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Different dosage applications of *Bacillus* sp. NP5 para-probiotic on the growth performance and resistance of Nile tilapia against *Streptococcus agalactiae* infection

Efek aplikasi dosis paraprobiotik *Bacillus* sp. NP5 yang berbeda terhadap kinerja pertumbuhan dan resistansi ikan nila terhadap infeksi *Streptococcus agalactiae*

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ABSTRACT

The study aimed to determine the effect of various dietary supplementation doses of *Bacillus* sp. NP5 para-probiotic for improving the immune responses and resistance of juvenile tilapia (*Oreochromis niloticus*) against *Streptococcus agalactiae* infection. *Bacillus* sp. NP5 para-probiotic was produced through heat-inactivation at 95°C for 1 h. This study used a completely randomized design, which consisted of four treatments and three repetitions. The test diet was supplemented with 1% para-probiotic at 10^8, 10^9, and 10^{10} CFU/ml density levels. Fish (10.29 ± 0.22 g) were reared for 30 days, before being challenged with *S. agalactiae* (10^7 CFU/ml) intraperitoneally for 14 days on the 31st day. For negative control treatment, fish were injected with PBS. The results showed that the dietary supplementation of para-probiotic treatments could significantly increase ($P<0.05$) the average final weight, specific growth rate (SGR), and feed conversion ratio (FCR), compared to the control treatment group after 30 days of rearing. Increased number of erythrocytes, number of leukocytes, hemoglobin, hematocrit, respiratory burst, and differential leukocyte in *Bacillus* sp. NP5 para-probiotic dietary supplementation at 10^{10} CFU/ml concentration obtained a higher survival rate ($P<0.05$), than the positive control, after *S. agalactiae* challenge. Therefore, dietary supplementation of para-probiotic *Bacillus* sp. NP5 at 10^{10} CFU/ml concentration can improve the growth performance, immune response, and disease resistance of Nile tilapia against *S. agalactiae* infection.

Keywords: *Bacillus* sp. NP5, Tilapia, para-probiotic, *Streptococcus agalactiae*

ABSTRAK

Penelitian ini bertujuan untuk menguji efektivitas pemberian berbagai dosis paraprobiotik *Bacillus* sp. NP5 melalui pakan dalam meningkatkan kinerja pertumbuhan, respons imun, dan resistansi benih ikan nila (*Oreochromis niloticus*) terhadap infeksi *Streptococcus agalactiae*. Pembuatan bakteri paraprobiotik *Bacillus* sp. NP5 dengan metode pemanasan pada suhu 95°C selama satu jam. Penelitian ini menggunakan rancangan acak lengkap terdiri dari empat perlakuan dengan tiga ulangan. Pakan uji diperkaya dengan 1% paraprobiotik dengan kepadatan 10^8, 10^9, dan 10^{10} CFU/ml. Benih ikan nila (10,29 ± 0,22 g) dipelihara selama 30 hari dan pada hari ke 31, ikan diuji tantang selama 14 hari dengan *S. agalactiae* (10^7 CFU/ml) melalui injeksi intraperitoneal kecuali perlakuan kontrol negatif yang dinjeksi dengan PBS. Hasil penelitian setelah 30 hari pemberian paraprobiotik *Bacillus* sp. NP5 menunjukkan rata-rata bobot akhir, laju pertumbuhan spesifik (LPS) dan rasio konversi pakan (RKP) yang berbeda signifikan ($P<0,05$) dibandingkan dengan kontrol. Peningkatan nilai total eritrosit, kadar hemoglobin, kadar hematokrit, total leukosit, aktivitas fagositik, respiratory burst dan diferensial leukosit pada semua perlakuan paraprobiotik *Bacillus* sp. NP5 khususnya pada perlakuan 10^{10} CFU/ml sehingga diperoleh tingkat kelangsungan hidup yang lebih tinggi ($P<0,05$) dibandingkan kontrol positif pasca uji tantang *S. agalactiae*. Pemberian paraprobiotik *Bacillus* sp. NP5 dengan dosis yang berbeda melalui pakan dapat meningkatkan kinerja pertumbuhan, respons imun dan resistansi ikan nila terhadap infeksi *S. agalactiae* dengan dosis terbaik 10^{10} CFU/ml.

Kata kunci: *Bacillus* sp. NP5, ikan nila, paraprobiotik, *Streptococcus agalactiae*

INTRODUCTION

Nile tilapia (*Oreochromis niloticus*) is a commercial freshwater fish, which is highly recommended as aquaculture commodity (FAO 2018) with high market demand (Uddin *et al*., 2021). Nile tilapia production in 2015-2018 continued to increase annually with the average of 12.85% (DJPB 2018). Indonesia becomes one of the most Nile tilapia producers, after China (Ali *et al*., 2020). Nile tilapia fish culture with an intensive system has many been performed to fulfill the market demand (Ottinger *et al*., 2016), however, good aquaculture practice applications can cause a negative impact on the environment, disturb the culture activities further (David *et al*., 2018), and increase the disease attack risk (Joffre *et al*., 2018).

Streptococcosis is the main disease in Nile tilapia, caused by *Streptococcus agalactiae* pathogenic bacteria (Xu *et al*., 2019). Several studies revealed some efforts that can be performed to control the streptococcosis disease by applying fish vaccines (Zhang *et al*., 2017) and probiotics (Kuebutornye *et al*., 2020). Vaccines are inactive or attenuated pathogens by heating or chemical exposure (Rodrigues & Plotkin, 2020). Vaccines are applied to assist specific and non-specific immune system formations for producing a long-term immune system memory (Laith *et al.,* 2019). However, vaccines are only effective to attack specific pathogen and ineffective for other pathogens (Van Hai, 2015). Moreover, vaccines are limited to apply as a preventive way in culture activities. Probiotics are living microbes that provide many benefits for their host by improving the microbial balance in the digestive tract (Hoseinifar *et al*., 2018). *Bacillus* sp. NP5 are probiotic-potential bacteria. *Bacillus* sp. NP5 bacteria were initially isolated from Nile tilapia digestive tract and could provide positive responses in improving the Nile tilapia growth (Putra & Widanarni, 2015), besides preventing Nile tilapia from streptococcosis disease (Widanarni & Tanbiyaskur, 2015).

Several studies regarding *Bacillus* sp. NP5 as probiotics revealed that these bacteria could promote the growth and increase the survival rate of catfish on *Aeromonas hydrophila* post-infection (Mustahal *et al*., 2021). *Bacillus* sp. NP5-supplemented diet could significantly induce the immune response and growth performance of striped-catfish (Tamamdusturi *et al*., 2016). Whiteleg shrimp fed with diets containing *Bacillus* sp. NP5 obtained an increased growth performance and a resistance against the disease due to immune system improvement (Widanarni *et al*., 2015). However, probiotics in aquaculture activities are still being concerned based on their function and application, namely the viability level in diet (Rosas-Ledesma *et al*., 2012), settling-capability in the intestine (Bernardeau *et al*., 2017), virulent-gene transfer possibility from pathogenic bacteria to probiotics (Li *et al*., 2020). According to Deshpande *et al*. (2018), dead microbes that can be utilized in the host as effective as the living microbes are called parabiotics. Therefore, parabiotics can be an alternative way to prevent the following concerns.

Para-probiotics are originated from good microorganisms that lose their viability due to exposure factors that alter microbial cell structures, such as DNA filament disconnection, cell membrane disruption, or enveloped-cell mechanical damage (de Almada *et al*., 2016). Parabiotics have been applied in several studies and known to demonstrate some bioactivity conditions, such as inflammation, immunomodulator, and antioxidative activity, which can positively affect several metabolism pathways and host immune system (Cuevas-González *et al*., 2020). Inactivated *Bacillus pumilus* probiotic could promote a significant increase on the growth of juvenile grouper (*Epinephelus coioides*) (Yan *et al*., 2016). *Bacillus amyloliquefaciens* could induce the immune system of Rohu carp (Singh *et al*., 2017). Moreover, *Lactobacillus plantarum* para-probiotic produced by heat-inactivation method could induce the immune response of *Macrobrachium rosenbergii* (Dash *et al.,* 2015). Based on the previous studies, para-probiotic applications through dietary supplementation is expected as an alternative way to improve the growth performance and immune response. This study aimed to determine the dietary supplementation effect of various *Bacillus* sp. NP5 para-probiotic doses in improving the growth performance, immune response, resistance of Nile tilapia against *S. agalactiae* infection.

MATERIALS AND METHODS

Para-probiotic preparations

Bacillus sp. NP5 bacteria were collected from Laboratory of Fish Health, Department of Aquaculture, FPIK, IPB University. Bacteria were marked with rifampicin as an antibiotic-resistant marker, thus called as *Bacillus* sp. NP5

Rf^R. *Bacillus* sp. NP5 Rf^R were mass-cultured on tryptic soy broth (TSB) media and shaken at 29°C and 140 rpm for 24 h. Bacteria were then harvested by centrifugation at 4°C and 10.000 rpm for 10 minutes, thus the final concentration was 10^{10} CFU/ml. Isolate suspension was diluted into 10^8, 10^9 dan 10^{10} CFU/ml. Furthermore, bacteria were inactivated at 95°C for 1 h (Yang *et al.* 2014). Bacteria were controlled by spreading them on a tryptic soy agar (TSA) and incubating them at 29°C for 24 h. Ungrown bacteria indicate that the para-probiotic has been created and can be used further.

Research design and test feed preparation

This study used a completely randomized design with four treatments and three replications. Para-probiotics were supplemented in the diet at 1‰ concentration with the densities of 10^8 CFU/ml (P8), 10^9 CFU/ml (P9), 10^{10} CFU/ml (P10), and 0 CFU/ml (control, without para-probiotic). A *781-2* commercial feed type was used as a diet with 31-33% protein content. Para-probiotics were supplemented to the diet through spraying with 1 mL syringe, after adding 2% egg whites as a binder (Djauhari *et al*., 2016). The diet was air-dried for 15-20 minutes, before packing in an airtight plastic and preserving in a refrigerator at 4°C.

Fish rearing

Samples used red Nile tilapia fish, that were obtained from a fish culturist in Bogor, West Java. The average weight and length of red Nile tilapia were measured at 10.29 ± 0.22 g and 8.93 ± 0.36 cm, respectively. After acclimatization for 3 days, the Nile tilapia seeds were stocked at 15 fish/aquarium. The tank used for rearing was a 60×30×40 cm^3 aquarium with 60 L water volume. Nile tilapia were fed with the treatment diets for 30 days three times a day at 08.00, 12.00, and 16.00 WIB until apparent satiation. Water exchange and siphon were performed every morning at 30%. Water quality was controlled at temperature of 25.9-26.9ºC, pH of 7.6-7.8, dissolved oxygen (DO) of 4.27 – 4.65 mg/L, and ammonia (NH_3) < 0.019 mg/L.

Challenge test with *Streptococcus agalactiae* bacteria

After feeding for 30 days, Nile tilapia were challenged with *S. agalactiae* bacteria, that were collected from Laboratory of Fish Health, Department of Aquaculture, FPIK, IPB. Challenge test contained five treatments, namely P8, P9, P10, K (+), and K (-). In the P8, P9, P10, and K (+) treatments, fish were challenged with *S. agalactiae* intraperitoneally at 0.1 mL of 10^7 CFU/ml bacterial density, while the K (-) treatment was injected with 0.1 mL PBS. Observation was performed for 14 days after challenge test and dead fish were counted for survival rate data on the final challenge test period.

Growth performance

Growth performance was measured on the final rearing period at 30th day. Growth performance parameters were composed of survival rate (SR), specific growth rate (SGR), and feed conversion ratio (FCR). These parameters were calculated following the formulas (Yan *et al.,* 2016): SR (%) = $N_t/N_0 \times 100$; SGR (%/day) = $100 \times [(\ln W_t - \ln W_0)/t]$; and FCR = $FI/(W_t - W_0)$, whereas N_t is final number of fish (g), N_0 is initial number of fish (g), ln W_t is average final fish weight (g), ln W_0 is average initial fish weight (g), t is rearing period duration (day), FI is feed intake (g), W_t is final fish biomass (g), and W_0 is initial fish biomass.

Enzyme activity

Nile tilapia digestive tract was measured at 0.5 g, before adding Tris-buffer solution (20 mM Tris-HCl, 1 mM EDTA, and 10 mM $CaCl_2$ pH 7.5) with 10% ratio (b/v). Furthermore, the mixture was moved to a microtube and centrifuged for 10 minutes at 12.000 rpm and 4°C. Supernatant was included in a microtube and preserved at -20°C for further use. Supernatant was supplied following the measured enzyme activities, namely amylase (Worthington, 1993), protease (Bergmeyer *et al.,* 1983), and lipase (Borlongan, 1990).

Immune responses

Immune response was observed on the 0th, 30th, 34th (3 days after challenge test), 37th (6 days after challenge test), and 41st (10 days after challenge test) days. The immune response parameters were composed of total erythrocytes (Blaxhall & Daisley, 1973), hemoglobin (Wedemeyer & Yasutake, 1977), hematocrit (Anderson & Siwicki, 1993), total leukocytes (Blaxhall & Daisley 1973), phagocytic activity (Anderson & Siwicki, 1993), respiratory burst (Cheng *et al*., 2004), and differential leukocytes (Amlacher, 1970).

Total *Streptococcus agalactiae* in target organs

S. agalactiae bacteria were counted using

the spreading dish method. The 0.1 g of each target organ (brain, eye, kidney, and liver) was homogenized in 0.9 mL sterile PBS for serial dilution. Then, 50 µL of each dilution serial was spread onto a Petri dish filled with brain-heart infusion agar (BHIA) medium. Total *S. agalactiae* in the target organs were counted on the 34th, 37th, and 41st days.

Data analysis

All data were tabulated in Microsoft Excel 2016. For growth performance and immune response data, Shapiro-Wilk and Levene tests were performed to confirm the normality and variance homogeneity. Then, data were analyzed with an analysis of variance (ANOVA) with SPSS 22.0. If there was a significant different found among the data, Duncan's multiple range test was used further.

RESULTS AND DISCUSSIONS

Growth performance

Based on the growth performance of Nile tilapia, the survival rate had no significant difference among treatments (P>0.05). SGR and FCR on para-probiotic treatments were significantly different (P<0.05) from the control treatment. The highest SGR value was found in the P9 treatment (1.67 ± 0.13 %/day), followed by the lowest FCR value in the similar treatment (1.33 ± 0.10). Growth performance of Nile tilapia on the rearing period is presented in Table 1.

Different doses of *Bacillus* sp. NP5 para-probiotic showed a significant different (P<0.05) to control on enzyme activities based on Table 2. The highest amylase and protease enzymes were found in the P10 treatment at 2.94 ± 0.04 IU/mL and 0.18 ± 0.00 IU/mL, respectively. Meanwhile, all para-probiotic treatments were significantly different (P<0.05) from the control treatment on lipase enzyme activity.

Immune responses

Fish health status and immune response are demonstrated from the blood profiles. Immune response after 30 days of rearing showed that the P8, P9, and P10 increased significantly (P<0.05), compared to the control treatment. The highest immune response parameter values were found in the P10 treatment. On *S. agalactiae* post-challenge test, the TE, Hb, and Ht were occurred on the 34th, 37th, and 41st days, whereas decreased parameter values were occurred on the 34th day, then continued to increase on the 37th and 41st day. Based on all parameter values, *Bacillus* sp. para-biotic treatments were higher and significantly different (P<0.05) from positive and negative control tretments (Figure 1A, B, and C).

Table 1. Growth performance of Nile tilapia (*O. niloticus*) fed with different concentrations of *Bacillus* sp. NP5 para-probiotic for 30 days.

	Treatment			
Parameter	K	P8	P9	P10
Initial weight (g)	10.43 ± 0.27^{a}	10.27 ± 0.31^{a}	10.15 ± 0.21^{a}	10.30 ± 0.07^{a}
Final weight (g)	15.17 ± 0.46^{a}	15.99 ± 0.10^{ab}	16.68 ± 0.95^{b}	16.41 ± 0.56^{b}
SR (%)	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}	100.00 ± 0.00^{a}
SGR (% hari^{-1})	1.26 ± 0.02^{a}	1.48 ± 0.08^{b}	1.67 ± 0.13^{b}	1.56 ± 0.14^{b}
FCR	1.60 ± 0.11^{b}	1.39 ± 0.12^{a}	1.33 ± 0.10^{a}	1.39 ± 0.16^{a}

Note: Different superscript letters on the same line show a significant different value (P<0.05). SR: survival rate, SGR: specific growth rate, and FCR: feed conversion ratio.

Table 2. Enzyme activity of Nile tilapia (*O.iloticus*) after feeding with different *Bacillus* sp. NP5 para-probiotic doses.

	Treatment			
Parameter	K	P8	P9	P10
Amylase (IU/mL)	2.49 ± 0.60^{a}	2.79 ± 0.01^{b}	2.84 ± 0.04^{bc}	2.94 ± 0.04^{c}
Protease (IU/mL)	0.11 ± 0.00^{a}	0.16 ± 0.02^{b}	0.16 ± 0.01^{b}	0.18 ± 0.00^{c}
Lipase (IU/mL)	0.11 ± 0.00^{a}	0.13 ± 0.00^{b}	0.13 ± 0.00^{b}	0.13 ± 0.00^{b}

Note: Different superscript letters on the same line show a significant different value (P<0.05).

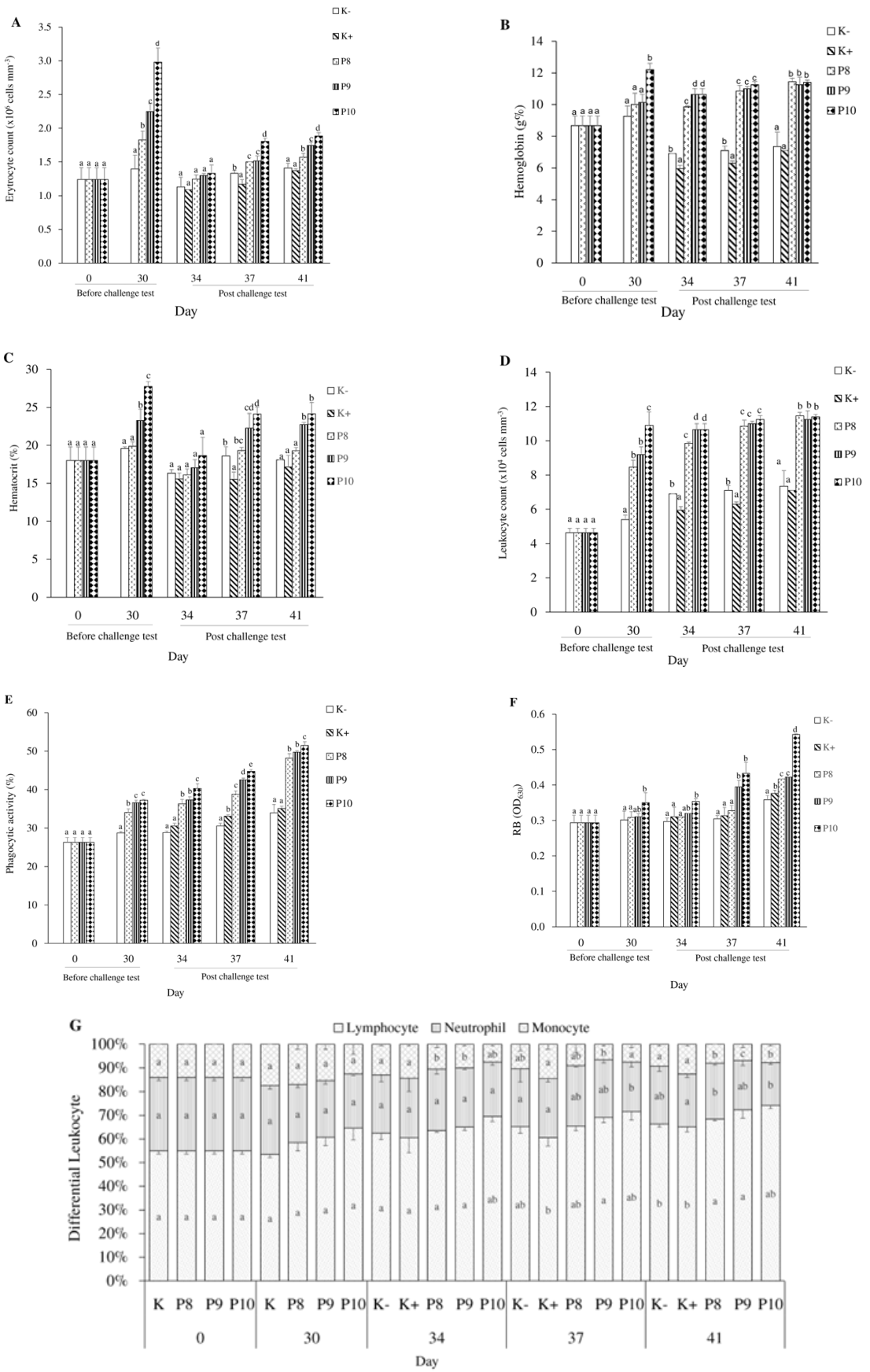

Figure 1. Total erythrocytes (TE) (A), hemoglobin (Hb) (B), hematocrit (Ht) (C), total leukocytes (TL) (D), phagocytic activity (AF) (E), respiratory burst (RB) (F), and leukocyte differential (G) of Nile tilapia before and after *S. agalactiae* challenge test. Different superscript letters on each bar (average value±standard deviation) show a significant different value (Duncan's multiple range test; P<0.05).

Immune response parameters, namely TL, AF, RB, and DL increased between 34th – 41st days with a significant different value found among *Bacillus* sp. NP5 para-biotic, positive control, and negative control treatments (Figure 1D, E, F, and G).

Survival rate

Survival rate of Nile tilapia after the challenge test was observed for 10 days and presented in Figure 2. The survival rate of P8 (83.33 ± 4.71%), P9 (83.33 ± 4.31%), and P10 (96.67 ± 4.71%) treatments were significantly higher (P<0.05) than the K (+) treatment (53.33 ± 0.00%).

Total *S. agalactiae* in target organs

The observation results of total *S. agalactiae* in target organs (eye, brain, kidney, and liver) before and after the challenge test are presented in Figure 3 (A, B, C, and D). The highest total *S. agalactiae* in target organs was found on the 34th day, then decreased on the 37th and 41st

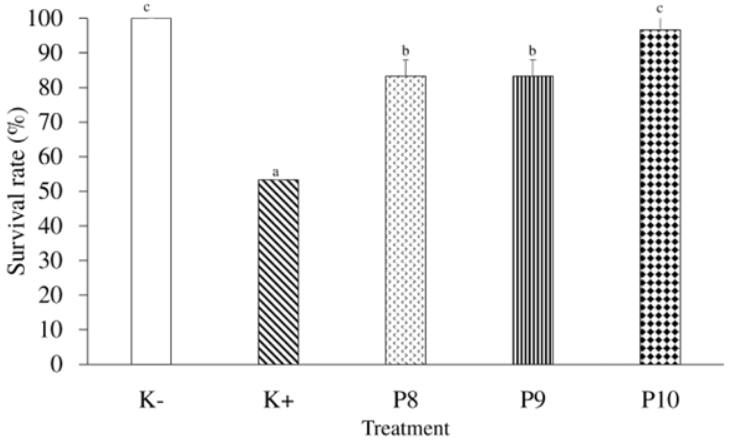

Figure 2. Survival rate of Nile tilapia after *S. agalactiae* infection. Different superscript letters on each bar (average± standard deviation) show a significant different value (Duncan's multiple range test; P<0.05).

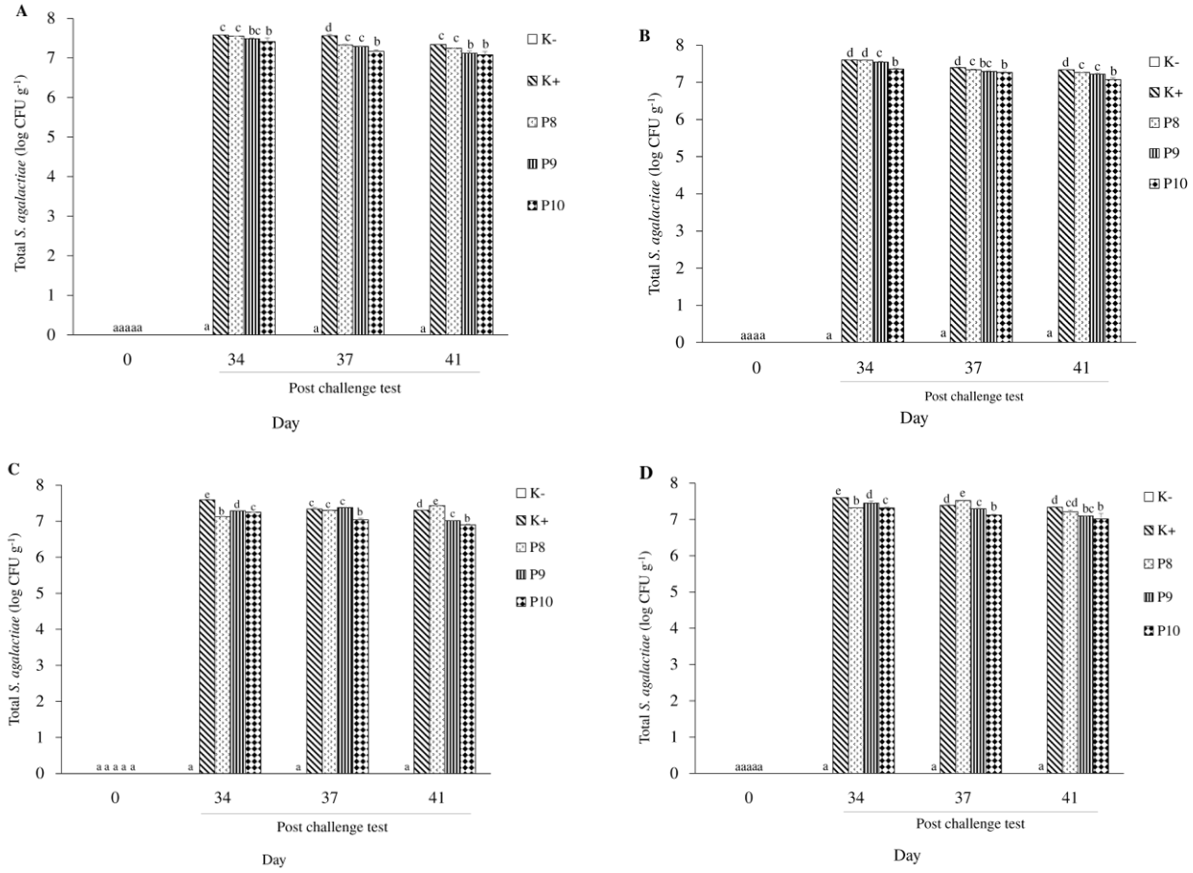

Figure 3. Total *S. agalactiae* in target organs, namely (A) eye; (B) brain; (C) kidney; and (D) liver, before and after the challenge test. Different letters on each bar (average±standard deviation) show a significant different value (Duncan's multiple range test; P<0.05).

days. Total *S. agalactiae* in the P8, P9, and P10 treatments was significantly lower (P<0.05) than the KP treatment. The P10 treatment showed the lowest total *S. agalactiae*, compared to the P8, P9, and KP in all target organs.

DISCUSSIONS

Specific probiotic uses that are killed or inactivated on several studies have been identified as a promotor or immunostimulant to induce feed utilization, thus producing a better growth performance and a greater non-specific immune response activity to resist disease attacks. The survival rate of Nile tilapia after 30 days of rearing fed with *Bacillus* sp. NP5 para-biotic diets was calculated at 100.00 ± 0.00 %. This means that para-probiotic supplementation has no negative response in Nile tilapia. According to Cuevas-González *et al*. (2020), para-probiotics as an immunostimulant could sustain the fish body against stress condition and pathogenic bacterial attacks. Dawood *et al.* (2019) dan Nguyen *et al.* (2019) reported the increased survival rate of Nile tilapia after applying the inactivated *L. plantarum*.

In this study, Nile tilapia seeds were reared for 30 days fed with para-probiotic supplemented diets could increase the specific growth rate and decrease the feed conversion ratio. In the para-probiotic supplemented diet treatment, the specific growth rate value was higher than the control treatment (P<0.05). Also, feed conversion ratio in para-probiotic supplemented diet treatment was lower than control diet treatment. SGR describes the good growth process due to good protein utilization in the diet as the main component for growth (Zaineldin *et al*., 2018), supported by the low FCR value, which demonstrates effective feed utilization (Wang *et al.*, 2020). This condition indicates a positive effect of *Bacillus* sp. NP5 para-probiotic dietary supplementation to improve the growth of Nile tilapia. Growth performance in fish will increase and affect the intestinal microflora, digestive enzymes, and appetite (Guo *et al*., 2017). According to Yang *et al*. (2014), inactivated-*B. pumilus* SE5 probiotics successfully form good intestinal microbiota of grouper (*E. coioides*) by activating the intestinal mucus. Specific component in bacteria, such as capsular polysaccharides, peptidoglycans, and lipoteichoic acids are stimulators for epithelial cells, dendritic cells, and other immune cells in the intestine (Piqué *et al.,* 2019). Mucus layer in digestive tract contains various protective and antimicrobial substances secreted by epithelial cells (Lazado & Caipang, 2014). Increased beneficial microflora in the intestine will have an impact on extracellular enzyme increase (Liu *et al*., 2017). Rodriguez Estrada *et al*. (2013) stated that the inactivated-*Enterococcus faecalis* dietary supplementation could improve the growth performance of rainbow trout (*Onchorhyncus mykiss*). Similarly, Yan *et al*. (2016) reported that the inactivated-*B. pumilus* supplementation provided a significant increase in the final weight and specific growth of juvenile grouper (*E. coioides*).

Digestive enzyme activity can become an indicator to support the growth performance improvement. Also, this activity is a comparative indicator in feed utilization, digestive capacity, and growth performance (Wang *et al*., 2019). Amylase, protease, and lipase enzymes in the P8, P9, and P10 treatments showed a significantly higher value (P<0.05) than the control treatment. This condition indicates that the dietary supplementation of *Bacillus* sp. NP5 para-probiotics can promote the digestive enzyme activity. Inactivated-beneficial bacteria supplementation can improve the nutrient utilization by the host due to digestive enzyme stimulation (Rodriguez-Estrada *et al*. 2013). Increased digestive enzyme activities are originated from both endogenous or exogenous enzymes in fish (Wu *et al*., 2012). These results were similar to Giri *et al*. (2020), who reported that the inactivated-*Pseudomonas aeruginosa* could induce the enzymatic activity in common carp. When enzymatic activity increased, after the fish were fed with heat-killed *L. plantarum* supplemented diets, feed utilization and growth performance in red sea bream could also be high (Dawood *et al*., 2015).

Dietary supplementation of para-probiotics in this study could induce the non-specific immune response of Nile tilapia by increasing total erythrocytes, hemoglobin, hematocrit, total leukocytes, phagocytic activity, respiratory burst, and leukocyte differential higher (P<0.05) than the control. Blood profiles can become a physiological biomarker to identify the fish health improvement after feeding with the supplemented diets (Dossou *et al*., 2019). In this study, total erythrocytes, hemoglobin, and hematocrit obtained increased values after fish were fed with the para-probiotic supplemented diets. The highest value of total erythrocytes, hemoglobin, and hematocrit of Nile tilapia in para-probiotic supplemented diet treatments were found in the

P10 treatment at 2.98 ± 0.21 x 10^6 sel mm, 9.27 ± 0.64 g%, dan 27.74 ± 0.64 %, respectively. Dawood *et al*. (2019) similarly mentioned that the hemoglobin level, hematocrit level, and total erythrocytes of Nile tilapia increased after feeding the fish with heat-killed *L. plantarum* (HK L-137) supplemented diets. However, total erythrocytes, hemoglobin, and hematocrit after challenge test obtained the lowest values on the 34th day, which then returned to increase on the 37th and 41st days. Decreased hematocrit and hemoglobin percentages are anemia indicators (Alsaid *et al*., 2015). This condition is suggested due to erythropoiesis inhibition after *S. agalactiae* infection, resulting in the lack of total erythrocytes (Sirimanapong *et al*., 2018). *S. agalactiae* from Nile tilapia has hemolytic characteristics (Lusiastuti *et al.*, 2014).

Leukocytes have roles in fighting the foreign particles that enter the body by showing a phagocytic activity. Leukocytes are important cells from the immune system that protect the body from foreign antigens that cause diseases (Kader *et al.,* 2018). Total leukocytes after fish were fed with *Bacillus* sp. NP5 para-probiotic supplemented diets for 30 days was found to increase compared to control diet treatment. The interaction between probiotic components and digestive epithelial cells is occurred through a receptor such as Toll-Like Receptor (TLR), which is an important fish immunology tissue that contains leukocyte population, namely phagocytes, lymphocytes, and cytotoxic cells (Zendeboodi *et al.,* 2020). Immune system activation process emerges as the inactive microbial components (peptidoglycan, polysaccharides, capsules, and lipoteichoic acid) were collected by the intestine enterocytes and brought by bloodstream to systemic lymphoid tissue, such as kidney and spleen (Kelly & Salinas, 2017). Total leukocytes increased significantly on post-infecttion. This condition indicates that there is an immune response to foreign materials (Biller & Takashi, 2018). The 40-50% increase in total leukocytes demonstrates the alerting level from pathogen attacks (Sirimanapong *et al*., 2018).

Phagocytic activity in para-probiotic diet treatments in this study increased significantly, compared to control diet treatment. This condition was similar to Nguyen *et al*. (2019), that Nile tilapia fed with inactivated-*L. plantarum* supplemented diets obtained an increased phagocytic activity. Increased phagocytic activity also occurred in sea cucumber (*Apostichopus japonicus*) after feeding with heat-killed *L. plantarum* L-137 supplemented diets (HK L-137) (Yang *et al*., 2016). Increased AF on post-challenge test was occurred due to the fighting process against pathogenic bacteria that has entered to the host body. Phagocytic activity contains neutrophil and macrophage activities that digest and kill bacteria, which are responsible for initial activation of inflammation response (Akhter *et al*., 2015).

The study results also showed an increased respiratory burst activity in para-probiotic treatments that were significantly higher than control treatments, mainly in the P10 treatment. These results were also similar to Kamilya *et al*. (2015), that the inactivated-*Bacillus subtilis* FPTB13 supplementation could induce the respiratory burst activity in Rohu carp. Dash *et al*. (2015) also reported that respiratory burst activity of giant freshwater prawn (*Macrobrachium rosenbergii*) increased significantly, after inactivated-*L. plantarum* supplementation. Leukocyte differential presents the leukocyte cell types in fish, namely lymphocytes, neutrophils, and monocytes. After the challenge test on the 34th day, lymphocytes increased, but neutrophils and monocytes decreased. Increased lymphocytes on post-challenge test were thought due to the effect of *S. agalactiae* injection and proliferation of T- and B-cells. Most antigen receptors are expressed on T- and B-lymphocytes (Kelly & Salinas, 2017).

The survival rate of Nile tilapia after being fed with different para-probiotic supplemented diets and challenged with *S. agalactiae* obtained a significant different value ($P<0.05$) to the positive control treatment. A significantly higher survival rate indicates that the para-probiotic concentration at 10^8, 10^9, and 10^{10} CFU/ml induces the immunity level to fight against pathogenic infections. A similar condition was reported by Nguyen *et al.* (2019), whereas Nile tilapia fed with inactivated-*L. plantarum* showed a highly resistance condition against the *S. agalactiae* infection. Also, heat-killed *P. aeruginosa* could increase the survival rate of common carp on *Aeromonas hydrophila* post-infection (Giri *et al*., 2020).

The *Bacillus* sp. NP5 para-probiotic bacteria capabilities in inducing the immune response can deteriorate the population of *S. agalactiae* bacteria in target organs, namely eye, brain, and liver. The highest total *S. agalactiae* bacteria was occurred on the 34th day, but then decreasing on the 37th and 41st days. On the 41st day, the lowest population in the target organ was found in the

P10 treatment and significantly different (P<0.05) from the positive control treatment. According to Agung *et al.* (2015), the dietary supplementation of *Bacillus* sp. NP5 bacteria in Nile tilapia could reduce total *S. agalactiae* in all target organs (eye, brain, kidney, and liver).

The existence of *S. agalactiae* in eyes causes several alterations, such as purulent, opacity, exophthalmia, and tightening (Phuoc *et al*., 2021). In brain, *S. agalactiae* causes abnormal swimming, i.e. gasping and whirling swimming (Assis *et al*., 2017). *S. agalactiae* that present on kidney will affect the hematological condition and immune system, which soon will impact on the fish capability in confronting the pathogen attack, which finally causes a mass mortality (Sirimanapong *et al*., 2018). Streptococcosis clinical symptoms in Nile tilapia were found during the challenge test period, namely slow swimming activity and spending more time in the base of the aquarium, irregular swimming, low appetite, exophthalmic and purulent eyes. These symptoms followed Verner-Jeffreys *et al*. (2018), that fish infected *S. agalactiae* bacteria will have several chronical signs, namely hemorrhage, exophthalmia, slow-swimming, thinning-caudal fin, and low appetite.

CONCLUSIONS

This study concludes that the dietary supplementation of *Bacillus* sp. NP5 para-probiotic significantly increases the growth performance and immune response in Nile tilapia. Increased immune response of Nile tilapia due to para-probiotic dietary supplementation is also followed by the increased fish resistance against *S. agalactiae* infection. Based on the *Bacillus* sp. NP5 para-probiotic supplementation results, the 10^{10} CFU/ml dose was observed to have the best results.

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