Growth performance, immune response, and resistance of Nile tilapia fed paraprobiotic *Bacillus* sp. NP5 against *Streptococcus agalactiae* infection

Kinerja pertumbuhan, respons imun, dan resistansi ikan nila yang diberi paraprobiotik *Bacillus* sp. NP5 terhadap infeksi *Streptococcus agalactiae*

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**ABSTRACT**

This study was aimed to evaluate the effectiveness of *Bacillus* sp. NP5 paraprobiotic administration through commercial feed on growth performance, immune response, and resistance of Nile tilapia against *Streptococcus agalactiae* infection. *Bacillus* sp. NP5 paraprobiotic was produced through heat-inactivation at 95°C for 1 h, then performed a viability test on tryptic soy agar (TSA) media and incubated for 24 hours. Paraprobiotics could be used whether the bacteria did not grow on the TSA media. This study used a completely randomized design, containing three treatments with five replications, i.e. 1% (v/w) probiotic addition, 1% (v/w) paraprobiotic addition, and no addition of probiotic or paraprobiotic (control). The experimental fish were reared for 30 days. On day 31 of rearing, fish were challenged with *S. agalactiae* (10⁷ CFU/mL) through intraperitoneal injection route, while the negative control was injected with PBS. This study results significantly improved growth performances and immune responses (P<0.05), compared to control after 30 days of probiotic and paraprobiotic *Bacillus* sp. NP5 administration. After challenge test, increased immune responses in probiotic and paraprobiotic of *Bacillus* sp. NP5 treatment had higher survival rates (P<0.05) than positive control. The administration of *Bacillus* sp. NP5 probiotic and paraprobiotic through commercial feed were effective in increasing growth performance, immune response, and resistance of Nile tilapia against *S. agalactiae* infection.

**Keywords:** *Bacillus* sp. NP5, Nile tilapia, paraprobiotic, *Streptococcus agalactiae*

**ABSTRAK**

Penelitian ini bertujuan mengevaluasi efektivitas pemberian paraprobiotik *Bacillus* sp. NP5 melalui pakan dalam meningkatkan kinerja pertumbuhan, respons imun, dan resistansi ikan nila terhadap infeksi *Streptococcus agalactiae*. Proses pembuatan bakteri paraprobiotik yaitu *Bacillus* sp. NP5 diinaktivasi panas pada suhu 95°C selama 1 jam, dilanjutkan dengan pengujian viabilitas dengan menyebarkannya pada media tryptic soy agar kemudian diinkubasi selama 24 jam. Jika bakteri tidak tumbuh, maka paraprobiotik dapat digunakan. Penelitian ini menggunakan rancangan acak lengkap dengan tiga perlakuan dan lima ulangan, yaitu penambahan probiotik 1% (v/w), penambahan paraprobiotik 1% (v/w), dan tanpa penambahan probiotik atau paraprobiotik (kontrol). Ikan perlakuan dipelihara selama 30 hari dan pada hari ke-31, ikan diuji tantang dengan *S. agalactiae* (10⁷ CFU/mL) melalui injeksi intraperitoneal, sementara perlakuan kontrol negatif diinjeksi dengan PBS. Hasil penelitian setelah 30 hari pemberian probiotik dan paraprobiotik *Bacillus* sp. NP5 menunjukkan kinerja pertumbuhan dan respons imun yang meningkat signifikan (P<0.05) dibandingkan dengan kontrol. Pascauji tantang, peningkatan respons imun pada perlakuan probiotik dan paraprobiotik *Bacillus* sp. NP5 menunjukkan tingkat kelangsungan hidup yang lebih tinggi (P<0.05) dibandingkan kontrol positif. Pemberian probiotik dan paraprobiotik *Bacillus* sp. NP5 melalui pakan dapat meningkatkan kinerja pertumbuhan, respons imun, dan resistansi ikan nila terhadap infeksi *Streptococcus agalactiae*.

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

Nile tilapia Oreochromis niloticus is an important freshwater species in Indonesia. The production of this fish in 2012 was 695,063 tons and kept increasing until 1,169,144 tons in 2018 (KKP, 2019). The increased production is supported by intensification of production and culture area expansion (Ottinger et al., 2016). The intensification of production can cause higher risk of disease attack (Joffre et al., 2018). One common disease appeared in Nile tilapia culture is streptococcus that caused by Streptococcus agalactiae (Xu et al., 2019).

One attempt to reduce the risk of streptococcus disease attack is by using probiotic. Probiotics are live microorganisms that if given in sufficient amounts will provide health for the host, therefore it can increase fish production (Hill et al., 2014). Bacillus spp. is a common bacteria used as probiotic agent. Bacillus spp. can survive longer due to their tolerance against high temperature and damage tissues compared to other probiotics (Kuebutornte et al., 2019). This study used Bacillus sp. NP5 as probiotic that has proven for preventing disease in fish and shrimp. Bacillus sp. NP5 has been tested in increasing growth rate and tilapia health status against streptococcus disease (Widanarni & Tanbiyaskur, 2015).

The survival rate of probiotic microorganisms during feed processing and feed storage is still a challenge in probiotic utilization. Ho et al. (2017) stated that the number of probiotic cells in feed is decreased around 10% after three weeks of storage. This was caused by probiotic cells are live microorganism that easily broken down or die because of various factors of production process (de Araújo et al., 2020). Another possibility that could happen is horizontally displacement of virulence factor genes from pathogen to the probiotic microorganism in the culture environment (Newaj-Fyzul et al., 2014).

Paraprobiotic could increase the immune system in gut mucus (Singh et al., 2017). Moreover, the application of Lactobacillus plantarum as paraprobiotic that has been inactivated by heating process could increase the immune response of large freshwater prawn Macrobrachium rosenbergii (Dash et al., 2015). According to the previous study result, the benefit of the application of paraprobiotic through feed is expected to be an alternative to increase fish health status. Hence, this study aimed to evaluate the efficacy of Bacillus sp. NP5 as paraprobiotic through feed to increase immune response and tilapia resistance against S. agalactiae infection.

MATERIAL AND METHOD

Tested fish

Fish used in this study was male tilapia weighed around 22.9 ± 0.47 g that obtained from production pond in Aquaculture Department, Faculty of Fisheries and Marine Science (FPIK), IPB University. The bacteria used in this study was Bacillus sp. NP5 as the probiotic and S. agalactiae as the pathogen, both of these bacteria were collected from the Fish Health Laboratory, Aquaculture Department, Faculty of Fisheries and Marine Science (FPIK), IPB University.

The preparation of probiotic and paraprobiotic bacteria

The probiotic bacteria used in this study was Bacillus sp. NP5 with rifampicin antibiotic marker resistant (Bacillus sp. NP5 RfR). The biomass production of Bacillus sp. NP5 RfR was done by culture technique in TSB (tryptic soy broth) media. The bacteria were cultured in shaker (140 rpm for 24 hours in 29°C). Meanwhile, the preparation for Bacillus sp. NP5 RfR refer as paraprobiotic was
used cultured bacteria in TSB media by harvesting the bacteria using centrifuge at 10,000 rpm for 10 minutes in 4°C, then it was washed twice using sterile PBS (phosphate buffered saline) (pH 7.2), until the bacteria density reached $10^6$ CFU/mL. The cultured bacteria was treated with water with high temperature (95°C) for an hour (Yang et al., 2014). After being inactivated, the bacteria were checked by spreading the inoculant in TSA (tryptic soy agar) media and was checked after 24 hours incubation in 29°C.

**Experimental design and feed preparation**

This study used three treatments and five replications. Every replication used 10 fishes. The three treatments in this study were probiotic (1% of Bacillus sp. NP5 Rf as probiotic), paraprobiotic (1% of Bacillus sp. NP5 Rf as paraprobiotic), and PBS as control. Feed used in this study was commercial feed contained 30% of protein. The addition of probiotic, paraprobiotic, and PBS for control treatment in the feed were mixed with 2% of egg white as a binder (Djauhari et al., 2016) and then were sprayed evenly by using a syringe. This feed then were dried, packed in an air-tight plastic bag, labeled, and stored in fridge with a temperature of 4°C before being used.

**Fish rearing**

The addition of Bacillus sp. NP5 Rf refer as probiotic and paraprobiotic in feed was conducted for 30 days. In day-31, the challenge test was conducted by feeding the fish with only commercial feed. The aquarium used in this study was measured of 60×30×40 cm3 with 60 L of water. Uneaten feed and fish feces were collected every day by siphoning. As much as 70% of the water was changed three times a day. Fish were fed by satiation three times a day (at 8.00; 12.00; and 16.00). The measurement by in-situ of water quality included water temperature and pH was conducted every day, meanwhile, the dissolved oxygen (DO), total ammonia nitrogen (TAN), and ammonia (NH3) (APHA, 1998) was measured at day-0, day-15, and day-30.

**Challenge test**

At day-31, the fish were challenged by using S. agalactiae. The challenge test was consisted of four treatments and three replications. During the challenge test, 12 aquariums with 60 L volume were used. The tilapia were reared with density of eight fishes of each aquarium taken from each treatment, then the fishes were acclimatized for 2–3 hours. Each fish in each treatment of probiotic, paraprobiotic, and positive control was challenged with S. agalactiae bacteria through intraperitoneal injection (dose of 0.1 mL from a bacteria density of $10^6$ CFU/mL), meanwhile, for negative control, the fish was injected with 0.1 mL of PBS. The observation was conducted for 14 days. The mortality of the fish was counted as survival rate data at the end of the challenge test.

**Growth performance**

The measurement of growth parameters were conducted at the end of the study, at day-30. The measured parameters were the survival rate (SR) with the following formula $SR(\%) = 100 \times \frac{N_{t}}{N_{0}}$ (Dawood et al., 2015a); the specific growth rate (SGR) with the following formula $SGR(\%/day) = 100 \times (ln AWG_{t} - ln AWG_{0}) t^{-1}$ and feed conversion ratio (FCR) with the following formula $FCR = F(BWG_{t} - BWG_{0}) t^{-1}$ (Ho et al., 2017). N0 showed total amount of fish at the end of the study (g), N0 showed total amount of fish at the initial of the study (g), AWG showed average weight of fish at the end of the study (g), AWG showed average weight of fish at the initial of the study (g), t showed time of rearing (days), F showed total amount of feed (g), BWG showed fish biomass at the end of the study (g), and BWG showed fish biomass at the initial of the study (g).

**The activity of enzyme**

The digestive tract of tilapia was weighed around 0.5 g, then was added with Tris buffer (20 mM Tris HCl, 1 mM EDTA, dan 10 mM CaCl\_2 pH 7.5) with the ratio of 10% (b/v), afterward, it was put into microtube and was centrifuged for 10 minutes (12000 rpm, 4°C). The supernatant was put into microtube and was stored in -20°C until it is ready to use for the activity of enzyme test. The parameters of the activity of enzyme that measured were the activity of amylase (Worthington, 1993), the activity of protease (Bergmeyer et al., 1983), and the activity of lipase (Borlongan, 1990).

**The total bacteria count and Bacillus sp. NP5 Rf\_ as probiotic in intestine**

The total bacterial count and Bacillus sp. NP5 Rf\_ probiotic in the intestine was done by using spread plate method. As much as 1 g of fish intestine was homogenized in 9 mL of sterile PBS for serial dilution. Then, as much as 50 µL from each dilution was spread in petri dish contained a culture medium. The culture media that used for the total bacteria count was TSA, meanwhile...
the culture media for total bacteria count of *Bacillus* sp. NP5 Rf was TSA with 50 µg/mL of rifampicin. The total bacteria count and total *Bacillus* sp. NP5 Rf in the intestine was done at the initial and at the end of the treatment, those were day-0 and day-30.

**Collecting sample of blood and serum**

Three fish were taken randomly from each treatment. An anesthetic was being performed at the time of collecting samples of blood by using 100 µL of clove oil. The fish blood was taken directly from linea literalis by using 1 mL of sterile syringe that has been rinsed by anticoagulant (3.8% of Na-citrate). The blood then was put into a microtube. The collecting sample of serum was done by using Singh *et al.* (2017) method. The blood was taken into 1 mL of sterile syringe without being rinsed with anticoagulant. This blood then was stored in 4°C for 12 hours, afterward, the blood was centrifuged in 5000 ×g for five minutes and the serum was put into microtube and was stored in -20°C.

**The observation of immune response parameter**

The observation of immune response parameter was done at day-0, day-30, and day-34 (three days after challenge test), day-37 (six days after challenge test), and day-41 (10 days after challenge test). The observed immune response parameters were total erythrocyte count (Blaxhall & Daisley, 1973), hematocrit (Blaxhall & Daisley, 1973), hemoglobin (Walter, 1988), total leukocyte count (Blaxhall & Daisley, 1973), phagocytic activity (Anderson & Siwicki, 1993), respiratory burst (Anderson & Siwicki, 1993), lysozyme activity (Hanif *et al.*, 2004), and total protein serum (Bradford, 1976).

**Total bacteria count of Streptococcus agalactiae in target organ**

The total bacteria count of *S. agalactiae* in the target organ was done by using a spread plate method. As much as 0.1 g of the target organ (brain, eyes, kidney, and liver) was homogenized in 0.9 mL of sterile PBS for serial dilution. Then, as much as 50 µL from each dilution was spread in a plate with BHIA (brain heart infusion agar) media. The total bacteria count of *S. agalactiae* in the target organ was done at day-34 (three days after challenge test), day-37 (six days after challenge test), and day-41 (10 days after challenge test).

**Data analysis**

The collected data was processed by using Microsoft Excel 2016. The data analysis of growth rate and immune response was analyzed by using analysis of variance (ANOVA) of SPSS ver.18, if it found significantly different, then the data was analyzed by using the Duncan test. The

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**Table 1. Growth performance of red Nile tilapia fed with the probiotic and *Bacillus* sp. NP5 paraprobiotic treatments.**

| Parameters   | Control          | Probiotic        | Paraprobiotic    |
|--------------|------------------|------------------|------------------|
| Initial weight (g) | 22.6 ± 0.36<sup>a</sup> | 22.8 ± 0.81<sup>a</sup> | 23.2 ± 0.24<sup>a</sup> |
| Final weight (g)   | 45.2 ± 1.73<sup>a</sup> | 51.0 ± 2.09<sup>b</sup> | 50.7 ± 0.91<sup>b</sup> |
| SGR (%/day)       | 1.98 ± 0.07<sup>a</sup> | 2.37 ± 0.07<sup>b</sup> | 2.24 ± 0.08<sup>b</sup> |
| FCR              | 1.52 ± 0.05<sup>b</sup> | 1.27 ± 0.06<sup>c</sup> | 1.36 ± 0.04<sup>c</sup> |
| SR (%)           | 100 ± 0<sup>b</sup> | 100 ± 0<sup>b</sup> | 100 ± 0<sup>b</sup> |

Numbers in the same column followed by the same superscript letters had insignificant difference at 5% degree levels (Duncan’s multiple range test). SGR: Specific growth rate, FCR: Feed conversion ratio, SR: Survival rate.

**Table 2. Digestive enzyme activity of red Nile tilapia fed with probiotic and *Bacillus* sp. NP5 paraprobiotic treatments.**

| Tested parameter | Control          | Probiotic        | Paraprobiotic    |
|------------------|------------------|------------------|------------------|
| Amylase (IU/mL)  | 2.13 ± 0.003<sup>a</sup> | 2.35 ± 0.006<sup>a</sup> | 2.37 ± 0.023<sup>a</sup> |
| Protease (IU/mL) | 0.09 ± 0.0004<sup>a</sup> | 0.10 ± 0.0002<sup>b</sup> | 0.15 ± 0.0007<sup>b</sup> |
| Lipase (IU/mL)   | 0.07 ± 0.001<sup>a</sup> | 0.13 ± 0.001<sup>b</sup> | 0.14 ± 0.004<sup>b</sup> |

Numbers in the same column followed by the same superscript letters had insignificant difference at 5% degree levels (Duncan’s multiple range test).
Figure 1. Total erythrocyte (TE) (A), hematocrit (Ht) (B), hemoglobin (Hb) (C), respiratory burst (RB) (D), total leukocyte (TL) (E), phagocytic activity (AF) (F), lysozyme activity (AL) (G), and total serum protein (TSP) (H). Different letters in each bar (average±standard deviation) show a statistical difference (Duncan’s multiple range test; P<0.05). Negatif control (K-); positive control (K+); 1% probiotic 1% (Pro) and 1% paraprobiotic (Para).
normality data was assessed by Shapiro-Wilk test and the homogeneity of variance was verified by Levene test.

RESULTS AND DISCUSSION

Results

Growth performance

The survival rate of the experimental fish after applying the probiotic and *Bacillus* sp. NP5 paraprobiotic treatments in this study showed an insignificant different (P>0.05). The SGR and FCR of probiotic and paraprobiotic treatments were also insignificantly different, but significantly different from the control treatment (P>0.05). The highest SGR value was at 2.28 ± 0.19 %/day and the lowest FCR value was at 1.27 ± 0.06, which were obtained from the probiotic treatment. The growth performance value of the Nile tilapia during maintenance period is presented in Table 1.

The probiotic and *Bacillus* sp. NP5 paraprobiotic treatments in this study were identified to improve the digestive enzyme activity compared to the control treatment. After 30 days of maintenance, the amylase enzyme activity in probiotic and paraprobiotic treatments were significantly different from the control treatment (P<0.05), while the protease and lipase enzymes were significantly different (P<0.05) among treatments. The enzyme activity measurement results are presented in Table 2.

Immune response

The fish health status and immune response can be evaluated through the blood profiles. The immune response parameter measurement results after 30 days of maintenance showed that the probiotic and *Bacillus* sp. NP5 paraprobiotic treatments increased significantly (P<0.05) compared to the control treatment. After the *S. agalactiae* challenge test, the immune response measurement results occurred a fluctuating condition in the 34th day (3 days of post-infection), 37th day (6 days of post-infection), and 41st day (10 days of post-infection). The lowest TE, Ht, and Hb values were obtained on the 34th day, and increased in the 37th and 41st days. The TE values in the probiotic and *Bacillus* sp. NP5 paraprobiotic treatments were significantly different (P<0.05) from the positive and negative control treatments (Figure 1A). For the Ht parameter values in the 34th and 41st days, the probiotic and *Bacillus* sp. NP5 paraprobiotic treatments were significantly different (P<0.05) from the positive and negative control treatments. Meanwhile, in the 37th day, the negative control, probiotic, and *Bacillus* NP5 paraprobiotic treatments were significantly different (P<0.05) from the positive control (Figure 1B). For the Hb parameter at the 34th day, the highest value was obtained from the paraprobiotic treatment and the lowest value was obtained from the positive control treatment. In the 37th day, the Hb values in the negative control, probiotic, and *Bacillus* sp. NP5 paraprobiotic treatments were insignificantly different (P>0.05), but showing a significant difference to the positive control treatment, while in the 41st day, the probiotic and *Bacillus* sp. NP5 paraprobiotic treatments were significantly different (P<0.05) from the positive and negative control treatments (Figure 1C). The immune response parameter values of TL, RB,
and AL increased with the highest value from the probiotic and *Bacillus* sp. NP5 paraprobiotic treatments in the 34th day, which were significantly different from the positive and negative control treatments (Figure 1D, 1E, and 1G). In the 37th day, the highest AF immune responses were in the probiotic and *Bacillus* sp. NP5 paraprobiotic treatments, and significantly different from the positive and negative control treatments (Figure 1F). The *Bacillus* sp. NP5 paraprobiotic treatment obtained the highest TSP value on the 37th day, while the paraprobiotic, positive control, and negative control treatments obtained the highest TPS value on the 41st day (Figure 1H).

**Survival rate in the post-challenge test**

The survival rate data in the post-challenge period were calculated on 14 days after injecting the red Nile tilapia with *S. agalactiae* bacteria at 10⁷ CFU/mL density. The SR data showed that Nile tilapia treated with probiotic, *Bacillus* sp. NP5 paraprobiotic, and negative control obtained the percentage values of 87.5 ± 0.00%, 87.5 ± 12.50%, 100 ± 0.00%, respectively, and were significantly different (P<0.05) from the positive control treatment at 54.2 ± 7.22% (Figure 2).

**Total bacteria and *Bacillus* sp. NP5 RfR probiotics in the intestine and total *S. agalactiae* bacteria in the target organ of red Nile tilapia**

The calculation results of the total intestinal bacteria after 30 days of maintenance, increased without a significant difference (P>0.05) among treatments. The total *Bacillus* sp. NP5 RfR probiotics in the intestine were only found in the probiotic treatment. The calculation results of total bacteria and *Bacillus* sp. NP5 RfR probiotics in the intestine are presented in Table 3.

The total *S. agalactiae* in the target organ in probiotic, paraprobiotic, and positive control treatments were fluctuating. In the 34th day (3 days of post-injection), the highest total *S. agalactiae* in the brain and eyes was occurred in the probiotic treatment, in the kidney was occurred in the probiotic and positive control treatments, while in the liver was occurring in all treatments, except the negative control treatment. The total *S. agalactiae* in the 37th day (6 days of post-injection) in the brain was significantly different (P<0.05) among treatments. The highest value was in the paraprobiotic treatment; the highest value in all treatments was obtained from eyes and kidney organs, except the negative control treatment, while the highest value in the liver organ was obtained from the positive control and paraprobiotic treatments. In the 41st day (10 days of post-infection), the highest total *S. agalactiae* in the brain was occur in the positive control treatment, in the eyes was occur in the probiotic and positive control treatments, while in the kidney and liver were occurred in the paraprobiotic treatment. The calculation results of total *S. agalactiae* in the target organs can be seen in Figure 3.

**Water quality**

The water quality measurement results during maintenance obtained the temperature of 25–28°C, pH of 6.8–7.2, dissolved oxygen (DO) of 4.9–7.7 mg/L, total ammonia nitrogen (TAN) of 0.13–0.49 mg/L, and ammonia (NH₃) of 0.0017–0.0063 mg/L.

**Discussion**

This study results showed that the supplementation of paraprobiotic obtained the similar results to the supplementation of probiotic in improving the growth performance of the red Nile tilapia. This condition was in line with Nguyen *et al.* (2019), that the application of heat-killed *L. plantarum* strain L-137 (HK L-137) could improve growth and diet nutrient in Nile tilapia. Dawood *et al.* (2015a) also reported that the application of heat-killed *L. plantarum* (LP20) could improve the growth performance of red sea bream juveniles. Furthermore, Dawood

### Table 3. Total intestinal bacteria and *Bacillus* sp. NP5 RfR probiotics in the intestine of red Nile tilapia

| Parameter                        | Day | Control     | Probiotic   | Paraprobiotic |
|----------------------------------|-----|-------------|-------------|--------------|
| Total intestinal bacteria (log CFU/g) | 0   |  7.69 ± 0.00<sup>a</sup> |  7.69 ± 0.00<sup>a</sup> |  7.69 ± 0.00<sup>a</sup> |
|                                  | 30  |  8.17 ± 0.56<sup>a</sup> |  9.12 ± 0.42<sup>b</sup> |  8.70 ± 0.49<sup>bc</sup> |
| Total probiotic(log CFU/g)       | 0   |  0 ± 0.00   |  0 ± 0.00   |  0 ± 0.00    |
|                                  | 30  |  0 ± 0.00   |  7.83 ± 0.09<sup>a</sup> |  0 ± 0.00    |

<sup>a</sup>Numbers in the same column followed by the same superscript letters had insignificant difference at 5% degree levels (Duncan’s multiple range test).
et al. (2016) presented that the dietary inactive *Pediococcus pentosaceus* supplementation could improve the growth and feed efficiency of red sea bream (*Pagrus major*) fish. The paraprobiotic application in this study could improve the specific growth rate and digestive enzyme activity of Nile tilapia compared to the control treatment. This condition followed the study of Dawood et al. (2019), that the dietary heat-killed *Lactobacillus plantarum* (HK L-137) supplementation could improve the growth rate and digestive enzyme activity of Nile tilapia. The improved digestive enzyme activity in the probiotic treatment occurred due to the exogenous enzyme produced from the probiotic bacteria. The *Bacillus* NP5 bacteria are amylolytic bacteria that excrete amylase enzyme (Putra & Widanarni, 2015).

The improved digestive enzyme activity and growth performance were also thought due to the microbiota composition occurred in the digestive tract, specifically related to the beneficial bacteria. The dietary *Bacillus* sp. NP5 probiotics supplementation can compete with the unbeneficial bacteria in the intestine, which results in the beneficial bacterial domination. According to Pandiyan et al. (2013), probiotics can compete with the pathogenic bacteria in the intestine. Paraprobiotics as inactive cells are incapable of competing with the intestinal microbiota, therefore a possible condition occurred in the paraprobiotic is to activate the immune response in the intestine, called GALT (gut associated lymphoid tissue) for suppressing the total pathogenic bacteria. The bacterial specific components, namely, capsular polysaccharides, peptidoglycans, and lipoteichoic acid, are stimulators for epithelial cells, dendritic cells, and immune cells in the intestine (Piqué et al., 2019).

The digestive mucosa layer contains protective and antimicrobial properties secreted by the epithelial cells (Lazado & Caipang, 2014). This condition was proven by Yang et al. (2014), who presented that the inactive *Bacillus pumilus* SE5 could decrease the bacterial diversity, specifically the unbeneficial bacteria by activating the intestinal mucosa. The mucosal immune system activation occurs due to the increased Toll-like receptor (TLR) expression induced by the microbe associated molecular patterns (MAMPs), namely, lipopolysaccharides.

![Graphs showing bacterial counts](image)

Figure 3. Total *S. agalactiae* bacteria in the brain (A), eyes (B), kidney (C), and liver (D) of red Nile tilapia on the post-challenge test. Different letters in each bar (average±standard deviation) show a statistical difference (Duncan’s multiple range test; P<0.05). Negatif control (K-); positive control (K+); 1% probiotic 1% (Pro) and 1% paraprobiotic (Para).
(LPS), peptidoglycan, flagellin, and microbial nucleic acids (Sánchez et al., 2013). Mohapatra et al. (2012) T2 (BF + Bacillus subtilis and Lactococcus lactis) also reported that the combined heat-inactivated probiotic application (Bacillus subtilis, Lactococcus lactis, and Saccharomyces cerevisiae) could significantly decrease the total unfavourable heterotrophic bacterial population in the Labeo rohita intestine. The digestive enzyme activity improvement in the paraprobiotic treatment was thought due to the role of intestinal beneficial microflora, following Dawood et al. (2016), that the commensal intestinal microflora could secrete the exogenous enzymes, which improved the digestive enzyme activity of red sea bream (P. major) fish.

The probiotic utilization in aquaculture is not only proposed to improve growth, but also expecting to increase the immune response, therefore the cultured fish become resistant against disease. The dietary paraprobiotic supplementation in this study could increase the non-specific immune response of red Nile tilapia as same as the probiotic treatment. This condition was thought as the intestinal microbiota performed a continuous direct contact with the intestinal mucosa layer, therefore the GALT tissue activated the defense mechanism to differ the microorganisms that could be pathogen-potential and commensal-potential. GALT contains important regulatory cells from the mucosal immune system, i.e. lymphocytes, which are capable of identifying and quickly responding, and selective against the dangerous pathogens and foreign phagocytes (Gomez et al., 2013). The commensal bacteria provides an important stimulus in the GALT tissue, especially cytokines, that involve in inducing the immune response (Lazado & Caipang, 2014). Increased non-specific immune response in fish fed with paraprobiotic was also reported by Singh et al. (2017), that the dietary supplementation of B. amyloliquefaciens could induce the immune response of catla (Labeo rohita) fish. The dietary supplementation of Enterococcus faecalis paraprobiotics could also induce the non-specific immune response of rainbow trout fish (Rodriguez-Estrada et al., 2013).

In this study, the total erythrocytes, hemoglobin level, and hematocrit level increased after feeding the fish with probiotic and Bacillus sp. NP5 paraprobiotic treatments compared to the control treatment. The blood hematology can become a physiological biomarker to identify the fish health improvement after feeding with a supplement (Dossou et al., 2019). The study results of Dawood et al. (2019) showed that the dietary supplementation of heat-killed L. plantarum (HK L-137) could increase the hemoglobin level, hematocrit level, total erythrocytes of Nile tilapia. Increased beneficial bacterial cells in the intestine causes the microbial balance (Rodriguez-Estrada et al., 2013) on rainbow trout Oncorhynchus mykiss: control (C, which impacts on the digestive system that is extremely required for attacking the pathogenic bacteria (Li et al., 2020). This condition followed the study of Nguyen et al. (2019), who reported that the Nile tilapia fed with the dietary supplementation of heat-killed L. plantarum (HK L-137) increased the phagocytic and lysozyme activities. The lysozyme activity also increased significantly in the catla fish fed with B. amyloliquefaciens probiotics (Das et al., 2013) and B. amyloliquefaciens paraprobiotics (Singh et al., 2017).

Leukocytes play a role in the fish non-specific immune response against the pathogenic infection. Leukocytes act of attacking the foreign particles that enter into the body as shown from the phagocytic activity. The total leukocytes in this study increased after feeding with probiotic or parabiotic treatments, and increased significantly in the post-infection period of S. agalactiae. The 40–50% increase from the total leukocytes indicates the danger level from pathogenic attack (Sirimanapong et al., 2018). The phagocytic and lysozyme activities in this study increased significantly either in the probiotic or Bacillus sp. NP5 paraprobiotic treatments compared to the control treatment. The phagocytic activity is responsible for the initial activation of the inflammatory response. Meanwhile, lysozyme is a bactericidal enzyme in the innate immune system that is extremely required for attacking the pathogenic bacteria (Li et al., 2020). This condition followed the study of Nguyen et al. (2019), who reported that the Nile tilapia fed with the dietary supplementation of heat-killed L. plantarum (HK L-137) increased the phagocytic and lysozyme activities. The lysozyme activity also increased significantly in the catla fish fed with B. amyloliquefaciens probiotics (Das et al., 2013) and B. amyloliquefaciens paraprobiotics (Singh et al., 2017).

Respiratory burst is one of the innate immunological parameters important for evaluating the fish defense mechanism. This study also showed the increased superoxide (H2O2) and anion (OH) productions either in the probiotic
treatment or *Bacillus* sp. NP5 paraprobiotic treatment. This condition followed the study of Kamiliya *et al.* (2015), that heat-inactivated *Bacillus subtilis* FPTB13 could increase the respiratory burst of catla fish. In Dash *et al.* (2015) also known as ghost probiotics, are non-viable microbial cells which, when administered in adequate amounts, confer a benefit on the host. However, the advantage of non-viable microbe over their viable counterparts is a much debated topic in aquaculture. Therefore, the present study was conducted to evaluate paraprobiotic effect of heat-killed *Lactobacillus plantarum* on giant freshwater prawn *Macrobrachium rosenbergii*. A 90-day feeding trial was conducted by feeding prawn juveniles (0.54 ± 0.03 g), the inactive *L. plantarum* treatment could significantly increase the respiratory burst activity in *M. rosenbergii*.

The main protein components in serum are albumin and immunoglobulin. The probiotic and *Bacillus* sp. NP5 paraprobiotic treatments were significantly higher (P<0.05) than the control treatment in 30 days of maintenance. This condition was thought due to the humoral immune system contribution (Singh *et al*., 2017). Increased total serum protein occurred in the red sea bream fish fed with dietary supplementation of inactivated *P. pentosaceus* (Dawood *et al*., 2016). Increased total serum protein occurred in the post-infection period of *S. agalactiae*, as thought due to the increased immunoglobulin and albumin levels in post-pathogenic bacterial attack. Immunoglobulins are an adaptive immune response system that may increase as a response against the *S. agalactiae* attack (Sirimanapong *et al*., 2018). The intestine epithelial cells (IEC), dendritic cells, and macrophages in the lamina propria present antigens from the microorganisms to T cells and B cell to form an adaptive immune system (Yahfoufi *et al*., 2018).

The probiotic bacteria and *Bacillus* sp. NP5 paraprobiotic could increase the immune response, therefore increasing the red Nile tilapia survival rate on the post-infection period of *S. agalactiae*. Increased immune response could reduce the total *S. agalactiae* bacteria, which decreased their infection level and caused better survival rate of red Nile tilapia on the post-infection period. This condition followed the Kuebutornye *et al.* (2020) that the dietary supplementation of *Bacillus* spp. (*B. subtilis, B. velezensis,* and *B. amyloliquefaciens*) could increase the Nile tilapia immune response, which increased the survival rate in the post-infection period of *S. agalactiae*.

The clinical symptoms of streptococcosis in red Nile tilapia show a slow swimming movement in the aquarium base, abnormal swimming, slow feeding response, exophthalmia, purulens, and rapid opercula opening. The disease attack caused by *S. agalactiae* could provide chronic effects, exophthalmia (popped eye) (Nguyen *et al*., 2021) hemorrhage (Verner-Jeffreys *et al*., 2018), fin loss (de Sousa *et al*., 2020), slow swimming and low appetite (Soto *et al*., 2015).

The total *S. agalactiae* values in the target organs were fluctuating, as the probiotic and *Bacillus* sp. NP5 paraprobiotic treatments tended to be lower than the positive control treatment. The highest total *S. agalactiae* values in the target organs (brain, eyes, kidney, and liver) in the *Bacillus* sp. probiotic was obtained on the 34th day, and decreased in the 37th and 41st days. This condition followed the study results of Agung *et al.* (2015), that the dietary supplementation of *Bacillus* sp. NP5 probiotic microcapsule in the Nile tilapia decreased the total *S. agalactiae* bacteria in all target organs (brain, eyes, kidney, liver). In another study, the dietary supplementation of *Bacillus* sp. NP5 probiotics increased the immune response, therefore decreasing the total *S. agalactiae* bacteria and resulted in lower target organ damages (Widanarni & Tanbiyaskur, 2015). In the paraprobiotic and positive control treatments, the significant increase in the total *S. agalactiae* bacteria occurred in the 37th day in the seyes and brain organs. Based on Su *et al.* (2017) *Streptococcus agalactiae* (also known as GBS, the *S. agalactiae* infected Nile tilapia resulted in an increased total bacteria in the 3rd to 7th day, specifically in the eyes and brain target organs. Brain and eyes organs are the target of *S. agalactiae* bacterial attack (Lusiastuti *et al*., 2014).

**CONCLUSION**

The dietary supplementation of probiotic and *Bacillus* sp. paraprobiotic was effective to improve growth performance, immune response, and Nile tilapia resistance against the *Streptococcus agalactiae* infection.

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