then cleaned and dried. The cleaned aquarium was placed in a fiber bath measuring 2 × 1 × 0.5 m^3 and filled with 10 L of water. The fiber tub was filled with 200 L of water, and 5 heaters were installed to keep the aquarium's temperature stable. Each aquarium was added with a hose and an aeration stone to supply oxygen.

Test Animal Preparation

This study examined catfish larvae from the Center for Development of Catfish and Catfish Cultivation (Balai Pengembangan Budidaya Ikan Patin dan Lele/BPBIPL) Cijengkol, Subang, West Java. Larvae were reared from newly hatched with an average weight and length of 1.52 ± 0.06 mg and 0.46 ± 0.007 cm. The stocking density of fish in each aquarium was 15 fish.L^{-1} (150 fish.aquarium^{-1}).

Feed Enrichment Process

This study employed natural and artificial feed. Natural food was in the form of *Artemia* sp. and silkworms. Meanwhile, the artificial feed was in the form of commercial feed. The *Artemia* sp. and commercial feed were enriched with probiotic *Bacillus* sp. NP5 according to the treatment; this probiotic *Bacillus* sp. NP5 were 10^6 CFU.mL^{-1}, 10^7 CFU.mL^{-1}, and 10^8 CFU.mL^{-1}. The enrichment process was carried out in each different container. The process of enrichment of *Artemia* sp. was done by centrifuging probiotics that had been cultured in Tryptic Soy Broth (TSB) and by discarding the liquid supernatant. The precipitated pellet was added with phosphate buffer saline (PBS) as much as the volume of the removed and vortexed supernatants. The ready probiotics were added to the *Artemia* sp. containing 1 L of water and 30 g.L^{-1} salinity and enriched with the treatment for 4 h [21]. Afterward, *Artemia* sp. was harvested using a plankton net and rinsed with water. The harvested *Artemia* sp. were given directly to the larvae or stored in the refrigerator at 4ºC for later use that day.

The commercial feed enrichment process was carried out by centrifuging the probiotics cultured on TSB media and discarding the liquid supernatant. The precipitated pellet was added with PBS as much as the volume of the removed and vortexed supernatants. After that, probiotics were given as much as 2% of the weight of the feed; these probiotics were mixed with a binder in the form of egg whites as much as 2% of the weight of the feed [22]. Next, the feed was sprayed with probiotics according to the treatment and air-dried. The excess feed can be stored in the refrigerator.

Maintenance and Challenge Test

Juvenile catfish received *Bacillus* sp. NP5 in feed with different concentrations, namely 10^6 CFU.mL^{-1}, 10^7 CFU.mL^{-1}, and 10^8 CFU.mL^{-1}; each concentration had three replicates. Meanwhile, the control was without the enrichment of *Bacillus* sp. NP5, but it consisted of positive control (tested on *A. hydrophile*) and negative control (without a test on *A. hydrophila*); each control has three replications. Test animals for this study follow [23] according to the standard guidelines of the IPB University animal ethics commission (ethical approval number 206–2021).

Probiotics were given in two stages. The first stage was giving *Artemia* sp. to the larvae aged 2–5 days, and the second stage was giving artificial feed to fish aged 14–28 days. Meanwhile, fish aged 6–13 days received silkworms without probiotics. Prior to treatment, catfish larvae had been sampled to determine initial weight and length. 18–24 $fish^{-1}$ larvae aged 2–5 days old were fed on the *Artemia* sp. every 2 h for 4 days of maintenance. Meanwhile, 0.575 $g.aquarium^{-1}$ of larvae aged 6–13 days were fed with silkworms without probiotics four times a day: at 8 a.m., 12 p.m., 4 p.m., and 8 a.m. Western Indonesian Time (WIB). Juvenile catfish aged 14–28 days were fed on artificial feed at satiation with a feeding rate (FR) of 11% of fish biomass 3 times a day: at 8 a.m., 12 p.m., and 4 p.m. WIB. The water of the rearing media was changed every other two days, starting from day 6 to 28 of rearing. The temperature (°C) ranged from 29.0-30.3, the dissolved oxygen (DO, $mg.L^{-1}$) ranged from 5.2–6.3, pH ranged from 6.19–7.12, and the Total Ammonia Nitrogen (TAN; $mg.L^{-1}$) ranged from 0.016–0.144.

After being reared with treated feed for 28 days, the fish were challenged with *A. hydrophila*. The challenge test process utilized a glass jar with a volume of 3 L for 15 fish. The jar was filled with water (1 L/jar) that had been deposited for 24 h. Juvenile catfish were reared at a density of 15 fish/L and adapted for 2–3 h in the container. The fish were then infected with *A. hydrophila* at a concentration of 10^7 $CFU.mL^{-1}$. The observation was conducted for seven days post-infection, and dead fish were counted as the fish survival rate data at the end of the challenge test. During the post-challenge fish rearing, the fish were fed artificial feed in the form of commercial feed without probiotic enrichment but with an FR of 11%. This artificial feed was given three times a day.

Observation Parameter

The survival rate of catfish was calculated at the end of the rearing using the following formulas [8].

Specific growth rate (SGR; %/day) = [ln final weight ln initial weight/days] x 100

Absolute length growth (cm) = Average length of fish at the end of rearing – the average length of fish at the beginning of rearing

Total feed consumption = Total weight of feed provided – Total weight of leftover feed

Feed conversion ratio (FCR) = (total food intake) × (weight gain) – 1

Survival rate (SR; %) = 100 × (final number of test fish)/(initial number of test fish)

Total Bacterial Count (TBC) and abundance of Bacillus **sp. NP5**

The total bacterial count (TBC) was performed using the plate count method of [24] at the beginning and end of the treatment on larvae (whole-body) and juveniles (intestines). Each treatment used two fish. Meanwhile, the observations were conducted using the Total Bacterial Count (TBC) to measure the larva and the abundance of *Bacillus* sp. NP5 to measure the juvenile. Meanwhile, the total bacteria employed

the Trypticase Soy Agar (TSA) media, while the abundance of *Bacillus* sp. NP5 employed the TSA + rifampin 50 µg/mL media. The bacteria was calculated using the formula [25].

Bacterial abundance = number of colonies x 1/diluent factor x 1/mL sample

Coefficient of Diversity

The coefficient of diversity measured various fish lengths by calculating the coefficient of variance, which was the percentage of the sample standard deviation to the mean value. This measurement used the following formula.

Coefficient of variance (%) = root of variance/sample mean x 100

Catfish Immune Response

Differential Leukocytes

The differential leukocyte was calculated before and after the challenge test. Blood was dripped on the object-glass. Then, another object glass was placed on the end of the first object glass that had already contained blood in a 30° shape. The glass object that formed the corner was pulled to the end. After that, the preparations were air-dried and fixed in methanol solution for five min. The preparations that had been immersed in the methanol solution were air-dried and immersed again in diluted Giemsa solution (1:20) for 15 min. The next steps were rinsing the preparations using distilled water and air-drying them. The finished preparations were observed under a microscope with a magnification of 400 times. Differential leukocytes were calculated according to their types, namely lymphocytes, neutrophils, and monocytes, using the following formula [26].

% Lymphocytes = (Number of Lymphocytes)/(Lymphocytes + Neutrophils + Monocytes) ×100

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Phagocytic Activity

The phagocytic activity had been calculated before and after the challenge tests. Fish blood was put into a 50 µL microtube, then 50 µL of *Staphylococcus aureus* suspension was added to the PBS and homogenized. After that, the blood was incubated for 20 min at 28□C. Then, a 5 µL solution was taken to make preparations for review. Then, the solution was fixed with methanol for 5 min and dried. The preparations were soaked for 15 min in Giemsa's solution, washed in running water, and dried. The solution was observed using a microscope with a magnification of 400 times. The phagocytic activity was calculated using the following formula [27].

Phagocytic Activity = (Number of phagocytic cells)/(Number of phagocytic cells) ×100

Respiratory Burst (RB) Activity

The respiratory burst activity was calculated using the reduction principle of nitroblue tetrazolium (NBT) which produces formazan—the sum of the sizes of superoxide anions. 50 µL of blood was put into the holes of the microplate titer and incubated at 37C for 1 h. The supernatant formed was discarded and rinsed three times with 50 µL of PBS. Then, 50 µL of 0.2% NBT solution was added and incubated at 37C for 1 h. Furthermore, the 0.2% NBT solution was discarded, and the blood was fixed with 50 µL 100% methanol solution for 2 min and rinsed with 30% methanol solution 3 times and dried. The formed formazans blue precipitate was dissolved using 60 L of 2 N KOH solution and 70 µL of dimethyl sulphoxide (DMSO) added to each titer microplate hole. The optical density (OD) of the formazan blue precipitate was then measured using a microplate reader at a wavelength of 630 nm [28].

Data analysis

The obtained data were processed using Microsoft Excel 2013 and Minitab 16. Meanwhile, the data were analyzed using the analysis of variance (ANOVA). When the ANOVA results were significantly different, the data were further tested using the Tukey Advanced Test.

Results

This study has revealed that juvenile catfish in all treatments have significantly different final biomass weight and daily growth rate from the control (Table 1). The final biomass weight and the highest daily growth rate of catfish seed were obtained at the treatment with 10^{8} CFU.mL^{-1} This treatment is not significantly different ($P > 0.05$) from the treatment of 10^{7} CFU.mL^{-1} but significantly different ($P < 0.05$) from the treatment with 10^{6} CFU.mL^{-1}. On day 14, the treatment with probiotic concentrations of 10^{8} CFU.mL^{-1} and 10^{7} CFU.mL^{-1} have resulted in significantly different ($P < 0.05$) body lengths from the result of the treatment with probiotic concentrations of 10^{6} CFU.mL^{-1} and the control. Meanwhile, on day 28, the treatment with a probiotic concentration of 10^{8} CFU.mL^{-1} has resulted in the highest body length which was significantly different ($P < 0.05$) from the results of other concentrations and the control. Moreover, the treatment with the probiotic concentration of 10^{8} CFU.mL^{-1} has produced significantly different absolute length growth ($P < 0.05$) from that of other concentrations and the control. The treatment with probiotic concentrations of 10^{8} CFU.mL^{-1} and 10^{7} CFU.mL^{-1} has resulted in significantly different absolute length growth of juvenile catfish from the result of the control. However, the treatment with probiotic concentrations of 10^{6} CFU.mL^{-1} has produced insignificantly different ($P > 0.05$) result from that of the control.

Table 1
Growth performance of juvenile *P. hypophthalmus* fed on various concentrations of *Bacillus* sp. NP5

Parameter	Treatment (CFU/mL)			
	Control	10^6	10^7	10^8
Initial biomass weight (g)	1.52 ± 0.06a	1.52 ± 0.06a	1.52 ± 0.06a	1.52 ± 0.06a
Final biomass weight (g)	177.67 ± 8.33a	303.33 ± 41.02b	401.67 ± 78.59bc	537.33 ± 35.00c
Specific growth rate (SGR; %/day)	18.54 ± 0.33a	20.81 ± 0.68b	22.00 ± 1.04bc	23.32 ± 0.39c
Length growth (H0) (cm)	0.46 ± 0.01a	0.46 ± 0.01a	0.46 ± 0.01a	0.46 ± 0.01a
Length growth (H14) (cm)	1.86 ± 0.14ab	1.63 ± 0.09a	2.08 ± 0.05bc	2.26 ± 0.04c
Length growth (H28) (cm)	2.64 ± 0.10a	2.82 ± 0.15a	3.33 ± 0.11b	4.00 ± 0.04b
Absolute length growth (cm)	2.18 ± 0.11a	2.35 ± 0.24a	2.87 ± 0.10b	3.54 ± 0.04c
Coefficient of diversity of fish length (H14) (%)	11.14 ± 0.77c	9.46 ± 1.42bc	6.49 ± 1.28ab	4.52 ± 1.16a
Coefficient of diversity of fish length (H28) (%)	7.69 ± 1.65ab	9.96 ± 2.9b	4.94 ± 0.70a	4.81 ± 2.00a
Total feed consumption	22.21 ± 3.90a	39.86 ± 7.00ab	50.40 ± 11.55b	59.80 ± 10.51b
Feed conversion ratio (FCR)	1.90 ± 0,17c	1.71 ± 0.06bc	1.47 ± 0.13b	1.11 ± 0.10a
Survival rate (SR; %)	78.44 ± 1.02a	81.33 ± 1.76ab	82.00 ± 1.76b	86.67 ± 0.67c
Data are mean ± SD. Different letters in the same line showing significantly different ($P < 0.05$)				

On day 14, the treatment with the concentration of probiotics 10^8 CFU.mL^{-1} has resulted in the lowest coefficient of the diversity of fish length. This result is not significantly different ($P > 0.05$) from that of the treatment with the concentration of probiotics 10^7 CFU.mL^{-1} but significantly different ($P < 0.05$) from that of the treatment with the concentration of probiotics 10^6 CFU.mL^{-1} and control. On day 28, each treatment has produced diverse lengths, but this result was not significantly different ($P > 0.05$) from that of the control. The amount of feed consumption, final biomass weight, and SGR at concentrations of 10^7 CFU.mL^{-1} and 10^8 CFU.mL^{-1} were not significantly different ($P > 0.05$), but the FCR of the two treatments was significantly different ($P < 0.05$). The survival rate of the treatment with a concentration of 10^8

CFU.mL^{-1} was significantly different ($P < 0.05$) from that of the other treatments and the control. The treatment with a probiotic concentration of 10^6 CFU.mL^{-1} was not significantly different ($P > 0.05$) from that of the control.

The total bacterial count (TBC) in larvae and the total of *Bacillus* sp. NP5 in juveniles are higher than those in the control (Table 2). The range of total bacteria count at the beginning of the treatment was 1.08–1.80 × 10^4 CFU.larva^{-1} while *Bacillus* sp. NP5 was not found. At the end of the treatment, the larvae has the highest number of bacteria obtained in the treatment of 10^8 CFU/mL and the lowest number of bacteria in the control. Probiotic *Bacillus* sp. NP5 in juveniles was found in the probiotic treatment, and the highest number of probiotic *Bacillus* sp. NP5 is found in the treatment of 10^8 CFU.mL^{-1}.

Table 2
Total bacterial count (TBC) of larvae and total of *Bacillus* sp. NP5 on juvenile *P. hypophthalmus*

Treatment	Total bacterial count (TBC)		Total *Bacillus* NP5	
	Initial (CFU/larva)	Final (CFU/larva)	Initial (CFU/ juvenile)	Final (CFU/ juvenile)
Control	1.08 x 10^4	1.3 x 10^7	0	0
10^6 CFU/mL	1.65 x 10^4	2.1 x 10^7	0	1.2 x 10^5
10^7 CFU/mL	1.41 x 10^5	3.7 x 10^7	0	1.0 x 10^5
10^8 CFU/mL	1.80 x 10^5	4.0 x 10^7	0	2.4 x 10^5
Data are presented as mean ($n = 2$).				

Before the challenge test, each treatment did not have a significantly different total of lymphocytes, neutrophils, and monocytes ($P > 0.05$). In contrast, after the challenge test, the treatment with the probiotic concentration of 10^8 CFU.mL^{-1} (Fig. 1) has produced the highest number of monocytes and neutrophils. Furthermore, before the challenge test, the highest percentage of the phagocytic activity was found in the probiotic treatment of 10^8 CFU.mL^{-1}, but after the challenge test with *A. hydrophila*, the highest value of the phagocytic activity is obtained at the treatment of 10^8 CFU.mL^{-1} with a significant difference of ($P < 0.05$) in the treatment and control (Fig. 2). The value of respiratory burst activity (RB) during the observation before the challenge test was not significantly different ($P > 0.05$) from that in each treatment. However, after the challenge test, the respiratory burst activity has increased, and the highest score is obtained at the treatment of 10^8 CFU/ mL. This score is significantly different ($P < 0.05$) from that at the control (Fig. 3). The juvenile catfish has the lowest survival rate after being infected with *A. hydrophila* in the positive control. This rate is significantly different ($P < 0.05$) from that in the negative control and the treatment added with the *Bacillus* sp. NP5.

Discussion

The final biomass weight and daily growth rate of juvenile catfish in all treatments are significantly different from those of the control (Table 1). It is suspected that the provision of probiotics in catfish seed feed can increase nutrient digestibility so that the feed can be easily absorbed by fish. Putra and Widanarni [29] have revealed that the provision of probiotic *Bacillus* sp. NP5 from the digestive tract of tilapia can increase nutrient digestibility, digestive enzyme activity, and growth performance of tilapia. Meanwhile, Tamamdusturi et al. [17] state that the *Bacillus* sp. NP5 can hydrolyze macromolecules in feed to be simpler molecules; thus, the nutrients can be easily absorbed by the intestinal wall of fish and will spread through the circulatory system of the body to serve as an energy source for the growth of catfish. According to Adineh et al. [30], probiotics of *Bacillus* species can more significantly grow fish larvae. Different doses in this study show different results on the final biomass weight and the highest daily growth rate of catfish parameters; the best treatment is obtained at 10^8 CFU.mL^{-1}. Silva et al. [31] assert that different doses of probiotics affect the growth performance of tilapia. Besides increasing the final biomass weight and daily growth rate, supplementation of probiotics could increase body length. Absolute length growth is obtained at the treatment with a probiotic concentration of 10^8 CFU.mL^{-1}.

The coefficient of diversity is one of the parameters of seed quality that indicates the level of uniformity of seed size. This study has found that the lowest coefficient of the diversity of fish seed length occurs on day 14. The fish length is classified as uniform if the coefficient of diversity is not more than 15% [32]. Postulate that the smaller the coefficient of fish diversity, the better the quality of the produced seeds; consequently, the selling value of the seeds will be higher [33]. The value of the coefficient of diversity increases because the fish cannot properly utilize the nutrients on feed for long growth.

The concentrations of 10^7 and 10^8 do not produce significantly different ($P > 0.05$) parameters of feed consumption, final biomass weight, and SGR but produce significantly different ($P < 0.05$) FCR. These findings show that the use of probiotic doses is necessarily considered in cultivation. This study has also discovered that the treatment of 10^6 CFU.mL^{-1} does not produce significantly different ($P > 0.05$) results from that of the control. However, probiotics have a lower feed conversion value than the control. This indicates that *Bacillus* sp. NP5 can increase the digestibility of feed and reduce the value of feed conversion. Djauhari et al. [15] state that *Bacillus* sp. NP5 can significantly increase the activity of digestive enzymes in carp because it can increase the digestibility of proteins, carbohydrates, and fats. *Bacillus* sp. NP5 can help the digestive process to produce extracellular enzymes, such as amylase, protease, and lipase. Thus, nutrient absorption and utilization become more efficient. Balcazar et al. [34] explain that digestive enzymes produced by probiotic bacteria, such as amylase, protease, and lipase, can help the digestive process. The lowest feed conversion ratio while using artificial feed is obtained at treatment with the probiotic concentration of 10^8 CFU.mL^{-1}.

A high growth rate and low FCR are influenced by the probiotic concentration that can be consumed by fish. Such a condition is denoted by the total bacterial count in larvae and the total of probiotics in juvenile fish (Table 2). A high concentration of probiotics also gives a higher value. The high total

bacteria in larvae and juvenile fish indicates that the probiotic *Bacillus* sp. NP5 is effectively administered through *Artemia* sp. and commercial feed. The effectiveness of probiotics depends on the ability of probiotics to survive during the journey to reach the target organs and act on the digestive system. The best fish growth performance is influenced by gut microbes as indicated by an increased total bacterial count and total *Bacillus* sp. NP5 [17]. However, the addition of high doses of probiotics is not always accompanied by an increase in total bacteria in the fish. This condition occurs because not all probiotics added to feed can be taken up by larvae [35]. It is suspected that during the bioencapsulation process into *Artemia* sp. and the coating on the feed, the amount of probiotic does not appropriate for the treatment dose. The interesting finding of this study is that *Bacillus* sp. NP5 probiotics were no longer found in all treatments after fish larvae had been fed on silkworms without *Bacillus* sp. NP5 for eight days. This indicates that probiotics should be given continuously during maintenance. Silva et al. [31] argue that the administration of probiotics gave effects after eight weeks of maintenance. The best survival rate is obtained in the treatment with a concentration of 10^8 CFU.mL^{-1}. Other studies have revealed that the addition of probiotic *Bacillus* sp. NP5 can increase the survival of tilapia [14] and white shrimp *L. vannamei* [16]. Meanwhile, this study has shown that probiotic supplementation could improve the survival of catfish *P. hypophthalmus*.

Besides improving growth performance, supplementation of *Bacillus* sp. NP5 could increase the immune response of catfish *P. hypophthalmus*. The immune system is a coordinating system that aims to protect individuals' identity and integrity and prevent the invasion of harmful organisms. Parameters or indicators to observe the immune response are leukocyte differential (Fig. 1), phagocytic activity (Fig. 2), and respiratory burst activity (Fig. 3). Differential leukocytes are grouped according to their types, namely lymphocytes, neutrophils, and monocytes. After the challenge test, the highest number of monocytes and neutrophils was found in the treatment of 10^8 CFU.mL^{-1} (Fig. 1). It is suspected that the phagocytic cells in the fish's body are ready to phagocytize the invading pathogenic bacteria. The differential value of leukocytes before and after the challenge tests has decreased the number of lymphocytes but has increased the number of neutrophils and monocytes. The higher number of monocytes and neutrophils in fish indicates that the fish can produce phagocytic cells, and the blood cells can perform phagocytosis when pathogenic microorganisms attack [14]. Consequently, administering probiotics can increase the body resistance of catfish.

Phagocytic activity is the first defense line of the cellular response carried out by monocytes (macrophages) and granulocytes (neutrophils). This process can occur when a foreign object enters the fish body which will be phagocytized by macrophages. These macrophages will destroy antigens by phagocytosis and send signals to the lymphocyte tissue to form specific antibodies. The formed antibodies will reduce the toxicity of the poison and weaken the pathogen from spreading; as a result, phagocytic cells will more easily attack the pathogen [36]. Unlike the control, the percentage value of phagocytic activity in the probiotic treatment has significantly increased after the challenge test (Fig. 2). The highest percentage value of phagocytic activity was found in the treatment of 10^8 CFU.mL^{-1} after the challenge tests. This shows that the addition of the *Bacillus* sp. NP5 can accelerate the phagocyte

process carried out by macrophages which is the first line of defense of the immune response. Meanwhile, Djauhari et al. [15] deliver that the percentage value of phagocytic activity has increased after the challenge test and in the probiotic treatment. Probiotics can interact with mononuclear phagocytic cells (monocytes and macrophages), polynuclear leukocytes (neutrophils), and natural killer cells. Probiotics can act as an effective trigger for phagocytic cells so that they can increase phagocytic activity.

The increase in the respiratory burst activity could increase the phagocytic cells of fish when challenged to fight pathogenic bacteria. Tamamdusturi et al. [17] have revealed that an increasing respiratory burst activity occurs after the challenge test which indicates the fish attempts to defend themselves from pathogenic bacterial infection. The probiotic treatment has a higher value of respiratory burst activity than the control. The respiratory burst activity is the basic building block of the antibacterial system found in the fish's body. The increased value of respiratory burst can be associated with an increased value of phagocytic cell activity [37]. The respiratory explosion can increase the amount of oxygen consumption and form superoxide anions. This process is accelerated by NADPH-oxidase and multi-component enzymes inside the plasma membrane after phagocytic activation [38].

This study has discovered that the supplementation probiotic *Bacillus* sp. NP5 could increase resistance to pathogenic *A. hydrophila*. Putra and Widanarni [29] state that probiotic *Bacillus* sp. NP5 has the strongest antagonistic activity against pathogenic bacteria, such as *Streptococcus* sp. Other studies have also discovered that the addition of the *Bacillus* sp. NP5 could increase the survival of tilapia infected by Streptococcosis [14] and goldfish injected with *A. hydrophila* [15]. Probiotics from *Bacillus* species can increase the fish's growth, immune response, and resistance to pathogenic infection [39], such as *A. hydrophila* in juvenile tilapia fish [40].

Conclusion

The administration of *Bacillus* sp. NP5 can increase the total bacterial count in the larvae of *P. hypophthalmus*. In addition, the administration of *Bacillus* sp. NP5 can increase survival, growth performance, and immune response (leukocyte differential, phagocytic activity, and respiratory burst activity) of juvenile *P. hypophthalmus* with the best results found in the dosage of 10^8 CFU.mL^{-1}.

Data Availability

The datasets of this study are available from the corresponding author upon reasonable request.

Declarations

Data Availability

The datasets of this study are available from the corresponding author upon reasonable request.

Ethical Approval

All the experimental studies considered the welfare of test animals according to the standard guidelines IPB University animal ethics commission (ethical approval number 206-2021).

Conflict of Interest

The authors declare that there are no competing interests.

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Figures

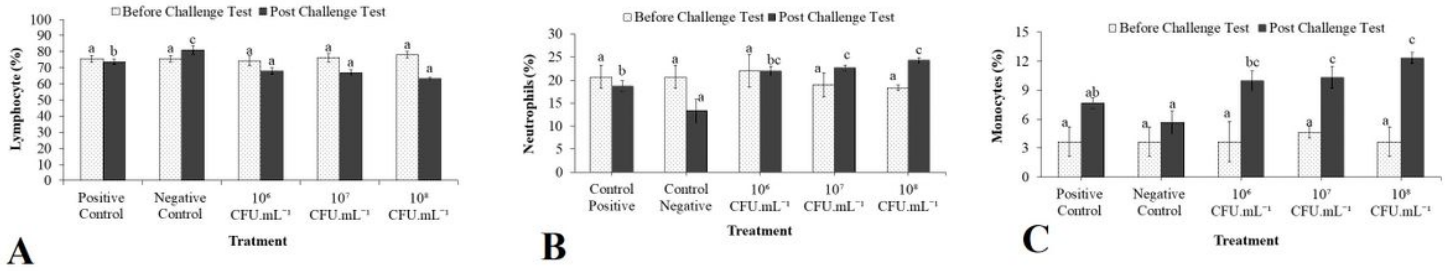

Figure 1

Percentages of lymphocytes (a), neutrophils (b), and monocytes (c) of juvenile catfish *P. hypophthalmus* supplemented with various doses of *Bacillus* sp. NP5 feed. Data are mean ± SD. Different letters in the same patterns showing significantly different results (P < 0.05).

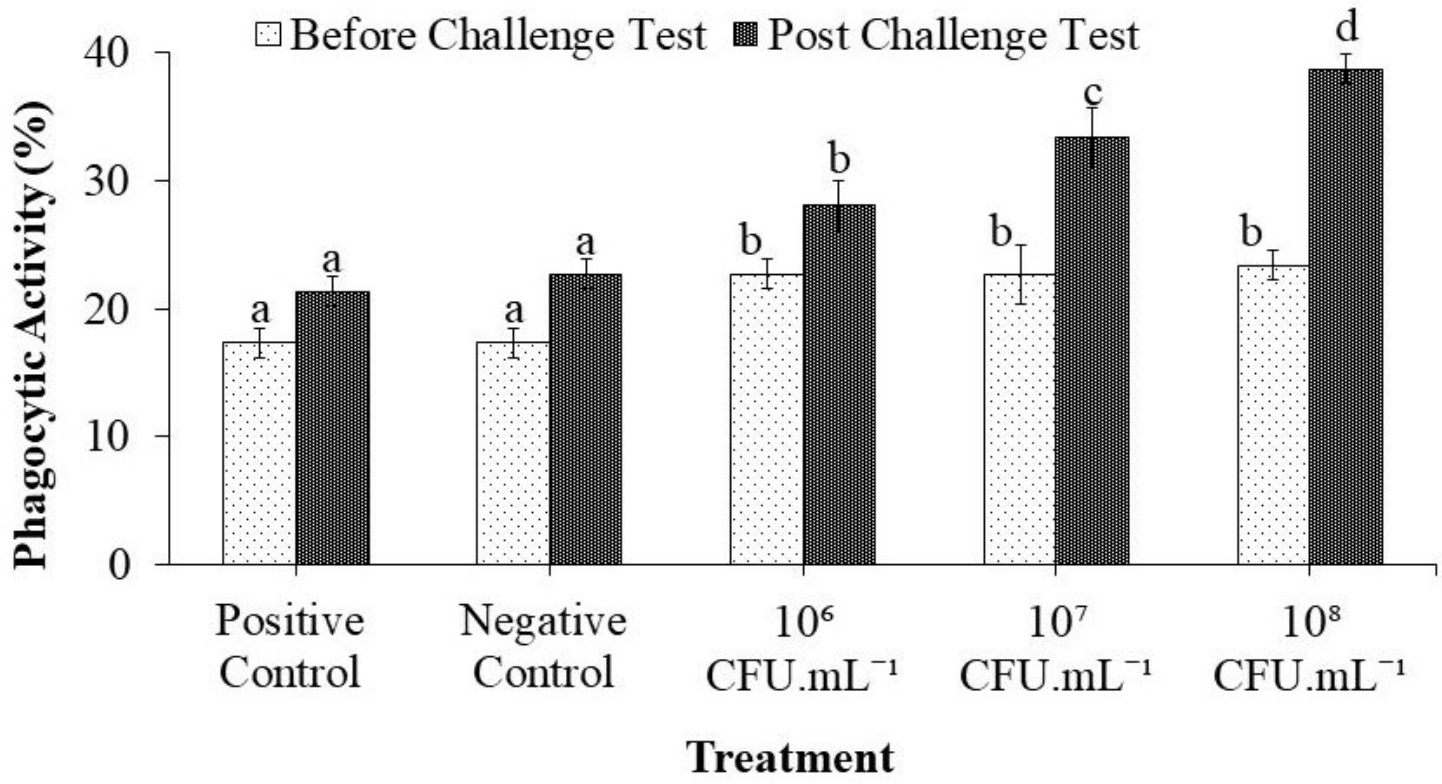

Figure 2

Percentages of the phagocytic activity of juvenile *P. hypophthalmus* catfish supplemented with different doses of *Bacillus* sp. NP5 on feed. Data are mean ± SD. Different letters in the same patterns showing significantly different results (P < 0.05).

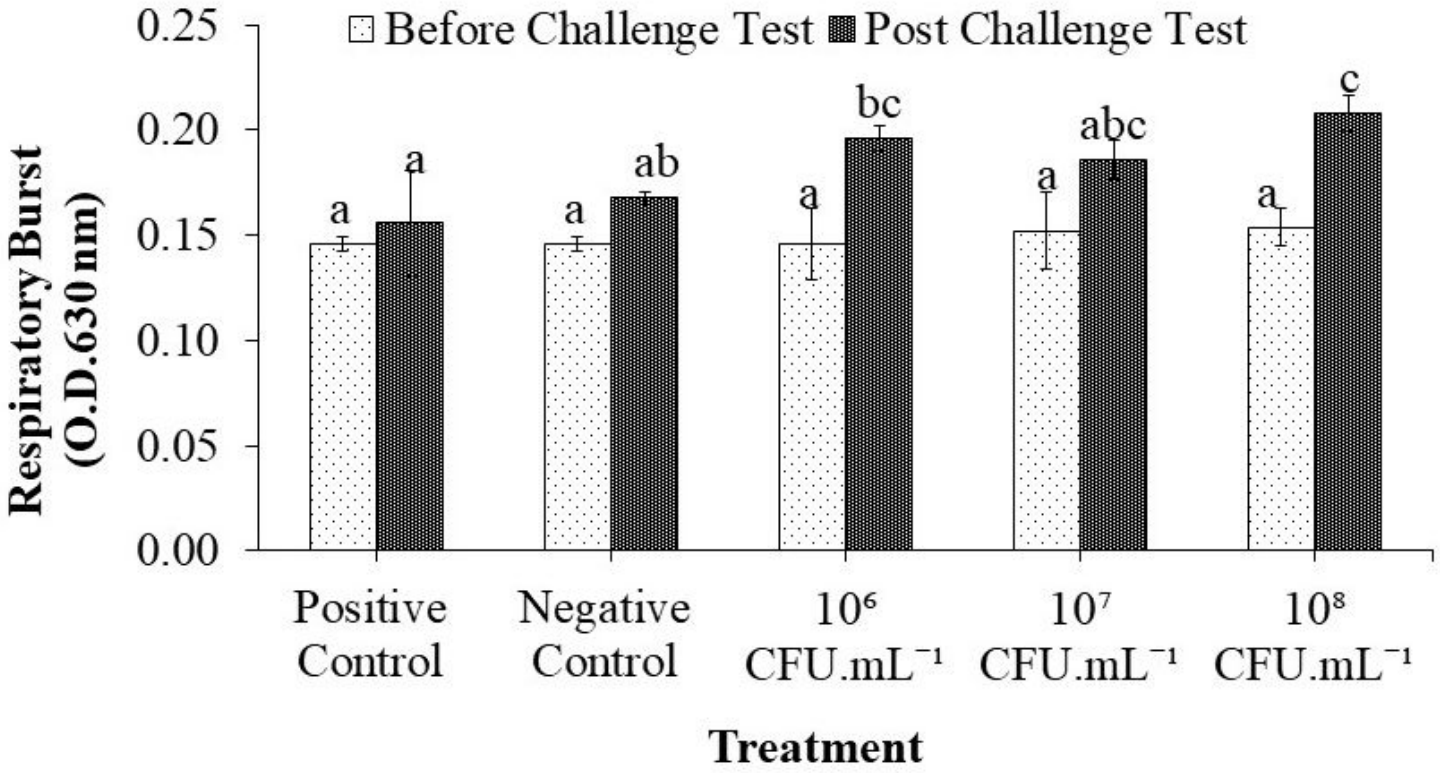

Figure 3

Respiratory burst activity of juvenile catfish *P. hypophthalmus* supplemented with different doses of *Bacillus* sp. NP5 on feed. Data are mean ± SD. Different letters in the same patterns showing significantly different results ($P < 0.05$).

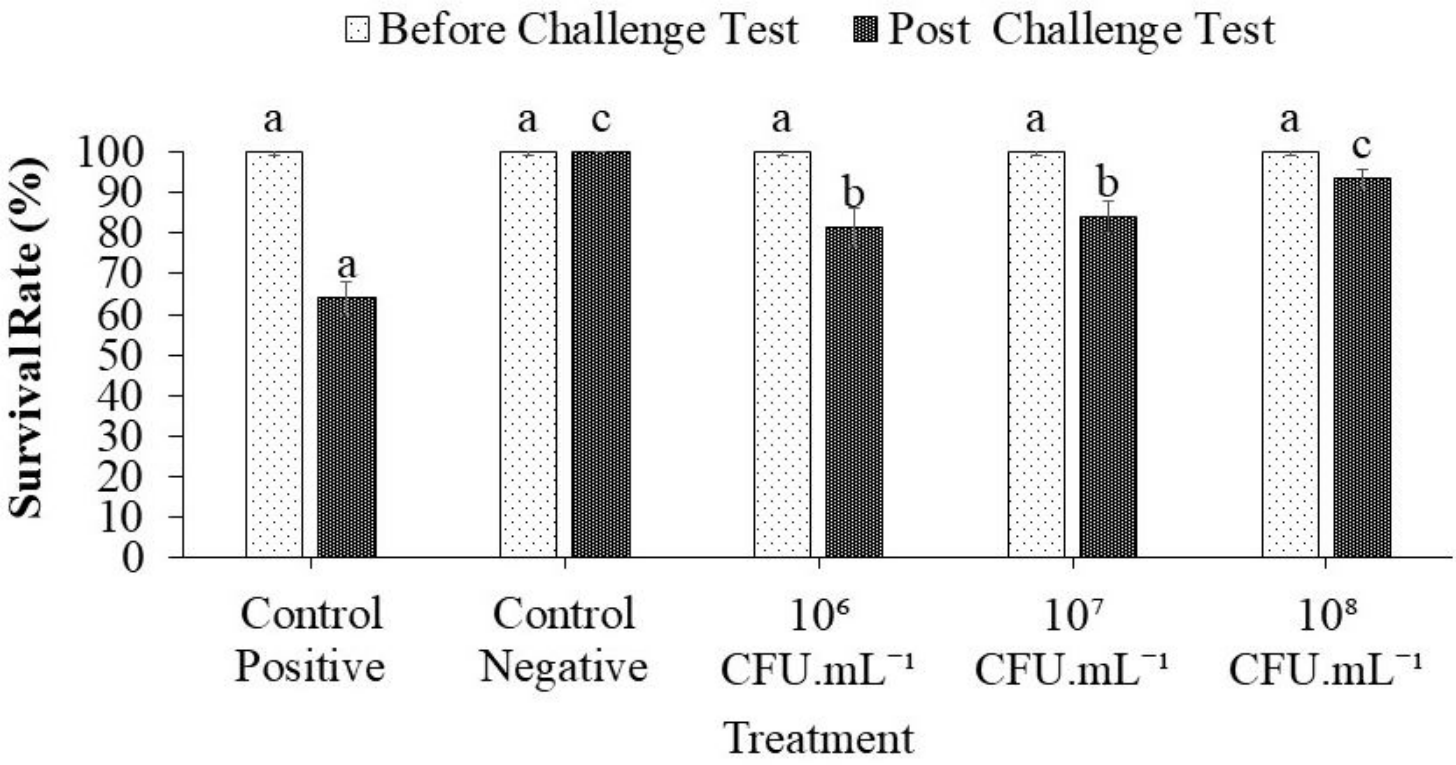

Figure 4

Survival rate of juvenile catfish *P. hypophthalmus* supplemented with different doses of *Bacillus* sp. NP5 on feed before and post challenge test with *A. hydrophila*. Data are mean ± SD. Different letters in the same patterns showing significantly different results ($P < 0.05$).