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Dietary supplementation of *Bacillus* sp. NP5 and dayak onion simplicia powder *Eleutherine bulbosa* (Mill.) Urb. for the prevention of *Aeromonas hydrophila* in catfish *Clarias* sp.

Suplementasi *Bacillus* sp. NP5 dan serbuk simplisia bawang dayak *Eleutherine bulbosa* (Mill.) Urb. untuk pencegahan *Aeromonas hydrophila* pada ikan lele *Clarias* sp.

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ABSTRACT

Aeromonas hydrophila is the main causative agent of ulcerative disease in catfish and causes considerable economic losses to Indonesian aquaculture. This study evaluates the prebiotic activity and the effect of feed supplementation of dayak onion simplicia powder (DOSP) on the immune response and survival of catfish infected with *A. hydrophila*. Five doses (0.20, 0.25, 0.30, 0.35, and 0.40 g/mL) of DOSP were tested *in vitro* to assess the prebiotic activity score. The results showed that a dose of 0.20 g/mL gave a significantly (P<0.05) higher probiotic stimulation value than other doses. In the in vivo test, the study used a completely randomized design with five treatments, namely simplicia (DOSP 20 g/kg), probiotic (PRO, *Bacillus* sp. NP5 108 CFU/mL, 1% v/w), combination (PRO+DOSP), and control (positive and negative). Fish were reared for 45 days and fed three times a day. On day 46, fish from all treatments, except negative control, were infected with an *A. hydrophila* dose of 106 CFU/mL injected intramuscularly. The results showed that the combination treatment (PRO+DOSP) gave better total erythrocytes, hemoglobin, hematocrit, total leukocytes and phagocytosis activity than probiotics, DOSP, and control. Administering the combination (PRO+DOSP) can reduce the total number of *A. hydrophila* lower than the probiotic, DOSP, and control treatments. In addition, the survival rate of catfish in the combined treatment (PRO+DOSP) was significantly (P<0.05) higher than probiotics, DOSP, and control. The results of this study can be a helpful reference and application for the early prevention of *A. hydrophila* infection.

Keywords: aquaculture, *Bacillus* sp. NP5, dayak onion, probiotics, simplicia powder

ABSTRAK

Aeromonas hydrophila adalah penyebab utama penyakit bercak merah pada budidaya ikan lele dan menyebabkan kerugian ekonomi cukup besar pada akuakultur Indonesia. Penelitian ini bertujuan mengevaluasi aktivitas prebiotik serbuk simplisia bawang dayak (SSBD) terhadap respons imun dan kelangsungan hidup ikan lele yang diinfeksi *A. hydrophila*. Lima dosis (0,20, 0,25, 0,30, 0,35, dan 0,40 g/mL) SSBD diuji secara *in vitro* untuk menilai skor aktivitas prebiotik. Hasil penelitian menunjukkan bahwa dosis 0,20 g/mL memberikan nilai stimulasi probiotik signifikan (P<0,05) lebih tinggi dibandingkan dosis lainnya. Pada uji in vivo, penelitian menggunakan rancangan acak lengkap terdiri dari lima perlakuan, yaitu simplisia (SSBD, serbuk simplisia bawang dayak 20 g/kg), probiotik (PRO, *Bacillus* sp. NP5 108 CFU/mL, 1% (v/w), kombinasi (PRO+SSBD), dan kontrol (positif dan negatif). Ikan dipelihara selama 45 hari dan diberi pakan tiga kali sehari. Pada hari ke 46, ikan pada semua perlakuan, kecuali kontrol negatif, di infeksi *A. hydrophila* dosis 106 CFU/mL secara intramuskular. Hasil penelitian menunjukkan bahwa kombinasi (PRO+SSBD) memberikan total eritrosit, hemoglobin, hematokrit, total leukosit dan aktivitas fagositosis lebih baik dibandingkan probitoik, SSBD, dan kontrol. Kombinasi (PRO+SSBD) mampu menekan total *A. hydrophila* lebih rendah dibandingkan probiotik, SSBD, dan kontrol. Selain itu, tingkat kelangsungan hidup ikan lele pada perlakuan kombinasi (PRO+SSBD) signifikan (P<0,05) lebih tinggi dibandingkan probitoik, SSBD, dan kontrol. Hasil penelitian ini bisa menjadi referensi dan aplikasi yang efektif untuk pencegahan dini infeksi *A. hydrophila*.

Kata kunci: akuakultur, *Bacillus* sp. NP5, bawang dayak, probiotik, serbuk simplisia

INTRODUCTION

Catfish (*Clarias* sp.) is one of the primary aquaculture commodities, as stated in the DJPB strategic plan 2020-2024, where production increased to fulfill national fish consumption demands. As a result, the catfish production target is 1,395 million tonnes in 2020 to 1,652 million in 2024 (DJPB, 2020). Based on this, the government continues intensively accelerating the aquaculture production sector development. Intensification is identical to high stocking density. The main problem in catfish production during the cultivation process is disease infection (Kartikaningsih *et al*., 2020). One of the primary diseases that attack freshwater fish is epidemic septicemia, mainly caused by *Aeromonas hydrophila*, an opportunistic pathogen (Xia *et al*., 2017; Chen *et al*., 2018).

It is a strong Gram-negative bacterium (Zhang *et al*., 2014; Jiang *et al*., 2016), is one of the economically important pathogens in modern aquaculture (Zhang *et al*., 2014), can infect a variety of freshwater fish, and cause substantial economic losses (Jiang *et al*., 2016). Furthermore, infection with *A. hydrophila* is associated with high levels of morbidity and mortality, most of which are acute but also chronic (Bebak *et al*., 2015; Chen *et al*., 2018). Therefore, this epidemic continues to have a negative impact on catfish production (Bebak *et al*., 2015). Catfish production failure caused by this bacterial disease can be prevented by applying suitable biocontrol agent (Yuhana, 2010) in fish farming methods, such as probiotics and prebiotics. Probiotics and prebiotics have become mainstream recently (Huynh *et al*., 2017; Knipe *et al*., 2021).

The synergistic combination of probiotics with prebiotics is known as "synbiotics" (Huynh *et al*., 2017). The application of probiotics and prebiotics is an alternative approach that can be done to increase fish growth, immune response, and significantly improved the stress and disease resistance (Yuhana *et al*., 2021). The results of several studies have proven the success of synbiotics in increasing growth, survival, immune response, fish resistance (Djauhari *et al*., 2016; Rahimnejad *et al*., 2018; Torrecillas *et al*., 2018; Dawood *et al*., 2019; Mohammadi *et al*., 2022) and shrimp resistance (Zubaidah *et al*., 2015; Febrianti *et al*., 2016; Yuhana *et al*., 2022; Nababan *et al*., 2022). This study used *Bacillus* sp. NP5 has been tested to increase growth performance, immune response, and survival as well as inhibit the growth of pathogenic bacteria in several aquaculture such as vannamei shrimp against Infectious Myonecrosis Virus infection (Widanarni *et al*., 2014), carp against infection *A. hydrophila* (Djauhari *et al*., 2016), tilapia against *Streptococcus agalactiae* infection (Agung *et al*., 2015), and catfish against *A. hydrophila* infection (Tamamdusturi *et al*., 2016).

The prebiotics used in this study were oligosaccharides (2.1% inulin, 10% fructooligosaccharides (FOS), 1% galactooligosaccharides (GOS), and 7.5% raffinose) contained in dayak onion plants which have been tested *in vitro* to stimulate the commodities growth of *Pseudoalteromonas piscicida* 1Ub and *Bacillus* sp. NP5 probiotics (Munaeni *et al*., 2020a). Another plant-derived prebiotic, fructooligosaccharides (FOS), has also been studied for animals, fish, and shrimp (Hong *et al*., 2022; Mustafa *et al*., 2019). In addition, dayak onions have antibacterial and antioxidant bioactive compounds, which have been tested to prevent *Vibrio parahaemolyticus* infection in vannamei shrimp (Munaeni *et al*., 2020b) and increase the immune response in catfish (Nugroho *et al*., 2018). Oligosaccharides are a source of prebiotics widely used in mammals and fish (Mahious *et al*., 2006).

The oligosaccharides contained in dayak onions are expected to be utilized by the probiotic *Bacillus* sp. NP5 for growth and activity so that when applied simultaneously, they can improve catfish's immune response and resistance. However, research on using the probiotic *Bacillus* sp. NP5 and dayak onion simplicia powder through feed on catfish is still limited. Therefore, research regarding the effective prevention of *A. hydrophila* infection between dayak onions simplicia powder and *Bacillus* sp. NP5 and their combination is necessary. In this study, the probiotics *Bacillus* sp. NP5, dayak onion simplicia powder (DOSP), and a combination of both were administered orally to catfish. Immune response and fish resistance to pathogens were evaluated to determine the increase in immunity after administering PRO+DOSP and *A. hydrophila* infection.

MATERIALS AND METHODS

Materials

The test materials in this study were fish, disease agents, and dayak onion simplicia powder (DOSP). The fish used was catfish (*Clarias* sp.)

(4.91 ± 0.18 g) obtained from farmers in Ciampea, Bogor, West Java. The pathogenic bacteria used were *Aeromonas hydrophila*. The probiotic bacteria used were *Bacillus* sp. NP5. Pathogenic and probiotic bacteria were identified using API 20 NE system (BioMérieux, France) and through biochemical tests referring to Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). These pathogenic and probiotic bacterial strains are collections of the Aquatic Organism Health Laboratory, Department of Aquaculture, IPB University. Dayak onion simplicia powder is obtained from farmers in Kembayan, Sanggau, West Kalimantan.

Prebiotic activity score test preparation (*in vitro*)

Dayak onion simplicia powder's (DOSP) activity test on probiotic growth stimulation refers to a modified spectrophotometric method (Huebner *et al*., 2008). The bacteria used consisted of two types, namely probiotic and enteric bacteria. The probiotic was *Bacillus* sp. NP5, while the enteric bacteria used *A. hydrophila*. DOSP with different concentrations (0.2; 0.25; 0.3; 0.35; 0.4 g/mL) was dissolved in 10 mL of sterile distilled water (w/v) and homogenized using a vortex.

As much as 1 mL of each concentration was taken and added to 10 mL TSB media, while only TSB media was used for the control. The bacterial suspensions of *Bacillus* sp. NP5 and *A. hydrophila* used had been previously cultured on TSB media and incubated for 14-24 hours at 29°C at 140 rpm. The bacterial suspension (density 10^8 CFU/mL) was inoculated with 5% of each treatment (v/v), then incubated in a water bath shaker at 140 rpm for 14-24 hours at 29°C (Munaeni *et al*., 2020a). The optical density of the suspension was analyzed using a spectrophotometer (600 nm wavelength) at 14, 18, and 24 hours. The prebiotic activity score was expressed quantitatively (Huebner *et al*., 2008):

$$\text{Prebiotic activity score} = \frac{\text{Log Ppt} - \text{Log Pp0}}{\text{Log Pgt} - \text{Log Pg0}} - \frac{\text{Log Ept} - \text{Log Ep0}}{\text{Log Egt} - \text{Log Eg0}}$$

Note:

Ppt = OD of probiotics in onion powder after t-hour
Pp0 = OD of probiotics in onion powder after 0 hours
Pgt = OD of probiotics in control after t-hour
Pg0 = OD of probiotics in control after 0 hour
Ept = OD of enteric in onion powder after t-hour
Ep0 = OD of enteric in onion powder after 0 hour
Egt = OD of enteric in control after t-hour
Eg0 = OD of enteric in control after 0 hour

Preparation of feed supplementation with probiotics

The probiotic bacteria used is *Bacillus* sp. NP5 was resistant to the antibiotic rifampicin, which functions as a genetic marker through the spontaneous mutation technique by growing the bacteria into TSA media containing 50 µg/mL of the antibiotic rifampicin (0.25 g rifampicin, 9.5 mL ethanol absolute, 0.5 mL aquabidest). Probiotic *Bacillus* sp. NP5 Rf^R cells were cultured on TSA medium and incubated for 24 hours at 37°C. Afterwards, *Bacillus* sp. NP5 Rf^R were inoculated onto the TSB medium and incubated in a water bath shaker at 140 rpm for 18-24 hours at 29°C. The total plate count (Madigan *et al*., 2017) was performed to determine the bacterial cells density.

Dilution was performed to achieve a cell density of 10^8 CFU/mL (Aswandi, 2017). The bacterial cell suspension was centrifuged at 5000 rpm for 10 min (Thermo Scientific). Probiotics are added to feed with the top-dressing method. As much as 1 mL of *Bacillus* sp. NP5 Rf^R suspension was mixed with 6 mL of distilled water and 2 mL of egg white as an adhesive. All ingredients were homogenized using a vortex and then sprayed evenly onto 100 g of commercial feed (contained 30% protein). The feed was stored at 4°C to keep the probiotics cells survived.

Preparation of feed supplementation with DOSP

Dayak onions used are three to four months old or have flowered. The tubers are cleaned of adhering dirt and thinly sliced. The slices were dried in an oven at 60°C for 48 hours. The dried slices were then milled using a blender and sifted (60 mesh/250 m) to produce simplicia powder (Munaeni *et al*., 2017). DOSP is added to the feed by the re-pelleting method (Munaeni *et al*., 2020b). The test feed was commercial feed (containing 30% protein), which was powdered and re-molded after adding 20 g/kg DOSP.

Preparation of feed supplementation with a combination of probiotic and DOSP

Commercial feed (containing 30% protein) powdered and re-molded after adding 20 g/kg of DOSP and mixed with 1% probiotic suspension of *Bacillus* sp. NP5 Rf^R 10^8 CFU/mL with the top-dressing method (Utomo *et al*., 2022). The feed was stored at 4°C to keep the probiotics alive.

Preparation of infectious agents

The pathogenic bacteria used were *A. hydrophila* YH1. This pathogenic bacteria has been tested for its pathogenicity in fish through Koch's postulate test. *A. hydrophila* YH1 was made resistant to the antibiotic rifampicin, which functions as a molecular marker through the spontaneous mutation technique by growing the bacteria into TSA media containing 50 µg/mL of the antibiotic rifampicin. Bacterial cells of *A. hydrophila* YH1 Rf^R were cultured in TSA medium and incubated for 24 hours at 37°C.

Next, one loop of bacterial colonies was inoculated onto the TSB medium and incubated in a water bath shaker at 140 rpm for 18-24 hours at 29°C. The bacterial cell suspension was centrifuged at 5000 rpm for 10 minutes (Thermo Scientific). A total plate count (Madigan *et al*., 2017) was performed to determine the density of bacterial cells. Then, dilution was performed to achieve a cell density of 10^6 CFU/mL (based on the LD50 test).

Experimental design and rearing of experimental animals

The fish were divided into five groups (A: feed supplementation with 20 g/kg DOSP; B: feed supplementation with 1% *Bacillus* sp. NP5 Rf^R 10^8 CFU/mL; C: feed supplementation with a combination of DOSP 20 g/kg and 1% *Bacillus* sp. NP5 Rf^R 10^8 CFU/mL; D: positive control; E: negative control) and three replicates with 30 fish in each container were reared for 45 days. The rearing container was aquarium with a dimension of 60×30×30 cm^3, each was filled with 30 L of fresh water equipped with aeration. During the rearing period, the aquarium was siphoned from feces, and water changes were performed every four days as much as 20-30% of the total water volume.

Feeding was given at satiation three times a day, at 08:00 am, 12:00 pm, and 05:00 pm. In addition, water quality monitoring was conducted three times during the rearing period (beginning, middle, and end) within the standard range for catfish based on SNI 6484.4 (2014), temperatures ranged from 29.7 to 31.4°C, pH ranges were between 6.8 to 7.6, dissolved oxygen ranges were between 5.0-6.2 mg/L, and total ammonia nitrogen (TAN) values were between 0.15-0.25 mg/L.

Challenge test

The challenge test was performed on day 46 after the fish were fed accordingly to the treatment feed. The intramuscular injection was carried out by injecting the *A. hydrophila* suspension at a cells concentration of 10^6 CFU/mL, and the volume was 0.1 mL for each fish (based on the LD50 test). The negative control (NC) was injected with 0.1 mL of PBS solution. The post challenge test observation was performed for seven days. During post infection, the fish were fed with the commercial feed without supplementation. Daily fish mortality was monitored for seven days, and survival rates were calculated. The dead fish were removed from the rearing tanks.

The measurement of parameters

Total Erythrocytes (Blaxhall and Daisley, 1973)

Fish blood was taken as much as 1 mL from the caudal area of the fish using a 1 mL syringe, put into the microtube, and stored in a cool box. Blood was sucked using a pipette to a scale of 0.5, and then Hayem's solution was added to a scale of 101. The pipette was shaken to the number eight-like form for three to five minutes. The first drop in the pipette was discarded, then the next drop dripped into the hemocytometer. The total erythrocytes number of cells was counted under a microscope with a magnification of 40×.

Hemoglobin (Wedemeyer and Yasutake, 1977)

Measurement of hemoglobin levels referred to the Sahli method with a Sahlinometer and expressed in % on the yellow scale. HCl 0.1N solution was put into the hemometer tube on a 10 (red scale) scale. The blood cells was added using a Sahli pipette to a scale of five and allowed to stand for three minutes. Aquadest was added until the color of the solution in the tube was the similar color as the both sides color of the hemometer tubes.

Hematocrit (Anderson & Siwicki, 1995)

Hematocrit levels were performed by sucking blood using a hematocrit tube up to ¾ of the volume. The blood was then centrifuged for five minutes at 8000 rpm. The ratio of pelleted-blood

cells and total blood volume were measured using a ruler.

Total Leukocytes (Blaxhall & Daisley, 1973)

Fish blood was taken as much as 1 mL from the base of the tail using a 1 mL syringe, put into a microtube, and stored in a cool box. Blood was sucked with a pipette to a scale of 0.5. Turk's solution was added to a scale of 11. The pipette was shaken to form an 8 for three to five minutes. The first drop in the pipette was discarded, and the next dripped on the hemocytometer. The total leukocytes were observed, and the number of cells was counted under a microscope with 40× magnification. The found white blood cells are counted using the following formulation:

$$\frac{\text{Total Leukocytes}}{(\text{cells mm}^{-3})} = \frac{\Sigma \text{ counted}}{\text{cells}} \times \frac{1}{\text{area counted (mm}^{3})} \times \frac{\text{dilution}}{\text{factor}}$$

Phagocytosis Activity (Anderson & Siwicki, 1995)

One loop of *Staphylococcus aureus* colonies was cultured 24 hours previously, put into a microtube containing 100 µL PBS, and then vortexed to make it homogeneous. As much as 50 µL of fish blood samples for each treatment were stored on a microplate. As much as 50 µL of *S. aureus* cells suspension was mixed into 50 µL of fish blood samples in each treatment until homogeneous and incubated for 20 minutes at room temperature. From this mixture, 5 µL was taken to make a smear preparation.

The smear preparations were dried and fixed with 100% methanol for five minutes and then redried. The preparations were soaked with Giemsa dye for 20 minutes, rinsed with distilled water and then dried. Observations were made using a microscope with a magnification of 40×. Phagocytosis activity was calculated based on the percentage of phagocytic cells showing phagocytosis.

Resistance

The resistance of catfish was measured based on the survival of the fish after a 7-day infection with *A. hydrophila*. Survival was calculated at the end of the challenge test (Dehaghani *et al*., 2015):

$$\text{Survival} = \frac{N_f}{N_0} \times 100$$

Note:
N_0 = Initial number of fish
N_f = Final number of fish

Total bacterial count, total *Bacillus* sp. NP5 Rf^R, and total *A. hydrophila* Rf^R

Total bacterial count, total *Bacillus* sp. NP5 Rf^R and total *A. hydrophila* Rf^R in catfish were calculated using the spread-plate method (Madigan *et al*., 2017). For total bacterial cells and *Bacillus* sp. NP5 Rf^R, fish intestine in each treatment was weighed as much as 0.1 g and homogenized in 0.9 mL of sterile PBS solution (0.8% NaCl, 0.15% K_2HPO_4, 0.02 Na_2HPO_4, and 0.02% KCl), serial dilutions were performed. As much as 50 µL of each dilution was spread into TSA medium for total bacterial counts. TSA medium was added with the antibiotic rifampicin (50 µg/mL) for total *Bacillus* sp. NP5 Rf^R. Meanwhile, for total *A. hydrophila* Rf^R, fish liver, and kidney in each treatment were weighed as much as 0.1 g and homogenized in 0.9 mL of sterile PBS solution, serial dilutions were performed. As much as 50 µL of each dilution was spread onto RS (Rimmler-Shotts) medium that was added with the antibiotic rifampicin (50 µg/mL).

Data analysis

Data were analyzed using Microsoft Excel 2010 and analyzed by variance (ANOVA) using SPSS version 20 software. If the results obtained were significantly different ($P<0.05$), further tests were carried out using Duncan's test with a 95% confidence interval.

RESULTS AND DISCUSSION

Result

Testing the prebiotic activity score showed that the highest score after 14 hours of incubation was at a concentration of 0.20 g/mL but was not significantly ($P>0.05$) different between treatments. After 18 hours of incubation, the concentration of 0.20 g/mL showed the highest value significantly ($P<0.05$) compared to other concentrations, except for concentrations of 0.25 g/mL and 0.30 g/mL. After 24 hours of incubation, the concentration of 0.20 g/mL showed the highest value significantly ($P<0.05$) compared to other concentrations, except for the concentration of 0.25 g/mL (Figure 1).

After 45 days of rearing, the TBC value of the PRO+DOSP treatment was significantly ($P<0.05$) higher than the other treatments, except for the DOSP treatment. Even so, the TBC value in the PRO+DOSP treatment was highfer than the other treatments both before and after infection. Meanwhile, the *Bacillus* sp. NP5 Rf^R value in the

PRO+DOSP treatment was significantly (P<0.05) higher than in other treatments before and after infection.

Post-infection, total *A. hydrophila* Rf^R in the liver in the PRO+DOSP treatment was not significantly (P>0.05) different compared to the DOSP and probiotic treatments but significantly (P<0.05) different than the positive control. Nonetheless, in general, the total *A. hydrophila* Rf^R in the liver in the PRO+DOSP treatment was lower than in the other treatments. In addition, the total *A. hydrophila* Rf^R in the kidneys in the PRO+DOSP treatment was significantly (P<0.05) lower compared to the DOSP, probiotic, and positive control treatments (Figure 2).

After 45 days of rearing, the TE and Ht values in the PRO+DOSP treatment were significantly higher (P<0.05) than in other treatments, except for the probiotic treatment. Even so, the TE and Ht values in the PRO+DOSP treatment were higher than the other treatments. Meanwhile, the Hb value in the PRO+DOSP treatment was significantly (P<0.05) higher than in the other treatments.

After infection with *A. hydrophila* Rf^R, TE values in the PRO+DOSP treatment on days 48, 50, and 52 were significantly (P<0.05) higher than the other treatments. Meanwhile, the Ht and Hb values in the PRO+DOSP treatment on days 48, 50, and 52 were significantly (P<0.05) higher

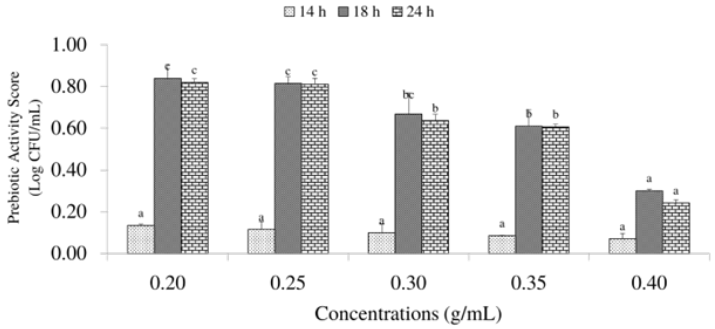

Figure 1. Prebiotic activity score. Values (mean ± SD) with different letters at the same hour were significantly different (P<0.05). Incubation 14, 18, and 24 hours.

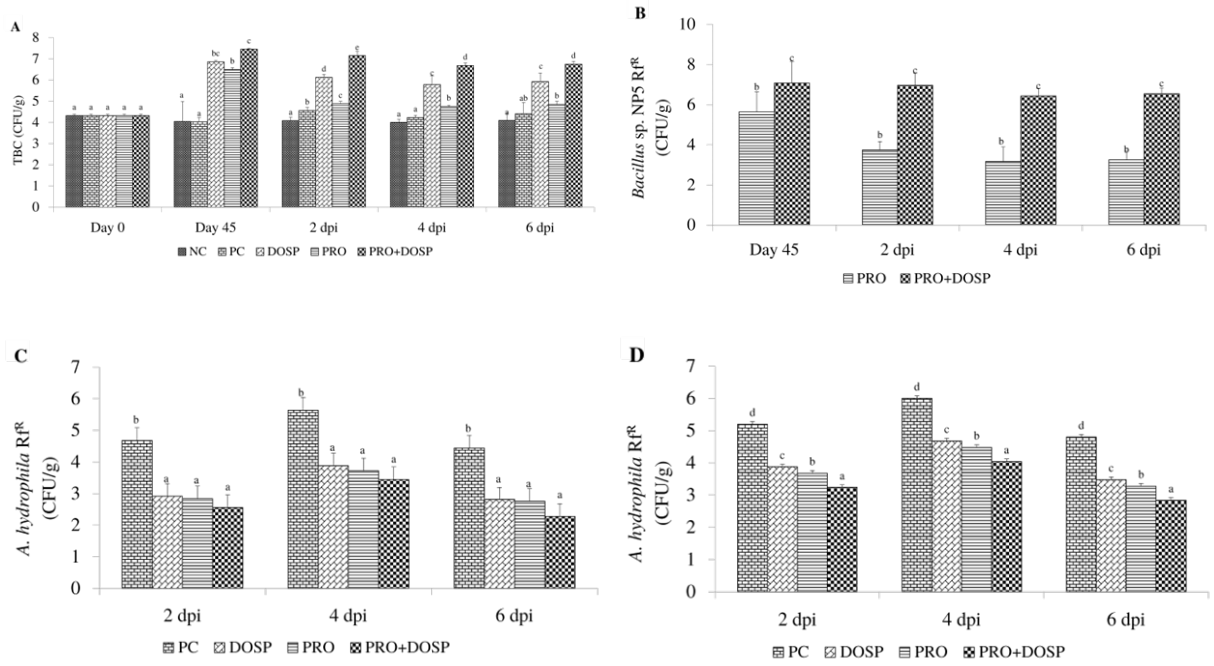

Figure 2. (A) Total bacterial count; (B) total *Bacillus* sp. NP5 Rf^R; (C) total *A. hydrophila* Rf^R in the liver; and (D) kidney. (NC) negative control; (PC) positive control; (DOSP) dayak onion simplicia powder; (PRO) probiotic; (PRO+DOSP) combination probiotic and dayak onion simplicia powder; (dpi) days post-infection. Different letters in each bar (mean value ± standard deviation) indicate a significant difference (P<0.05). Days post infection.

than the other treatments, except for the probiotic treatment. Nonetheless, the Ht and Hb values in the PRO+DOSP treatment were higher than the other treatments (Figure 3).

After 45 days of rearing, the TL value of the PRO+DOSP treatment was significantly (P<0.05) higher than the other treatments, except for the probiotic treatment. Meanwhile, the AF value in the PRO+DOSP treatment was not significantly (P>0.05) different compared to the other treatments but significantly (P<0.05) different from the control. Nonetheless, the TL and AF values in the PRO+DOSP treatment were higher than in the other treatments. After infection with *A. hydrophila* Rf^R, the TL and AF values of the PRO+DOSP treatment were not significantly (P>0.05) different with the DOSP and probiotic treatments but significantly (P<0.05) different with the positive control. Nonetheless, the TL and AF values in the PRO+DOSP treatment were higher than in the other treatments (Figure 4).

The survival of catfish before infection in all treatments was 100%. Post-infection for seven days, the survival rate of the PRO+DOSP treatment

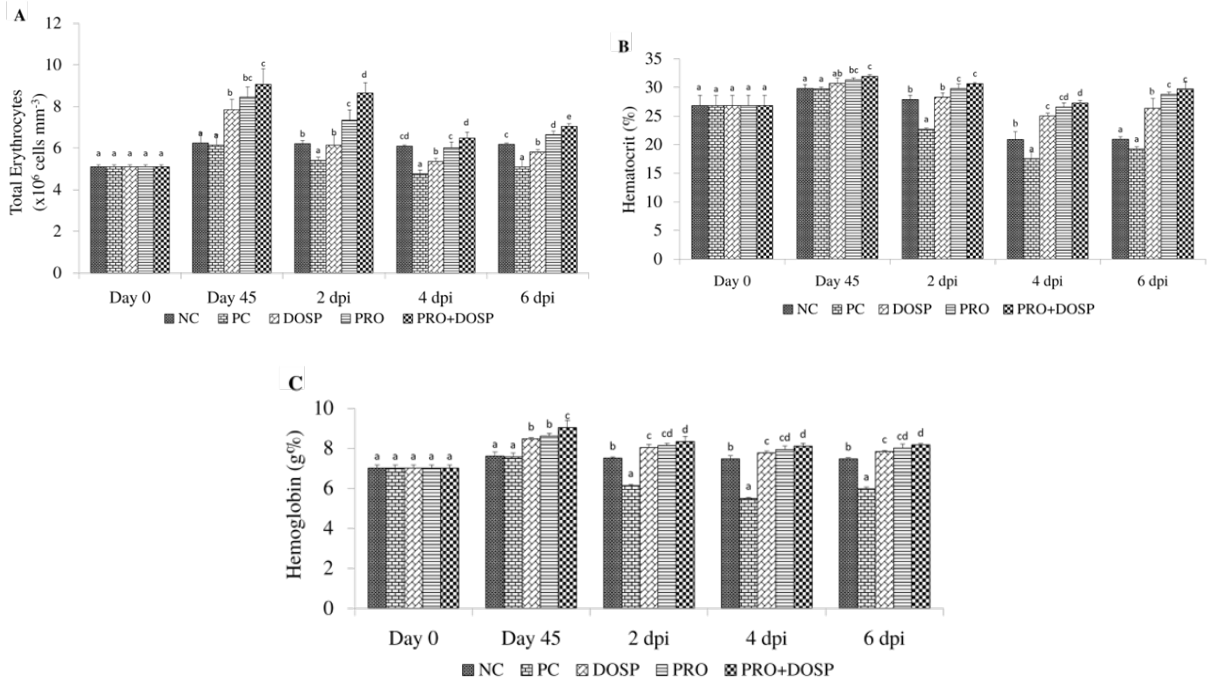

Figure 3. (A) Total erythrocyte (TE); (B) hematocrit (Ht); and (C) hemoglobin (Hb) catfish before and post-infection with *A. hydrophila*. (NC) negative control; (PC) positive control; (DOSP) dayak onion simplicia powder; (PRO) probiotic; (PRO+DOSP) combination probiotic and dayak onion simplicia powder; (dpi) days post-infection. Different letters in each bar (mean value ± standard deviation) indicate a significant difference (P<0.05).

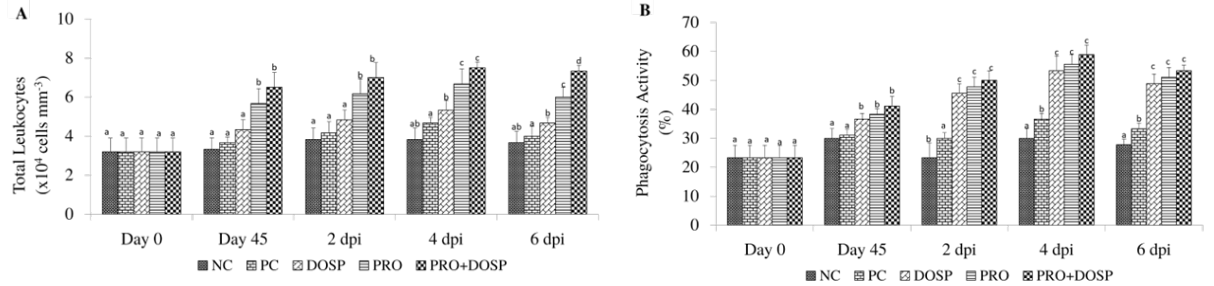

Figure 4. (A) Total leukocytes (TL) and (B) phagocytic activity (PA) of catfish before and post-infection with *A. hydrophila*. (NC) negative control; (PC) positive control; (DOSP) dayak onion simplicia powder; (PRO) probiotic; (PRO+DOSP) combination probiotic and dayak onion simplicia powder; (dpi) days post-infection. Different letters in each bar (mean value ± standard deviation) indicate a significant difference (P<0.05).

was significantly (P<0.05) higher than the positive control and DOSP but not significantly (P>0.05) different with the probiotics. The highest survival in the treatment infected with *A. hydrophila* Rf^R was found in the PRO+DOSP treatment (79.01%), followed by the probiotic treatment (75.31%) and DOSP (70.37%). The lowest value was found in the positive control of 59.26% (Figure 5).

Discussion

In this study, we combined probiotic strains of *Bacillus* sp. NP5 with dayak onion simplicia powder (DOSP). We evaluated their effect on immune response and resistance to *A. hydrophila* infection in catfish. The probiotic dosage used referred to the optimal dose obtained from previous research (Aswandi, 2017), and the DOSP dosage used referred to obtained from the optimal dose *in vitro* tests. Studies on *Bacillus* sp. NP5 combination supplementation with dayak onion simplicia powder is still limited.

The dayak onion used in this study was in the form of simplicia powder without extraction. The *in vitro* test results showed that the probiotic activity score decreased with increasing incubation time with the higher concentration of DOSP. It is due to the role of the phytochemical compounds from the DOSP as an antibacterial which is more significant than its prebiotic function (Munaeni *et al*., 2020a). In this study, the concentration of 0.20 g/mL was the optimal concentration for using DOSP, which stimulated the probiotic *Bacillus* sp. NP5 starting from 14 to 24 hours of incubation (Figure 1).

Sawangwan *et al*. (2018) found that oligosaccharide prebiotics from mushroom extracts stimulated the growth of the probiotics *Lactobacillus acidophilus* and *Lactobacillus plantarum* for 24 hours. An effective synbiotic formulation must contain both prebiotics and beneficial microorganisms (Romano, 2021). Our research results show that *Bacillus* sp. NP5 concentration of 10^8 CFU/mL 1% combined with 20 g/kg DOSP significantly increased fish defense against *A. hydrophila* infection. Fish supplemented with PRO+DOSP had an average survival rate of 79.01%, while the control only had 59.26%.

Survival in the PRO+DOSP group was also higher compared to the single administration of probiotics (75.31%) or DOSP (70.37%). This study showed that the probiotic *Bacillus* sp. NP5 and DOSP increased the resistance of catfish. Compared with single probiotic or DOSP supplementation, the administration of a PRO+DOSP combination strengthens the beneficial effect on catfish. Balcazar *et al*. (2006) stated that the beneficial effect of synbiotics on the host occurs due to the colonization of some beneficial bacteria in the host's digestive tract.

It is supported by the research results of Total Bacterial Count (TBC) and total *Bacillus* sp. NP5 Rf^R in the PRO+DOSP treatment was higher than in the other treatments (Figures 2A and 2B). Therefore, combining probiotics and DOSP can increase resistance and protect fish against pathogenic infections. Hematological parameters are usually measured to determine fish-fed functional feed additives' general immunity and health status (Dawood *et al*., 2019). This study showed that fish supplemented with PRO+DOSP had higher TE, Ht, and Hb values than other treatments.

The increase in TE, Hb, and Ht after administration of the PRO+DOSP indicated that the catfish's health status was good. Djauhari

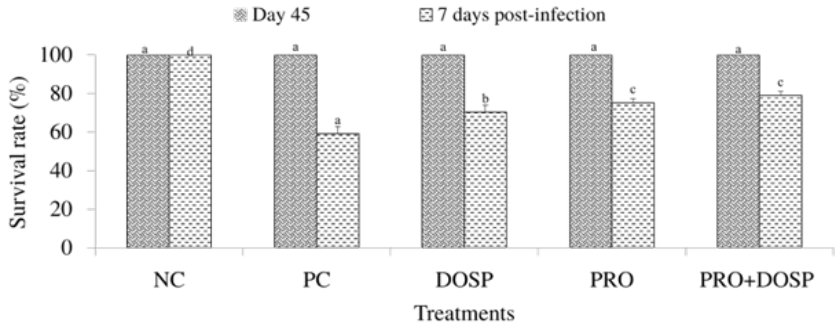

Figure 5. The survival rate of catfish before infection (Day 45) and seven days post-infection with *A. hydrophila*. (NC) negative control, (PC) positive control, (DOSP) dayak onion simplicia powder, (PRO) probiotic, (PRO+DOSP) combination probiotic and dayak onion simplicia powder Different letters in each bar (mean value ± standard deviation) indicate a significant difference (P<0.05).

(2016) reported that administering synbiotics through the feed for 30 days increased carp's TE, Ht, and Hb. After infection, there was a decrease in TE, Ht, and Hb values in all treatments except the negative control. The decrease peak occurred on day 50 (four days post-infection) due to the fish experiencing kidney infection due to an attack by *A. hydrophila*, causing anemia (Korni *et al*., 2017).

Sirimanapong *et al*. (2018) stated that the inhibition of red blood cell production caused by *S. agalactiae* infection caused the number of red blood cells to decrease. *A. hydrophila* infection causes significant hematological changes in fish (Yu *et al*., 2015). *A. hydrophila* infection damages internal organs, especially hematopoietic organs such as the spleen, liver, and kidney (Stratev *et al*., 2015). In fish, the kidney, especially the head kidney, is the main erythropoietic organ (Chen *et al*., 2013).

Meanwhile, the liver is the main organ of nutrient and energy metabolism, detoxification, and immunity (Hajirezaee *et al*., 2020; Jia *et al*., 2019). The liver and kidney have a functional relationship, interference with the liver and kidney causes impaired function. *A. hydrophila* produces toxins, namely β-hemolysin, and aerolysin. It can lyse erythrocytes, causing a decrease in erythrocytes, hemoglobin, and hematocrit levels (Harikhrisnan *et al*., 2003).

Hemoglobin and hematocrit are related, a low red blood cell count causes hemoglobin and hematocrit in the blood also to be low (Susanti *et al*., 2016). On day 52 (six days post-infection), TE, Ht, and Hb began to increase. It is presumably due to the decreased incubation period of *A. hydrophila* bacteria in the fish's body. It is supported by data on the total *A. hydrophila* in the target organs four days post-infection decreased. In this condition, the fish returns its body to its normal condition.

The increase in TE indicated an effort to maintain homeostasis in the fish body after infection. The body will re-produce erythrocytes to replace the decreased erythrocytes due to infection. Probiotics, prebiotics, and synbiotics are known for their substantial immunostimulating effects, starting with activating mucosal immunity in the intestine, serum immunity, antioxidant response, then whole-body immunity (Romano, 2021). Administration of PRO+DOSP to catfish for 45 days also increased TL and PA significantly ($P<0.05$) than other treatments.

The increase in TL and PA after administering PRO+DOSP is due to *Bacillus* sp. NP5 contains substances of immunogenic character, which are recognized as foreign bodies by the fish body so that they can induce and activate immune cells (Djauhari, 2016). Leukocytes are a critical component of the immune system and play an essential role in defense against pathogens (Yu *et al*., 2015). This role is played by phagocytic cells (monocytes and neutrophils), which are indicated by the value of the phagocytic activity (Deleo & Quinn, 2019). According to Akhter *et al*. (2015), the increase in the immune response after administering synbiotics is due to the immunomodulatory effects of probiotics and prebiotics. Probiotics and prebiotics in the digestive tract will interact with pattern recognition receptors (PRRs) through microbial-associated molecular patterns (MAMPs), which can trigger an immune response (Song *et al*., 2014).

Djauhari (2016) reported that administering synbiotics through the feed for 30 days increased the TL and PA of carp. After infection, the TL and PA values increased from day 48 (two days post-infection) and experienced the highest peak on day 50 (four days post-infection), then decreased on day 52 (six days post-infection). The increase in TL and PA values after infection both on day 48 and day 50 is a form of resistance from catfish immune cells against an *A. hydrophila* bacteria attack. This form of resistance also can be seen from the SR data of catfish given PRO+DOSP, which was higher than DOSP, probiotics, and controls after being infected with *A. hydrophila* (Figure 5).

On day 52, the TL and PA decreased. It is possible because *A. hydrophila* was successfully eliminated by immune cells. It can be seen from the total number of *A. hydrophila* bacteria in the target organs (liver and kidney) on day 52, where the population decreased compared to days 48 and 50 post-infection (Figures 2C and 2D). It is also suspected that the fish experienced tissue repair then, and their immune system started to recover. In addition, the ability of PRO+DOSP to increase the immune response of catfish can see from the total number of *A. hydrophila* in target organs (liver and kidney) (Figures 2C and 2D).

In fish, the kidney and liver are internal organs sensitive to *Aeromonas* disease (Rozi *et al*., 2018). The total *A. hydrophila* in the target organs in the PRO+DOSP treatment showed a lower value

than in the other treatments. Based on several studies, the increase in the immune response of catfish in this study is expected to be related to the performance of the fish immune system, which is triggered by the synergistic action of probiotics and DOSP. Furthermore, it is possible due to the role of oligosaccharides which can stimulate the growth of *Bacillus* sp. NP5 so that bacteria can dominate, colonize, compete, and inhibit in the end, the number (*quorum*) needed to express the virulence factor of *A. hydrophila* was not achieved. In addition, it is suspected that there is a role for antibacterial compounds from DOSP in helping to suppress *A. hydrophila* populations in target organs.

CONCLUSION

Combination of probiotic strains *Bacillus* sp. NP5 10^8 CFU/mL 1% with DOSP 20 g/kg showed a positive effect on catfish immune response and protection against *A. hydrophila* infection. The significantly reduced fish mortality after being infected with *A. hydrophila* is related to the ability of the PRO+DOSP to modulate the immune response. Our results also show that supplementation of the *Bacillus* sp. NP5 and DOSP significantly improve the overall performance of the parameters compared to the single administration of the probiotic *Bacillus* sp. NP5 or DOSP in catfish.

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