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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 streptococcosis that caused by *Streptococcus agalactiae* (Xu *et al*., 2019).

One attempt to reduce the risk of streptococcosis 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 (Kuebutornye *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 streptococcosis 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). Probiotic cells that are dead or inactive is called paraprobiotic (Zendeboodi *et al*., 2020). This problem encourages for further study about the application of inactive probiotic bacteria.

The concept of paraprobiotic is the utilization of non-viable probiotic cells (inactive probiotic cells with whole cell structure or broken cell structure) that provide certain benefits for the host. Paraprobiotic comes from good microorganism that lost their viability after being exposed to some factors that can change the microbe cells structure, such as severed DNA filaments, a disorder in the cell membrane, or mechanical damage in cell's envelope (de Almada *et al*., 2016). The application of paraprobiotic in feed can provide certain benefit compared to other probiotic, as none interaction found within bacteria and feed product component that can affect the age of feed store. Paraprobiotic can be added before heating process, allowing in remain at doses required to generate some benefits for the host and ease in storage (de Almada *et al*., 2016).

Some previous study related to the application of paraprobiotics are the application of *Bacillus pumilus* SE5 that able to modulate gut microbe and able to increase immune system in gut mucus of grouper (Yang *et al.,* 2014), the application of *B. amyloliquefaciens* as paraprobiotic could increase the immune system in *Labeo catla* (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 Rf^R). The biomass production of *Bacillus* sp. NP5 Rf^R 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 Rf^R 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^{10} 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^R as probiotic), paraprobiotic (1% of *Bacillus* sp. NP5 Rf^R 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^R 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 cm^3 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 at 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 (NH_3) (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 10^7 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 (%)= $N_t / N_0 \times 100$ (Dawood *et al*., 2015a); the specific growth rate (SGR) with the following formula SGR (%/day) = 100 ($\ln AWG_t – \ln AWG_0$) t^{-1} and feed conversion ratio (FCR) with the following formula FCR = $F(BWG_t – BWG_0)^{-1}$ (Ho *et al*., 2017). N_t showed total amount of fish at the end of the study (g), N_0 showed total amount of fish at the initial of the study (g), AWG_t showed the average weight of fish at the end of the study (g), AWG_0 showed the 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_t showed fish biomass at the end of the study (g), and BWG_0 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^R probiotic in intestine

The total bacterial count and *Bacillus* sp. NP5 Rf^R 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^R was TSA with 50 μg/mL of rifampicin. The total bacteria count and total *Bacillus* sp. NP5 Rf^R 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/L 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

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

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

[a]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	Treatments		
	Control	Probiotic	Paraprobiotic
Amylase (IU/mL)	2.13 ± 0.003^a	2.35 ± 0.006^b	2.37 ± 0.023^b
Protease (IU/mL)	0.09 ± 0.0004^a	0.10 ± 0.0002^b	0.15 ± 0.0007^c
Lipase (IU/mL)	0.07 ± 0.001^a	0.13 ± 0.001^b	0.14 ± 0.004^c

[a]Numbers in the same column followed by the same superscript letters had insignificant difference at 5% degree levels (Duncan's multiple range test).

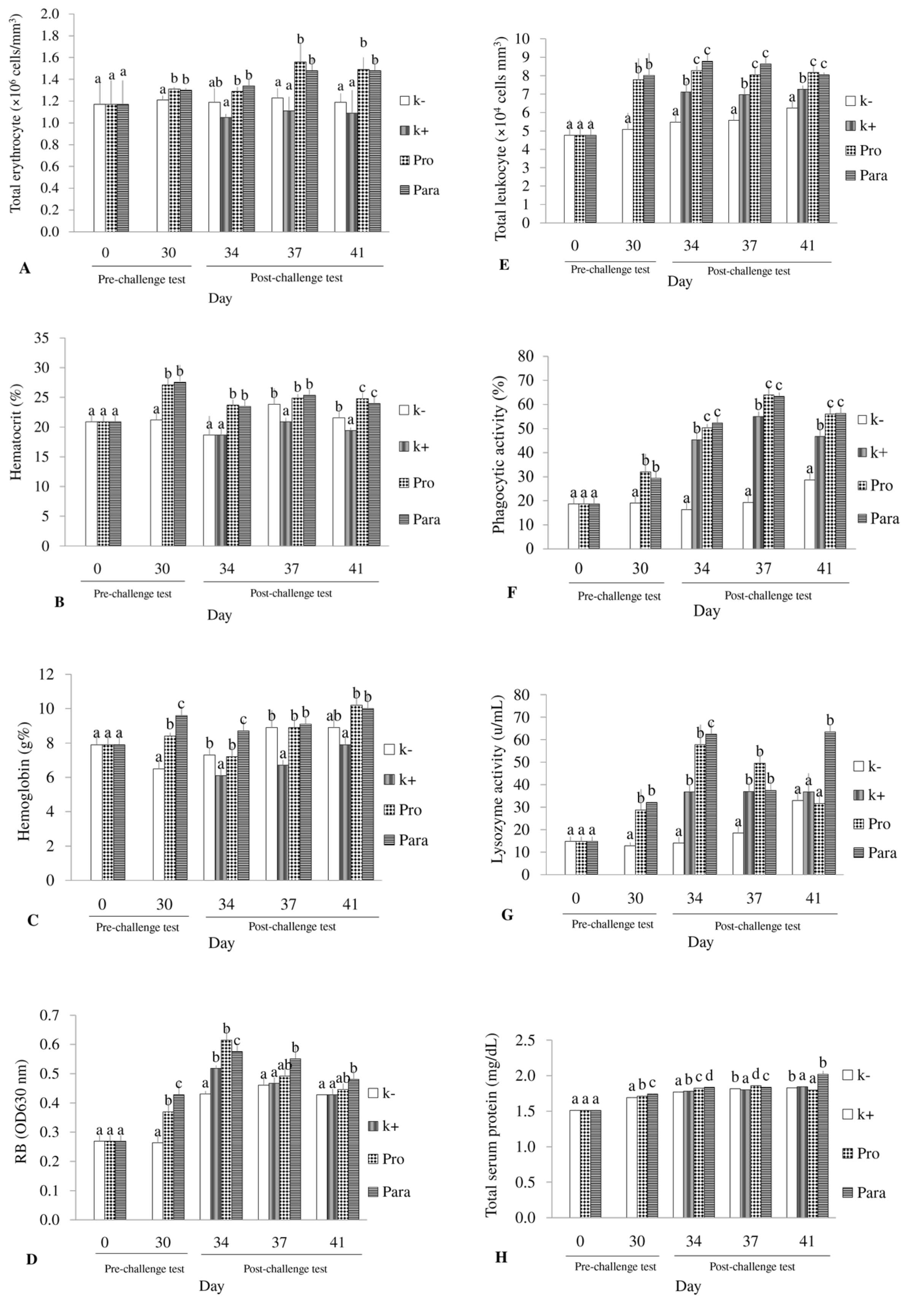

Figure 1. Total erythrocyte (TE) (A), hematocrit (Ht) (B), hemoglobin (Hb) (C), respiratory burst (RB) (D), total leukocyte (TL) (E), phagocytic activity (AF) (F), lysozyme activity (AL) (G), and total serum protein (TSP) (H). Different letters in each bar (average±standard deviation) show a statistical 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 Rf^R 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,

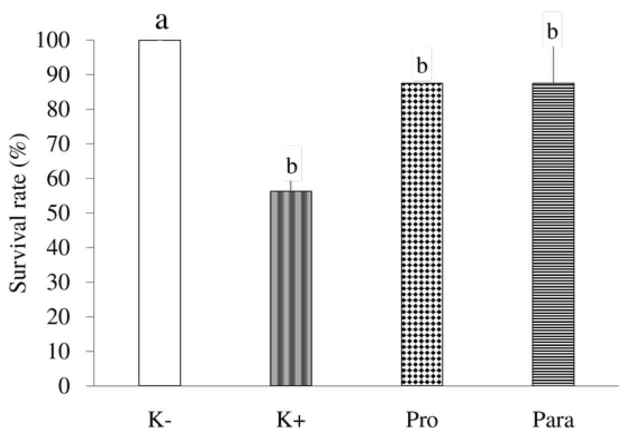

Figure 2. Survival rate of red Nile tilapia after infected with the *S. agalactiae*. Different letters in each bar (average±standard deviation) show a statistical difference (Duncan's multiple range test; P<0.05). Negatif control (K-); positive control (K+); 1% probiotic 1% (Pro) and 1% paraprobiotic (Para).

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 I 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 10^7CFU/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 Rf^R 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 Rf^R probiotics in the intestine were only found in the probiotic treatment. The calculation results of total bacteria and *Bacillus* sp. NP5 Rf^R 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 occur 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 (NH_3) 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	Treatments		
		Control	Probiotic	Paraprobiotic
Total intestinal bacteria (log CFU/g)	0	7.69 ± 0.00^a	7.69 ± 0.00^a	7.69 ± 0.00^a
	30	8.17 ± 0.56^a	9.12 ± 0.42^a	8.70 ± 0.49^a
Total probiotic(log CFU/g)	0	0 ± 0.00	0 ± 0.00	0 ± 0.00
	30	0 ± 0.00^a	7.83 ± 0.09^b	0 ± 0.00^a

^{a}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* sp. 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

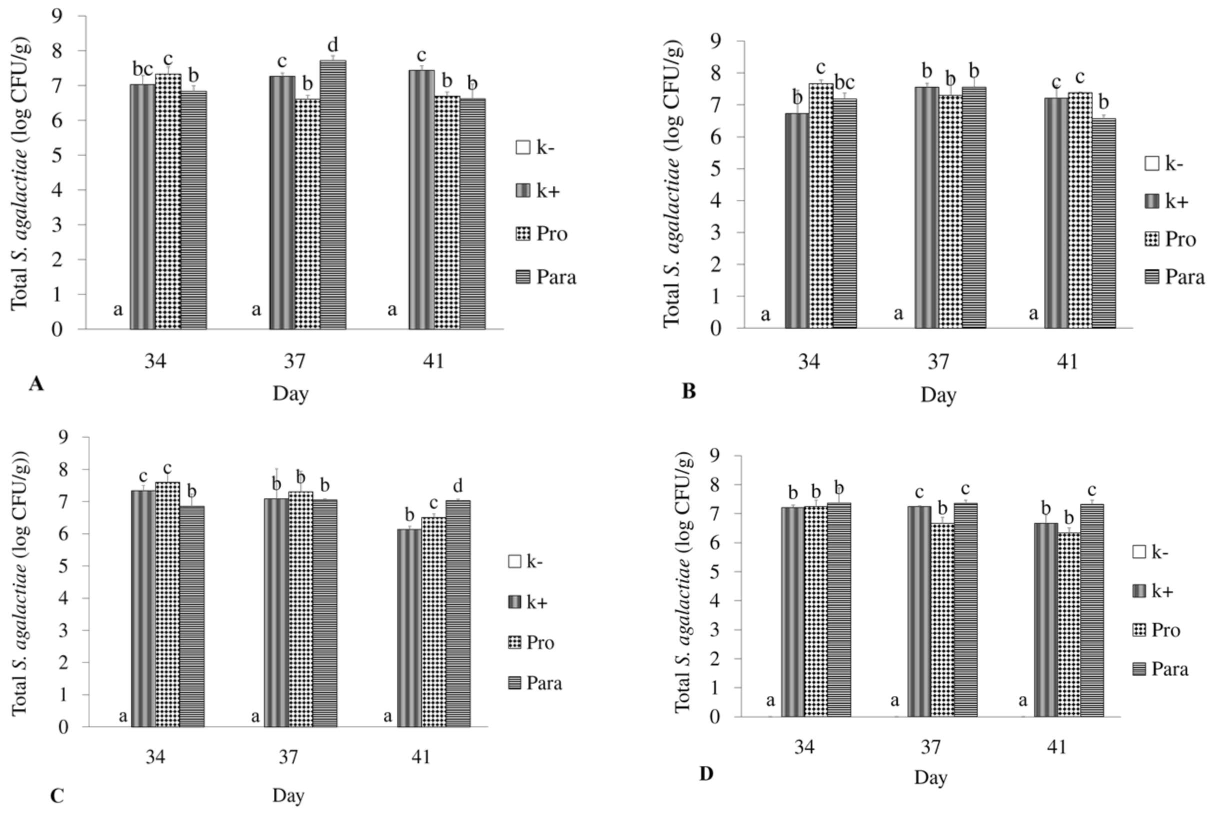

Figure 3. Total *S. agalactiae* bacteria in the brain (A), eyes (B), kidney (C), and liver (D) of red Nile tilapia on the post-challenge test. Different letters in each bar (average±standard deviation) show a statistical difference (Duncan's multiple range test; P<0.05). Negatif control (K-); positive 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 unbeneficial 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 increased absorption and nutrient utilization, including Fe (iron), that is required in blood formation (Dawood *et al*., 2015b). The parameter measurement results of TE, Hb, and Ht obtained the lowest values in the 34th day, and increased in the 37th and 41st days. This condition was thought due to the inhibition of erythropoiesis caused by the *S. agalactiae* infection, which decreased the total erythrocytes value (Sirimanapong *et al*., 2018).

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 esponse. 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 evaluationg the fish defense mechanism. This study also showed the increased superoxide (H_2O_2) and anion (OH) productions either in the probiotic treatment or *Bacillus* sp. NP5 paraprobiotic treatment. This condition followed the study of Kamilya *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 microbes 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 propia 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.

REFERENCES

Agung LA, Widanarni, Yuhana M. 2015. Application of micro-encapsulated probiotic *Bacillus* NP5 and prebiotic mannan oligosaccharide (MOS) to prevent streptococcosis on tilapia *Oreochromis niloticus*. Research Journal of Microbiology 10: 571–581.

Anderson DP, Siwicki AK. 1993. Basic hematology and serology for fish health programs. Paper presented in second symposium on disease in asian aquaculture "Aquatic Animal Health and the environment". Phuket, Thailand. October 25-29th, 1993. p185–202.

APHA. 1998. Standard methods for the examination of the water and wastewater. American public health association. Washington DC.

Bergmeyer HU, Grossi M, Walter HE. 1983. Samples, reagents, assessment of results. Bergmeyer HU, editor. Methods of Enzymatic Analysis 3rdedition. Michigan (US): Academic Press, The University of Michigan. 274–275.

Blaxhall PC, Daisley KW. 1973. Routine haematological methods for use with fish blood. Journal of Fish Biology 5: 577–581.

Borlongan TG. 1990. Studies on the lipase of milkfish *Chanos chanos*. Aquaculture 89: 315–325

Bradford MM. 1976. A rapid sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry 72: 248–254.

Das A, Nakhro K, Chowdhury S, Kamilya D. 2013. Effects of potential probiotic *Bacillus amyloliquifaciens* FPTB16 on systemic and cutaneous mucosal immune responses and disease resistance of catla (*Catla catla*). Fish and Shellfish Immunology 35: 1547–1553.

Dash G, Raman RP, Pani Prasad K, Makesh M, Pradeep MA, Sen S. 2015. Evaluation of paraprobiotic applicability of *Lactobacillus plantarum* in improving the immune response and disease protection in giant freshwater prawn, *Macrobrachium rosenbergii* (de Man, 1879). Fish and Shellfish Immunology 43: 167–174.

Dawood MAO, Koshio S, Ishikawa M, Yokoyama S. 2015a. Interaction effects of dietary supplementation of heat-killed *Lactobacillus plantarum* and β-glucan on growth performance, digestibility and immune response of juvenile red sea bream, *Pagrus major*. Fish and Shellfish Immunology 45: 33–42.

Dawood MAO, Koshio S, Ishikawa M, Yokoyama S. 2015b. Effects of partial substitution of fish meal by soybean meal with or without heat-killed *Lactobacillus plantarum* (LP20) on growth performance, digestibility, and immune response of amberjack, *Seriola dumerili* juveniles. BioMed Research International 2015: 11.

Dawood MAO, Koshio S, Ishikawa M, Yokoyama S. 2016. Effects of dietary inactivated *Pediococcus pentosaceus* on growth performance, feed utilization and blood characteristics of red sea bream, *Pagrus major juvenile*.Aquaculture Nutrition 22: 923–932.

Dawood MAO, Magouz FI, Salem MFI, Abdel-Daim HA. 2019. Modulation of digestive enzyme activity, blood health, oxidative responses and growth-related gene expression in GIFT by heat-killed *Lactobacillusplantarum* (L-137). Aquaculture 505: 127–136.

de Almada CN, Almada CN, Martinez RCR, Sant'Ana AS. 2016. Paraprobiotics: evidences on their ability to modify biological responses, inactivation methods and perspectives on their application in foods. Trends in Food Science & Technology 58: 96–114.

de Araújo Etchepare M, Nunes GL, Nicoloso BR, Barin JS, Moraes Flores EM, de Oliveira Mello R, Ragagnin de Menezes C. 2020. Improvement of the viability of encapsulated probiotics using whey proteins. LWT-Food Science and Technology 117: 108601.

de Sousa EL, Assane IM, Santos-Filho NA, Cilli EM, de Jesus RB, Pilarski F. 2020. Haematological, biochemical and immunological biomarkers, antibacterial activity, and survival in Nile tilapia *Oreochromis niloticus* after treatment using antimicrobial peptide LL-37 against *Streptococcus agalactiae*. Aquaculture 533: 736181.

Djauhari R, Widanarni, Sukenda, Suprayudi MA, Zairin Jr. M. 2016. Characterization of *Bacillus* sp. NP5 and its application as probiotic for common carp *Cyprinus carpio*. Research Journal of Microbiology 11: 101–111.

Dossou S, Koshio S, Ishikawa M, Yokoyama S, El Basuini MF, Zaineldin AI, Mzengereza K, Moss A, Dawood MAO. 2019. Effects of replacing fishmeal with fermented and non-fermented rapeseed meal on the growth, immune and antioxidant responses of red sea bream *Pagrus major*.Aquaculture Nutrition 25: 508–517.

Gomez D, Sunyer JO, Salinas I. 2013. The mucosal immune system of fish: The evolution of tolerating commensals while fighting pathogens. Fish and Shellfish Immunology 35: 1729–1739.

Hanif A, Bakopoulos V, Dimitriadis GJ. 2004. Maternal transfer of humoral specific and non-specific immune parameters to sea bream *Sparus aurata* larvae. Fish and Shellfish Imunology17 : 411–435.

Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME. 2014. Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nature Reviews Gastroenterology and Hepatology 11: 506–514.

Ho TTT, Tri NN, Quy OM, Fotedar R, Kannika K, Unajak S, Areechon N. 2017. Effects of the dietary supplementation of mixed probiotic spores of *Bacillus amyloliquefaciens* 54A, and *Bacillus pumilus* 47B on growth, innate immunity and stress responses of striped catfish *Pangasianodon hypophthalmus*. Fish and Shellfish Immunology 60: 391–399.

Joffre OM, Poortvliet PM, Klerkx L. 2018. Are shrimp farmers actual gamblers? An analysis of risk perception and risk management behaviors among shrimp farmers in the Mekong Delta. Aquaculture 495: 528–537.

Kamilya D, Baruah A, Sangma T, Chowdhury S, Pal P. 2015. Inactivated probiotic bacteria stimulate cellular immune responses of catla, *Catla catla* (Hamilton) in vitro. Probiotics and Antimicrobial Proteins 7: 101–106.

[KKP] Kementerian Kelautan dan Perikanan. 2019. Data Produksi Nasional Perikanan dan Kelautan Tahun 2018. [diunduh 2020Agustus 5]. Tersedia pada https://satudata.kkp.go.id/dashboardproduksi.

Kuebutornye FKA, Abarike ED, Lu Y. 2019. A review on the application of *Bacillus* as probiotics in aquaculture. Fish and Shellfish Immunology 87: 820–828.

Kuebutornye FKA, Tang J, Cai J, Yu H, Wang Z, Abarike ED, Lu Y, Li Y, Afriyie G. 2020. In vivo assessment of the probiotic potentials of three host-associated *Bacillus* species on growth performance, health status and disease resistance of *Oreochromis niloticus* against *Streptococcus agalactiae*. Aquaculture 527: 735440.

Lazado CC, Caipang CMA. 2014. Mucosal immunity and probiotics in fish. Fish and Shellfish Immunology 39: 78–89.

Li L, Cardoso JCR, Félix RC, Mateus AP, Canário AVM, Power DM. 2020. Fish lysozyme gene family evolution and divergent function in early development. Development and Comperative Immunology 114: 103772

Lusiastuti AM, Textor M, Seeger H, Akineden Ö, Zschöck M. 2014. The occurrence of *Streptococcus agalactiae* sequence type 261 from fish disease outbreaks of tilapia *Oreochromis niloticus* in Indonesia. Aquaculture Research 45: 1260–1263.

Mohapatra S, Chakraborty T, Prusty AK, Das P, Paniprasad K, Mohanta KN. 2012. Use of different microbial probiotics in the diet of rohu, *Labeo rohita* fingerlings: effects on growth, nutrient digestibility and retention, digestive enzyme activities and intestinal microflora. Aquaculture Nutrition 18: 1–11.

Newaj-Fyzul A, Al-Harbi AH, Austin B. 2014. Review: Developments in the use of probiotics for disease control in aquaculture. Aquaculture 431: 1–11.

Nguyen VN, Onoda S, Tran V K, Hai PD, Nguyen TT, Hoang L, Koshio S. 2019. Evaluation of dietary Heat-killed *Lactobacillus plantarum* strain L-137 supplementation on growth performance, immunity and stress resistance of Nile tilapia *Oreochromis niloticus*. Aquaculture 498: 371–379.

Nguyen NP, Nguyen THL, Crestani C, Zadoks RN. 2021. Effect of strain and enviromental conditions on the virulence of *Streptococcus agalactiae* (Group B Streptococcus; GBS) in red tilapia (*Oreochromis* sp.). Aquaculture 534: 736256.

Ottinger, M., Clauss, K., Kuenzer, C., 2016. Aquaculture: relevance, distribution, impacts and spatial assessments – a review. Ocean & Coastal Management 119: 244–266.

Pandiyan P, Balaraman D, Thirunavukkarasu R, George EGJ, Subaramaniyan K, Manikkam S, Sadayappan B. 2013. Probiotics in aquaculture. Drug Invention Today 5: 55–59.

Piqué N, Berlanga M, Miñana-Galbis D. 2019. Health benefits of heat-killed (Tyndallized) probiotics: An overview. International Journal of Moleculer Sciences 20: 1–30.

Putra AN, Widanarni. 2015. Screening of amylolytic bacteria as candidates of probiotics in tilapia *Oreochromis* sp.. Research Journal of Microbiology 10: 1–13.

Rodriguez-Estrada U, Satoh S, Haga Y, Fushimi H, Sweetman J. 2013. Effects of inactivated *Enterococcus faecalis* and mannan oligosaccharide and their combination on growth, immunity, and disease protection in rainbow trout. North America Journal of Aquaculture 75: 416–428.

Sánchez de Medina F, Ortega-González M, González-Pérez R, Capitán-Cañadas F, Martínez-Augustin O. 2013. Host-microbe interactions: The difficult yet peaceful coexistence of the microbiota and the intestinal mucosa. British Journal of Nutrition 109: 12–20.

Singh ST, Kamilya D, Kheti B, Bordoloi B, Parhi J. 2017. Paraprobiotic preparation from *Bacillus amyloliquefaciens* FPTB16 modulates immune response and immune relevant gene expression in *catla catla* (Hamilton, 1822). Fish and Shellfish Immunology 66: 35–42.

Sirimanapong W, Thompson KD, Shinn AP, Adams A. 2018. *Streptococcus agalactiae* infection kills red tilapia with chronic *Francisella noatunensis* infection more rapidly than the fish without the infection. Fish and Shellfish Immunology 81: 221–232.

Soto E, Wang R, Wiles J, Green C, Plumb J, Hawke J. 2015. Characterization of isolates of *Streptococcus agalactiae* from diseased farmed and wild marine fish from the U.S. Gulf coast, Latin America, and Thailand. Journal of Aquatic. Animal Health 27: 123–134.

Su Y, Feng J, Liu C, Li W, Xie Y, Li A. 2017. Dynamic bacterial colonization and microscopic lesions in multiple organs of tilapia infected with low and high pathogenic *Streptococcus agalactiae* strains. Aquaculture 471: 190–203.

Verner-Jeffreys DW, Wallis TJ, Cano Cejas I, Ryder D, Haydon DJ, Domazoro JF, Dontwi J, Field TR, Adjei-Boteng D, Wood G, Bean T, Feist SW. 2018. *Streptococcus agalactiae* Multilocus sequence type 261 is associated with mortalities in the emerging Ghanaian tilapia industry. Journal of Fish Diseases 41: 175–179.

Walter HE. 1988. Method with haemoglobin, casein and azocoll as substrate. In: Bergmeyer HU, editor. Methods of enzymatic analysis. ED ke-3. London (UK): Academia Press. P270–277

Widanarni, Tanbiyaskur. 2015. Application of probiotic, prebiotic and synbiotic for the control of streptococcosis in tilapia *Oreochromis niloticus*. Pakistan Journal of Biological Science 18: 59–66.

Worthington V. 1993. Worthington enzyme manual. Enzymes and related biochemicals. New Jersey (US): Worthington Biochemical Corp.

Xu J, Xie YD, Liu L, Guo S, Su YL, Li AX. 2019. Virulence regulation of cel-EIIB protein mediated PTS system in *Streptococcus agalactiae* in Nile tilapia. Journal of Fish Diseases 42: 11–19.

Yahfoufi N, Mallet JF, Graham E, Matar C. 2018. Role of probiotics and prebiotics in immunomodulation. Current Opinion in Food Science 20: 82–91.

Yang HL, Xia HQ, Ye YD, Zou WC, Sun YZ. 2014. Probiotic *Bacillus pumilus* SE5 shapes the intestinal microbiota and mucosal immunity in grouper *Epinephelus coioides*. Disease of Aquatic Organisms 111: 119–127.

Zendeboodi F, Khorshidian N, Mortazavian AM, da Cruz AG. 2020. Probiotic: conceptualization from a new approach. Current Opinion in Food Science 32: 103–123