BIODIVERSITAS
Volume 22, Number 1, January 2021
Pages: 253-261

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d220131

Dietary *Bacillus* NP5 supplement impacts on growth, nutrient digestibility, immune response, and resistance to *Aeromonas hydrophila* infection of African catfish, *Clarias gariepinus*

ACHMAD NOERKHAERIN PUTRA[1, 2,♥], MUSTAHAL[1,2], MAS BAYU SYAMSUNARNO[1], DODI HERMAWAN[1], DEVIA GUSNUR FATIMAH[1], PRAMODITA BALITA PUTRI[1], SEVIA[1], RINA ISNAINI[1], MUHAMAD HERJAYANTO[1]

[1]Department of Fisheries, Faculty of Agriculture, Universitas Sultan Ageng Tirtayasa. Jl. Raya Jakarta Km. 04, Pakupatan, Serang 42121, Banten, Indonesia. Tel.: +62-254-280330, Fax.: +62-254-281254, ♥email: Putra.achmadnp@untirta.ac.id

[2]Indonesia-Center of Excellence for Local Food Innovation, Universitas Sultan Ageng Tirtayasa. Jl. Raya Jakarta Km. 04, Pakupatan, Serang 42121, Banten, Indonesia

Manuscript received: 9 October 2020. Revision accepted: 21 December 2020.

Abstract. *Putra AN, Mustahal, Syamsunarno MB, Hermawan D, Fatimah DG, Putri PB, Sevia, Isnaini R, Herjayanto M. 2021. Dietary Bacillus NP5 supplement impacts on growth, nutrient digestibility, immune response, and resistance to* Aeromonas hydrophila *infection of African Catfish,* Clarias gariepinus. *Biodiversitas 22: 253-261.* This study aims to investigate the effects of *Bacillus* NP5 supplementation as a probiotic on growth, immune response, and resistance of African catfish to *Aeromonas hydrophila* infection. Catfish with an initial weight of 6.8 ± 0.1 g were fed with different doses of *Bacillus* NP5 (0%, 1.1%, 1.2% B, 1.3%, and 1.4%) with three replications and reared for 60 days. The first 45 days were used to observed growth performance, and the last 15 days were used to perform challenge tests against *A. hydrophila* infection. The results showed that the treatment of 1.2% *Bacillus* NP5 results in the highest specific growth rate (2.55±0.28 day^{-1}) and increased protein and lipid digestibility significantly ($P<0.05$). Treatment of 1.1-1.3% *Bacillus* NP5 increase amylase and lipase activity that significantly higher than 0% *Bacillus* NP5 treatment. Supplementation of *Bacillus* NP5 significantly increased the leukocyte, phagocytic index, and survival rate in African catfish after *A. hydrophila* infection. Therefore, the supplementation of 1.2% *Bacillus* NP5 in the feed increased the growth, immune response, and African catfish resistance to the infection.

Keywords: African catfish, *Bacillus* NP5, growth, probiotic, response immune

INTRODUCTION

Indonesia is the second-largest producer of aquaculture products in the world after China, with a total of 9.3 million tons in 2018 (FAO 2020). African catfish (*Clarias gariepinus*) is one of the common commodities found in its freshwater fishery. According to the Ministry of Marine Affairs and Fisheries (2019), catfish production in Indonesia was the 3rd largest after shrimp and tilapia, with a total production of 19.6 thousand tons in 2017. Catfish farming has been intensively and sustainably carried out to fulfill the increasing demand for fish. Zhang et al. (2018) stated that various negative impacts, such as the emergence of disease attacks, decrease in environmental quality, and retardation of fish growth, are associated with the intensification of aquaculture. *Aeromonas hydrophila* is an opportunistic gram-negative bacterium. It causes Motile *Aeromonas* Septicemia (MAS) in fish (Korni and Khalil 2017; Shoemaker et al. 2018), which leads to an increase in mortality rate (Maji et al. 2017; Jung-Schroers et al. 2018; Li et al. 2019a). Over the past few decades, fish farmers have adopted the use of antibiotics, a conventional method to control disease attacks (Lee et al. 2019). However, antibiotics and other chemical compounds lead to the improvement of resistance in pathogenic bacteria (Hoseinifar et al. 2018) and increase chemical residues in water and fish (Lee et al. 2016; Hassani et al. 2019). Therefore, an environmentally friendly approach is needed to overcome the problems in aquaculture intensification to maintain sustainability.

Probiotics are environmentally friendly applications to improve fish health and growth (Carnevali et al. 2017). According to Ramesh et al. (2015), it is a substitute for antibiotics and can suppress pathogens' growth without being detrimental to the host and its environment. Furthermore, Li et al. (2019b) reported that it has been widely applied in aquaculture and used as a substitute for fish and shrimp antibiotics. Previous studies showed that probiotics supplementation in aquaculture results in increasing growth and feed digestibility. Probiotics increase digestive enzyme activity (Enferadi et al. 2018; Valiallahi et al. 2018; Arani et al. 2019), increase immunity against pathogens (Amir et al. 2019; Zhu et al. 2019), and improve the water quality of culture media (Elsabagh et al. 2018; Kewcharoen and Srisapoome 2019).

Bacillus NP5 is a Gram-positive bacteria, oxidation fermentation, and catalase-positive (Putra and Widanarni 2015). Previous studies showed that supplementation of *Bacillus* NP5 in fish increase growth (Putra et al. 2015; Utami et al. 2015), increase immunity against fish pathogens (Widanarni et al. 2014; Febrianti et al. 2016; Tamamdusturi et al. 2016) and improve water quality

(Putra et al. 2020a). The addition of 1% and 1.5% of *Bacillus* NP5 results in increasing African catfish' growth performance (Putra et al. 2020b). In this study, a probiotic dose range of 1-1.5% was used to obtain the correct dose of *B.* NP5 in the diet. Besides, there were still a few data related to the effect of *Bacillus* NP5 addition to catfish's immune system. Therefore, this study was carried out to investigate *Bacillus* NP5 probiotics' supplementation on growth performance, immune response, and African catfish resistance to *A. hydrophila* infection.

MATERIALS AND METHODS

Probiotic and feed preparation

Bacillus NP5 is a probiotic bacteria originating from the intestine of *Oreochromis niloticus* (Putra and Widanarni 2015). Bacteria were cultured according to the method by Putra and Romdhonah (2019). *Bacillus* NP5 was cultured aseptically in 250 mL of tryptic soy broth (TSB) medium and incubated for 18 hours at room temperature. After incubation, the bacterial culture was centrifuged (HITACHI himac CT6E/CT6EL) at the speed of 1000 *x g* for 10 minutes at 4ºC, and the bacterial supernatant was washed and homogenized by adding 100 mL of phosphate buffer saline (PBS, pH 7.2). Furthermore, serial dilutions with PBS were carried out to obtain a 106 CFU/mL bacterial density. Bacterial supernatant was stored at 4ºC before being mixed in the feed.

Commercial catfish feed (781, INDONESIA) was used as a basal diet grounded into flour, sieved with 320 µm mesh, and thoroughly mixed with chromium oxide of 0.5% as the digestibility indicator and 3% tapioca as a binder. Then, 30% of warm water was added and printed in a 1-2 mm feed diameter in a pellet machine. Subsequently, the feed was oven heated at 50ºC for 24 hours, followed by homogenization of 2% egg yolk (Putra et al. 2015) and sprayed with *Bacillus* NP5 supernatant according to the treatment dose. The proximate composition of the experimental diet is presented in Table 1.

Table 1. Proximate composition (g/100 g feed) of experimental diet

Proximate	Value
Crude protein (%)	33.95
Crude lipid (%)	7.73
Crude fiber (%)	4.16
Ash (%)	9.9
Moisture (%)	6.49
Organic matter (%)*	83.61
Nitrogen-free extracts**	37.77
Dry matter (%)	93.51
Energy (Kcal g feed^{-1})***	275.863
Size feed (mm)	1-2

Note: *Dry weight – ash, **100 - protein-lipid - fiber - ash – moisture, **Digestible energy according to NRC (1993), calculated as protein 118.8 Kcal g feed^{-1}; lipid 62.6 Kcal g feed^{-1}, and carbohydrate: 94.4 Kcal g feed^{-1}

Fish rearing and experimental procedure

A total of 375 African catfish obtained from the Baros Seed Center, Serang, Indonesia, were used in this study. Fish rearing was carried out at the Laboratory of Aquaculture, University of Sultan Ageng Tirtayasa, Indonesia. The initial weight of the catfish was 6.8 ± 0.1 g were randomly scattered into the round tank with a diameter of 72 cm diameter and fish density of 25 fish /tank with a water volume of 40 L. The aerator was placed at the center of the tank at 15 cm from the bottom. The acclimatization process was carried out for seven days, and the fish was unfed for 24 hours before treatment. Feeding was carried out three times daily, i.e., at 08.00, 12.00, and 17.00 Western Indonesian time zone through a satiation method. Approximately 20% of the total water volume was changed every three days and 50% every two weeks. The rearing process was carried out for 60 days, of which the first 45 days were used for growth tests and the remaining 15 days for a challenge test with the injection of the pathogen *A. hydrophila*.

A completely randomized design with five different levels of *Bacillus* NP5 probiotic (0% or negative control, 1.1%, 1.2%, 1.3%, and 1.4%) with three replications was used in this study. The in-situ water quality monitoring such as temperature (RESUN, Indonesia) was carried out daily, while pH (LUTRON 208) and dissolved oxygen (LUTRON DO550) were observed weekly. Ammonia measurements were carried out based on the standard Phenate method (APHA 1998) at the beginning and the end of the rearing period. In the present study, water temperature was in the range of 27.1-28.8ºC, while the pH value during rearing before and after water change was 5.17-7.23. The dissolved oxygen was 5.17-7.23 mg L^{-1}, while the ammonia value was below 2 mg L^{-1}. The value of water quality showed that it was in the normal range for catfish farming, according to Bhatnagar and Devi (2013).

Growth parameters

The number and weight of African catfish were measured at the beginning and the 45 days to determine the growth performance, which was calculated based on the equation proposed by Huisman (1987) as follow: (i) Feed intake (g) = the initial weight of feed – final weight of feed. (ii) Specific growth rate (% day^{-1}) = 100 × ((ln final weight of catfish − ln initial weight of catfish /50 days)). (iii) Feed conversion ratio = Total feed consumption/(final weight of catfish − the initial weight of catfish). (iv) Survival rate (%) = 100 × (final number of catfish/initial number of catfish)

Collection of feces was carried out 2 hours after feeding by shipon from the 7th day until the treatment was complete. Feces was stored at -4°C. Nutrient digestibility was calculated based on the equation proposed by Takeuchi (1988) as follows: (i) Dry matter digestibility (%) = 100 × (1 – (% chromium content in the diet/% chromium content in the feces)). (ii) Protein/lipid digestibility (%) = 100 × (1 – ((% chromium content in the diet/% chromium content in the feces) × (% nutrient content in the feces/% nutrient content in the feed)))

Digestive enzyme activity was determined on day 45. A total of 5 fish from each treatment were randomly selected, and their total intestines (anterior and posterior) were surgically removed and weighed. The intestinal samples were crushed to obtain the substrate. The Bergmeyer et al. (1983) method was used to determine the protease activity with casein as a substrate in 0.05 M phosphate buffer, pH 7, and 5 mmol/L of tyrosine. Amylase activity was determined using 1% starch solution as a substrate in 20 mM sodium phosphate buffer, pH 6.9, and 20 mM of sodium phosphate according to the method by Worthington (1993). Lipid enzyme activity was calculated based on the procedure described by Borlongan (1990), using olive oil as a substrate and Tris-HCL as a buffer.

Chemical analysis

Proximate analysis of feed and fish was carried out using the standard method of AOAC (2005). A total of 5 catfish were randomly selected for analysis with the micro-Kjeldahl method to determine crude protein content. Moisture was estimated with an oven at 105°C for 6 hours, while the sample was analyzed through extraction with a Soxhlet to determine crude lipid content. The percentage of ash was determined by combustion at 550 °C for 24 hours, and crude fiber was estimated by an Automatic analyzer.

Experimental infection

Aeromonas hydrophila was obtained from the Fish Health Laboratory of IPB University for challenge tests carried out on the 45th day. Before the challenge test, Koch's Postulates were performed to increase the virulence of *A. hydrophila*. Catfish were reared according to the treatment, and the fish density was uniform (15 individuals/tank). Meanwhile, *A. hydrophila* were cultured on 200 ml of TSB media for 24 hours and incubated at 29 °C. After incubation, the culture was centrifuged at 1000 *x g* for 10 minutes and diluted using PBS (pH: 7.2) to obtain 10^7 CFU/mL density. Furthermore, it was injected intramuscularly into catfish as much as 0.1 mL per fish (Putra and Widanarni 2015).

Hematological and immune response

Evaluations of the hematological and immune response were carried out on days 0, 45, and 60. Five fish from each treatment were randomly selected to determine the number of erythrocytes and leukocytes according to the method by Blaxhall and Daisley (1973). Blood was mixed with Hayem's and Turk's solutions until it was homogeneous. Erythrocytes were counted using a Neubauer-type hemocytometer under a microscope with a magnification of 400x. Hematocrit level was calculated using a microhematocrit tube following the method by Anderson and Siwicki (1995) Determination of hemoglobin levels in the blood refers to Sahli's method (Wedemeyer and Yasutake 1977). Phagocytosis index was measured by mixing 20 µL of the blood sample with 20 µL of *A. hydrophila* suspension as an antigen with a density of 10^7 cells mL^{-1}. The phagocyte cells are counted based on the percentage during the phagocytosis process (Ispir and Dorucu 2005).

Statistical analysis

Data of growth and immune response were analyzed by Analysis of Variance (ANOVA) in Microsoft Office Professional Plus 2016. Duncan Multiple Range Test (DMRT) analyzed significant differences between treatments with a 95% confidence interval.

RESULTS AND DISCUSSION

Nutrient digestibility and digestive enzyme activity of African catfish

Table 2 showed that the dry matter digestibility in treatment 1.1-1.2% *Bacillus* NP5 was significantly higher ($P < 0.05$) compared to 0% *Bacillus* NP5. However, the dry matter digestibility in the treatment of 1.3-1.4% *Bacillus* NP5 was not significantly different compared to the control (0%) ($P > 0.05$). Treatment of 0% and 1.3% *Bacillus* NP5 results in the lowest protein digestibility, while the highest was in 1.2% *Bacillus* NP5 ($P < 0.05$) treatment. The lowest lipid digestibility was obtained from 0% and 1.4% *Bacillus* NP5 treatment, and the highest one was in 1.1-1.3% *Bacillus* NP5 ($P < 0.05$). Furthermore, the highest protease enzyme activity was obtained from 1.2% *Bacillus* NP5, significantly different from other treatments ($P < 0.05$). The highest amylase and lipase enzyme activity were obtained from 1.1-1.3% *Bacillus* NP5 treatment.

Growth performance

The growth performance of catfish was presented in Table 3. The highest specific growth rate was in 1.2% *Bacillus* NP5 treatment, which significantly different from other treatments ($P < 0.05$), while treatments of 0%, 1.1%, 1.3%, and 1.4% *Bacillus* NP5 were not different. The final weights of 1.1-1.3% *Bacillus* NP5 treatments were significantly higher than 0% *Bacillus* NP5. However, feed intake and survival rates in all treatments were not significantly different ($P > 0.05$). Furthermore, the highest feed conversion was obtained from 0% *Bacillus* NP5 treatment, which was significantly different from the treatment of 1.1-1.4% *Bacillus* NP5 ($P < 0.05$).

Whole-body composition

The whole-body composition of catfish was presented in Table 4. The highest crude protein was obtained from 1.2% *Bacillus* NP5 treatment ($P < 0.05$). Crude lipids in treatments of 1.2-1.4% *Bacillus* NP5 were significantly higher than 0-1.1% *Bacillus* NP5 treatment. However, the application of *Bacillus* NP5 does not have any effect on moisture and ash content ($P > 0.05$).

Table 2. Nutrient digestibility and digestive enzyme activity of African catfish with five experimental diets containing the different doses of *Bacillus* NP5

Dietary of probiotic	Digestibility and digestive enzyme parameters*					
	Dry matter digestibility (%)	Protein digestibility (%)	Lipid digestibility (%)	Protease activity (U. mg protein^{-1})	Amylase activity (U. mg protein^{-1})	Lipase activity (U. mg protein^{-1})
0% *B.* NP5	66.86±0.97^{a}	76.06±1.31^{a}	56.85±4.39^{a}	0.007±0,00^{a}	1.40 ±0.04^{a}	0.282±0.00^{a}
1.1% *B.* NP5	69.09±1.70^{b}	78.10±1.11^{c}	67.75±0.94^{bc}	0.014±0.00^{b}	2.07±0.034^{b}	0.325±0.016^{bc}
1.2% *B.* NP5	73.01±1,27^{c}	85.52±1.14^{d}	70.30±2.34^{c}	0.057±0.00^{d}	2.38 ±0.05^{b}	0.358±0.00^{c}
1.3% *B.* NP5	67.75±0.94^{ab}	76.16±0.47^{ab}	70.86±0.45^{c}	0.037±0.00^{c}	2.35 ±0.08^{b}	0.354±0.03^{c}
1.4% *B.* NP5	65.78±0.33^{a}	77.39±0.72^{bc}	62.32±6.46^{ab}	0.010±0.00^{a}	1.28±0.26^{a}	0.308±0.01^{ab}

Note: *mean ± SD values in the same column with similar superscript letters are not significantly different (*P > 0.05*)

Table 3. Growth performance of African catfish with five experimental diets containing the different doses of *Bacillus NP5*

Dietary of probiotic	Growth parameters*					
	Initial weight (g fish^{-1})	Final weight (g fish^{-1})	Feed intake (g)	Specific growth rate (% day^{-1})	Feed conversion ratio	Survival rate (%)
0% *B.* NP5	6.77±0.02	19.53 ± 1.18^{a}	321.37±57.62	2.12±0.12^{a}	1.82±0.07^{c}	96.00± 0.00
1.1% *B.* NP5	6.74±0.02	23.78 ± 2.89^{bc}	330.37±26.75	2.16±0.14^{a}	1.29±0.02^{b}	94.67±2.30
1.2% *B.* NP5	6.75±0.01	26.26 ± 1.14^{c}	328.33±9.28	2.55±0.28^{b}	1.15±0.04^{a}	96.00 ± 0.00
1.3% *B.* NP5	6.84±0.26	23.91 ± 1.34^{bc}	329.62±8.85	2.35±0.10^{ab}	1.17±0.02^{a}	96.00±4.00
1.4% *B.* NP5	6.99±0.01	21.98 ± 1.01^{ab}	324.93±28.55	2.34±0.06^{ab}	1.28±0.02^{b}	97.33 ±2.30

*mean ± SD values in the same column with similar superscript letters are not significantly different (*P > 0.05*)

Table 4. Whole-body composition of African catfish with five experimental diets containing different doses of *Bacillus* NP5

Dietary of probiotic	Body composition*			
	Crude protein (%)	Crude lipid (%)	Moisture (%)	Ash (%)
0% *B.* NP5	10.35±0.05^{a}	5.01±0.02^{a}	71.07±0.46	2.82±0.05
1.1% *B.* NP5	10.07±0.03^{a}	5.11±0.09^{a}	71.15±0.02	2.80±0.12
1.2% *B.* NP5	13.16±0.28^{c}	5.52±0.10^{b}	72.66±0.35	2.86±0.03
1.3% *B.* NP5	11.52±0.85^{b}	5.54±0.33^{b}	73.96±0.40	2.77±0.06
1.4% *B.* NP5	12.05±1.12^{bc}	5.12±0.10^{b}	73.36±0.98	2.87±0.07

*mean ± SD values in the same column with similar superscript letters are not significantly different (*P* > 0.05)

Hematological and immune response

Table 5 showed no significant difference in the values of blood profile and phagocyte index of African catfish on day 0. The same results were also obtained on day 45, except for the hematocrit value in the probiotic treatment, which was higher than the control (P <0.05). At the end of rearing (day 60), the highest hemoglobin value was obtained from 1.2% *Bacillus* NP5, which was higher than other treatments (P <0.05). Erythrocyte value in the treatment of 1.1, 1.2, and 1.4% *Bacillus* NP5 was not significantly different, but it significantly higher than the 0% *Bacillus* NP5 (P <0.05) treatment. Probiotic treatment had a higher value of hematocrit than 0% *Bacillus* NP5 (P <0.05). Supplementation of 1.1-1.2% *Bacillus* NP5 results in higher leukocytes than other treatments (P <0.05). The highest phagocytes index was obtained from 1.2% *Bacillus* NP5 (P <0.05). The lowest survival rate was at 0% *Bacillus* NP5 treatment in weeks 1 and 2 (Figure 1). The highest survival rate after *A. hydrophila* infection was obtained from 1.2% *Bacillus* NP5 treatment, namely 75.46% and 66.67% in weeks 1 and 2, respectively.

Discussion

Bacteria of the genus *Bacillus* have long been used as probiotics in aquaculture with numerous beneficial effects on their hosts, such as increasing growth performance, enhancing feed digestibility, the production of exogenous enzymes, enhancing immunity against pathogens, and stimulating microbiota populations to inhibit pathogenic colonization (Kuebutornye et al. 2019; RingØ 2020). The high activity of digestive enzymes indicates an increase in the development of the intestine of fish and its ability to digest feed (Darafsh et al. 2019). According to Dawood and Khosio (2016), protease, lipase, and amylase are the main enzymes responsible for feed digestion in the digestive tract of fish. This study showed that *Bacillus* NP5 supplementation increased protease activity, amylase, and lipase in the intestine of catfish compared to controls, especially at doses of 1.1-1.3%. It increases is due to the production of exoenzyme by probiotics. Sankar et al. (2016) reported that the high activity of digestive enzymes in feed treated with probiotics is due to the exoenzyme production by probiotics and its stimulation to the digestive tract of the host. The results obtained in this research are in

line with the previous studies by Putra et al. (2015 and 2020b) that the administration of *Bacillus* NP5 increases the activity of protease, lipase, and amylase enzymes in the digestive tract of the host. Similar results were found on *L. vannamei* (Zokaeifar et al. 2012; Zokaeifar et al. 2014), Javanese carp (Allameh et al. 2015), and tilapia (Midhun et al. 2019).

The results showed that supplementation of 1.1-1.2% *Bacillus* NP5 increased nutrient digestibility compared to 0% *Bacillus* NP5. A study by Lin et al. (2004) showed that supplementation of *Bacillus* spp. increased the digestibility of dry matter and protein in white shrimp. Mohapatra et al. (2012) reported the same results, which showed that dietary probiotics in feed significantly (P <0.05) enhance dry matter, protein, and lipid digestibility compared to controls on the rearing of juvenile rohu, *Labeo rohita*. In this study, the supplementation of 1.2% *Bacillus* NP5 significantly increased the digestibility of dry matter and protein (P <0.05), which were considered to be related to the protease enzyme activity in 1.2% *Bacillus* NP5 treatment. Meanwhile, increasing protease enzyme activity in probiotic treatment has been reported in other fish species, such as *Litopenaeus vannamei*, with the application of *LactoBacillus pentosus* AS13 (Zheng and Wang 2016) and rainbow trout with *L. plantarum* supplementation (Enferadi et al. 2018).

Table 5. The hematological and immune response of African catfish with five experimental diets containing the different doses of *Bacillus* NP5

Parameter/day	Dietary of probiotic*				
	0% *Bacillus* NP5	**1.1% *Bacillus* NP5**	**1.2% *Bacillus* NP5**	**1.3% *Bacillus* NP5**	**1.4% *Bacillus* NP5**
0 day					
Hemoglobin (g%)	6.20±0.00	6.23±0.12	6.24±0.70^{ab}	6.21±0,.9	6.21±0.69
Erythrocytes (x 10^6 cell/mm^3)	2.89±0.09	2.85±0.04	2.95±0.05	2.81±0.08	2.81±0.08
Hematocrit (%)	17.88±0.3	17.60±0.17	17.96±0.30	20.17±2.49	20.17±2.49
Leukocytes (x 10^4 cell/mm^3)	6.32±0.02	6.35±0.06	6.34±0.03	6.16±0.36	6.16±0.36
Phagocyte index (g%)	40.33±4.51	40.33±4.73	40.00±4.36	40.07±1.53	40.67±1.53
45 days**					
Haemoglobin (g%)	7.03±0.42	6.94±0.53	7.00±0.20	7.09±0.62	6.95±0.64
Erythrocytes (x 10^6 cell/mm^3)	2.16± 0.14	2.60±0.55	2.70±0.02	2.38±0.11	2.66±0.33
Hematocrit (%)	7.82±0.64^{a}	11.27±0.37^{b}	11.24±0.69^{b}	11.36±1.29^{b}	11.36±1.16^{b}
Leukocytes (x 10^4 cell/mm^3)	8.74±0.02	8.63±0.02	8.95±0.03	8.49±0.28	8.86±0.22
Phagocyte index (g%)	41.67±4.04	40.67±4.51	40.67±7.37	40.00±3.46	41.33±4.51
60 days***					
Hemoglobin (g%)	4.40±0.40^{a}	5.60±0.72^{ab}	6.00±0.20^{b}	5.26±1.16^{ab}	5.38±1.22^{ab}
Erythrocytes (x 10^6 cell/mm^3)	1.25±0.04^{a}	1.81±0.03^{b}	1.45±0.05^{b}	1.35±0.09^{ab}	1.45±0.05^{b}
Hematocrit (%)	7.80±0.40^{a}	14.20±0.25^{b}	12.56±0.39^{b}	12.32±0.30^{b}	15.40±5.90^{b}
Leukocytes (x 10^4 cell/mm^3)	8.49±0.12^{a}	9.54±0.02^{b}	9.45±0.03^{b}	9.53±0.50^{b}	9.40±0.39^{b}
Phagocyte index (g%)	51.33±2.52^{a}	46.67±1.53^{a}	66.00±3.00^{b}	62.67±5.03^{b}	58.67±4.73^{ab}

Note: *mean ± SD values in the same row with similar superscript letters are not significantly different (*P* >0.05). **before *Aeromonas hydrophila* infection, ***after *A. hydrophila* infection.

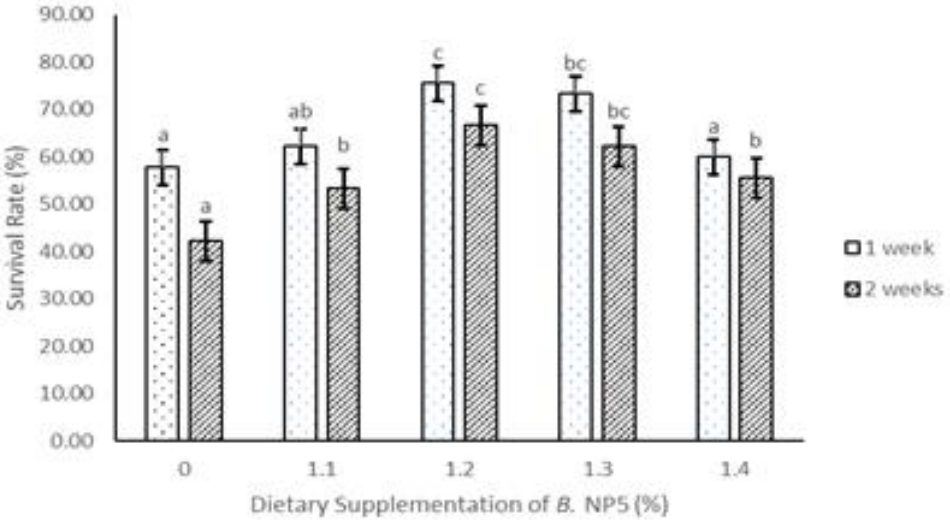

Figure 1. The survival rate of African catfish after *Aeromonas hydrophila* infection

The application of probiotics in feed has resulted in the increasing growth of fish and shrimp (Liu et al. 2017; Arani et al. 2019; Xie et al. 2019). Jang et al. (2019) stated that probiotics increase the growth of the host by improving the digestibility of the feed. The highest final weight, specific growth rate, and feed conversion ratio were obtained from 1.2% *Bacillus* NP5 treatment ($P < 0.05$) was considered to be associated with high nutrient digestibility due to increased enzyme activity. According to Wu et al. (2012), probiotic supplementation improves the digestion process of feed and the beneficial microbial population and digestive enzyme activity in the digestive tract of the host. These results were consistent with previous studies, which showed that probiotics have a positive effect on enzyme activity, feed digestibility, and growth performance in hybrid groupers (He et al. 2016), tilapia (Poolsawat et al. 2020), and *L. vannamei* (Tsai et al. 2019).

The results showed that the feed intake was the same for all treatments, indicating that *Bacillus* NP5 supplementation in the feed did not affect its palatability (Tantikitti 2014). Similar results were observed in tilapia (Elsabagh et al. 2018), juvenile olive flounder (Lee et al. 2019), and rainbow trout (Nargesi et al. 2019). Supplementation of 1.2-1.4% *Bacillus*. NP5 increased crude protein and lipids in the body composition of African catfish. It was consistent with the results obtained in several other species, such as goldfish, by administering *L. delbrueckii* (Zhang et al. 2018) and *L. vannamei* through the use of *B. subtilis* and *B. licheniformis* (Madani et al. 2018). Increasing protein and lipids in the body composition can be caused by the increased digestibility and food absorption of feed. As a result, there were more protein and fat deposits in the body (Abdel-Tawwab et al. 2008; Reda and Selim 2015). The results showed no difference in moisture and ash content in all treatments ($P > 0.05$). Similar results were discovered in *L. vannamei* (Adel et al. 2017), parrotfish (Liu et al. 2017), and snakehead (Dai et al. 2018).

Several studies have reported that probiotic supplementation reduces the mortality rate of fish and improves its immune system against pathogens (Adel et al. 2017; He et al. 2017; Hassani et al. 2019). Dawood et al. (2016) stated that probiotic supplementations in feed positively affect fish blood profile. This study showed that the erythrocyte and hemoglobin number in African catfish decreased after the challenge test compared to the pre-challenge test. Olivier et al. (1981) stated that the decrease in erythrocyte and hemoglobin is related to the ability of pathogens to damage red blood cells. A study by Kumar et al. (2015) showed that the number of erythrocytes and hemoglobin in Catla fingerling after experiencing a challenge test with *A. hydrophila* bacteria was decreased. The hematocrit number was the ratio of erythrocyte to total blood volume influenced by the number of cells (Soltani et al. 2017). In this study, the hematocrit value obtained by probiotic treatment was higher before and after pathogenic bacterial infection compared to those without probiotics. This result indicates that probiotic has improved the immune system of African catfish. Hematocrit value is related to heath status of fish (Nandi et al. 2017) and administration probiotics stimulated the innate immunity of fish (Adorian et al. 2018). The same results demonstrated by Ahmadifar et al. (2020) who reported the addition of probiotic *Pediococcus pentosaceus* 10^8-10^9 CFU g^{-1} increased the general hematocrit value compared to the controls in carp. Zhu et al. (2019) also reported that the hematocrit value in tilapia given probiotic *B. subtilis* LT3-1 was increased compared to the controls.

In this study, *Bacillus* NP5 supplementation in feed significantly increased leukocytes after infection with *A. hydrophila*. These results were in line with the study carried out by Reda and Selim (2015) that supplementation of *B. amyloliquefaciens* increased the leukocyte of Nile tilapia. A study by Pourgholam et al. (2017) showed that the addition of *L. plantarum* as a probiotic increased the leukocyte number in juvenile Siberian sturgeon. The leukocyte was one of the fish's non-specific defense systems (Uribe et al. 2011). The increase in fish leukocytes was related to the body's defense system response to disease infections (Tanbiyaskur et al. 2015). Phagocytes have been widely used to determine the defense against pathogenic infections (Giri et al. 2012). Tamamdusturi et al. (2016) stated that phagocytosis is the initial cellular immune system after a pathogenic infection was provided by monocytes and granulocytes. According to Djauhari et al. (2016), probiotic supplementation increases the cellular immune system of fish and increasing its resistance to pathogenic infections. The phagocyte index of African catfish increased significantly in the treatment of *Bacillus* NP5 after the challenge test. This result was similar to the result of a study by Doan et al. (2015) on Pangasius catfish that was challenged with *A. hydrophila* and Zhao et al. (2019) on giant freshwater prawns given *B. pumilus* probiotic.

The increase in blood profile values, especially the leukocytes and phagocyte index in probiotic treatment, indicated an increase in the immune system of African catfish. It was confirmed by increasing the survival rate after the challenge test with *A. hydrophila* at the *Bacillus* NP5 treatments. The results showed that *Bacillus* NP5 supplementation increased African catfish' resistance to *A. hydrophila* infection and increased their survival rate compared to treatment without probiotics. It implies that *Bacillus* NP5 supplementation increases the cellular and humoral defenses of African catfish. Similar results were also reported by Zokaeifar et al. (2012), with the addition of *B. subtilis* in the feed, which was used to increase the survival rate of *L. vannamei* infected with *V. harveyi*. This is also in accordance with the result in freshwater prawn through the addition of *B. licheniformis* in the feed (Kumar et al. 2013), *Cyprinus carpio* with administration *L. delbrueckii* (Zhang et al. 2017), and white shrimp with *Pediococcus pentosaceus* supplementation in the diet (Adel et al. 2017).

In conclusion, dietary *Bacillus* NP5 supplement positively increased the growth, immune response, and resistance of African catfish against *A. hydrophila* infection

with the optimal dose of *Bacillus* NP5 supplementation in the feed of African catfish was 1.2%.

ACKNOWLEDGEMENTS

The authors express gratitude to LPPM-UNTIRTA for funding this study through the research internal grant scheme of Penelitian Terapan Internal 2020 (Nomor: B/135/UN.43.9/PT.01.03/2020).

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