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Comparative study between probiotics isolated from giant freshwater prawns and giant tiger prawns in improving the health of Nile tilapia (Oreochromis nilotius)

F Feliatra*, I Lukistyowati, N Nursyirwani, D Melina, M Ramadhani
Marine Microbiology Laboratory, Department of Marine Science, Fisheries and Marine Sciences Faculty, University of Riau, Pekanbaru, Riau, Indonesia
*Email: feliatra@yahoo.com

Abstract. One of bacterial diseases frequently attacking Nile tilapia (Oreochromis niloticus) is caused by Streptococcus iniae. This research aims to analyse the impact of immersion of probiotic bacteria isolated from giant freshwater and giant tiger prawns, towards the health of Nile tilapia. The research was conducted from February to June, 2017. The completely randomized design was employed method, with 5 treatment phases and 3 repetition. The probiotic provision treatment to Nile tilapia was executed by immersing it for 5 minutes in every 10 days for 30 days of culture. The probiotic density used was $10^5$ CFU/ml. Subsequent to the 30 days of culture, an infection with Streptococcus iniae bacteria was carried out in an intramuscular technique with 0.1 ml/fish and $10^5$ CFU/ml density. The research result reveals that the probiotic provision, either from giant freshwater or giant tiger prawn shows a positive indication towards the health of Nile tilapia, observed from parameters like the number of red blood cells, hemoglobin level, hematocrit level, total leucocytes and survival rate. The P3 treatment is the best treatment with total erythrocytes as $174.67 \times 10^4$ sel/mm$^3$, 7.47 g/dL hemoglobin, 33.67%, hematocrit, 80.00$ \times 10^3$ sel/mm leucocytes. The immersion with probiotic bacteria could enhance the health quality of Nile tilapia.

I. Introduction
Indonesia has a tendency to improve its pisciculture activity, together with other society activities near the pisciculture area, which bring negative consequences toward the water quality in the public waters where the pisciculture is carried out. The situation is aggravated by the emergence of upwelling which at that time has catalyzed a massive death to fish in Sumatera [1].

An attempt to improve the stamina and immunity of the fish without any side effects has currently been developed. A new and latest study requiring meticulous investigation is the one using probiotic. The probiotic provision to fish is beneficial to enhance the cellular non-specific immunity systemin a form of kidney macrophage increased number and the phagocytosis activity. Probiotic bacteria might play a role in suppressing the growth of pathogenic microbial populations. Probiotic bacteria including lactic acid bacteria had an ability to produce several antimicrobial compounds such as lactic acid, diacetyl, hydrogen peroxide, carbondioxide and bacteriocins [2].

The probiotic of Bacillus sp. type has extensively been applied for the biotechnolology interest, including its various enzymes and produced amino acid. It is as well used to produce an antibiotic to control the pathogens [3]. This type of probiotic also has been implemented on the pisciculture media within an aquarium with $10^4$ CFU/ml dosage, which proves to inhibit the growth of Aeromonas hydrophila to African catfish (Clarias gariepinus) [4].
This research uses probiotic isolated from giant freshwater prawn and giant tiger prawns digestion from Riau [5]. The use of probiotic of Bacillus sp. type from giant freshwater prawns and giant tiger prawns originate from Riau has not yet been considerably employed. Therefore this research aims to analyse the impact of immersion of probiotic bacteria isolated from giant freshwater prawns and giant tiger prawns, towards the health of Nile tilapia.

2. Material and Methods
The research was carried out from February to June 2017 in Marine Microbiology Laboratory and Laboratory of Parasites and Fish Diseases, Fisheries and Marine Sciences Faculty, University of Riau. The method used was an experiment by using completely randomized design with 5 treatment phases and 3 times repetition.

2.1. Experimental design
The treatment given used collection of probiotic isolate, both found in giant freshwater prawn (Macrobrachium rosenbergii) or giant tiger prawn (Penaeus monodon). The followings are the treatment applied:
P0 : Control (without probiotic)
P1 : GFP4 isolate Bacillus sp probiotic provision (the best isolate from giant freshwater prawn)  
P2 : Bacillus sp. probiotic provision originated from the combination of isolate from giant freshwater prawn (GFP1+GFP12+GFP13+GFP4+GFP5)  
P3 : TP4 isolate Bacillus sp probiotic provision (the best isolate from giant tiger prawn)  
P4 : Bacillus sp probiotic provision originated from the combination of isolate of giant tiger prawn (TP1+TP2+TP3+TP4+TP5)

Both Bacillus sp. probiotic provision from giant freshwater prawn or giant tiger prawn with 10^5 CFU/ml density was immersed for 5 minutes in every ten days until 30 days of culture. It was then infected by using 5 S. iniae. During the culture, the fish were given F-999 ad satiation of commercial fodder for 3 times a day. After 30 days of culture, the fish were infected with S. iniae bacteria having 10^5 CFU/ml density by intramuscular injection with 0.1 ml/fish dosage. The fish blood extraction was accomplished after the culture (day 30) and after the infection (day 44). The measured parameters include the total of erythrocytes, the level of hemoglobin and hematocrit, total leucocytes and survival rate.

The bacteria sample of S. iniae used were the collections available in the Laboratory of Parasites and Fish Diseases, Laboratory of Fish Quarantine Station Class I, Sultan Syarif Kasim II, Pekanbaru. Total of 150 samples Nile tilapia (each aquarium containing 10 fish) were gained from Fish Seed Halls Sipungguk, Kampar Regency.

The used probiotic isolate originates from giant freshwater prawn and giant tiger prawn were sequenced with 16S rDNA. They were afterwards recultured by using Nutrient Agar (NA) media and Nutrient Broth (NB). As for the anesthesia, cloves oil was used. Last, regarding to the blood check, substances like Na-Sitrat (anticoagulan), Turk solution, Hayem solution, HCl 0,1 %, Aquades, vortex candle, and F-999commercial pellet were used.

2.2. Materials
The treatment container utilized was 15 aquariums with 40 x 30 x 30 cm in size. Before being used, they were initially cleaned up to get rid of pathogenic microorganisms, through KMnO₄ solution for 24 hours. Thenceforth, the aquariums were rinsed and dried. Once they were certainly hygienic, water for 25 cm high (30 L) was filled and aerated. Finally, Nile tilapia with 8-12 cm in size, were put into the aquarium with 10 fish/aquarium.

2.3. Preparation
Probiotic coming from giant freshwater prawn or giant tiger prawn wasre-grown on the NA media, and incubated in 27-28°C temperature for 24 hours. It was then harvested by means of taking the
probiotic growing on the agar NA for 1 ose, and put to the NB (Nutrient Broth) media with 50ml (as starter bacteria) volume. Lastly, a 24-hours incubation was conducted.

The starter bacteria flourishing on the NB media was subsequently mixed to the NB media for about 250 ml so that the probiotic volume reached 300 ml. Next, in order to ensure and obtain 10^5 CFU/ml probiotic bacteria density, a gauge using spectrophotometer was carried out.

Further, the mixed probiotic from the digestive tract of giant freshwater prawn and giant tiger prawn were organized. Each isolate with the same amount was taken and brought into the erlenmeyer flask (2 elenmeyer flasks are utilized) for every mixed probiotic of giant freshwater and giant tiger prawns.

Pisciculture was executed for 30 days. The fish was taken care in 28°C temperature using a heater. Siphoning was done everyday to scour the remaining fodder and feces by reducing the water volume to 50%. Every 10 day, a 5-minute immersion of tested fish to a given-probiotic container was completed with 10^5 CFU/ml probiotic density. The immersion procedure was accomplished by taking the fish out of the aquarium and moving them to the immersion container. After being immersed, the fish were again transferred to the aquarium. At the last stage of the pisciculture, there was a blood drawing and an infection by using _S. Iniae_ bacteria. At the post infection activity, the fish blood test was done all over again.

The total of erythrocytes measurement was conducted by absorbing the blood sample using 0.5 scaled pipette, and was then continued by absorbing the Hayem solution until 101 scale. After that, it was homogenized by way of shaking to form an 8 figure. The first drop was discarded and the following drops were put into the hemocytometer and covered with cover glass [6]. The measurement was done within 5 small boxes of hemocytometer. The total was calculated with the following formulation:

\[
Total \ erythrocytes = total \ of \ counted \ erythrocytes \ cell \times 10^4 \ cell/mm^3
\]

Total of hemoglobin was gauged by filling the salinometer with HCl 0.1 N solution until it reached 0 (the lowest scale line on salinometer). Hereafter, the tube was positioned between 2 tubes with standard color. For 0.02 fish blood was then taken by using a microtube with sahli pipette. The blood was put into salinometer and stilled for 3 minutes after the tip of the pipette was firstly sterilized. Moreover, aquades was added by using a drip pipette bit by bit while being stirred with a glass stirrer until the color is exactly similar to the standard color. The level of hemoglobin is stated in g/dL [6].

The blood sample was put into the hematocrit capillary tube until approximately four-fifth of the tube. Then, blocked the tip (red sign) with critoseal and centrifuge for 15 minutes by 3500 rpm speed. After that, the percentage of the hematrocit was measured. The hematrocit level is presented as % of blood cell volume [6].

The measurement of total leucocytes was carried out by absorbing the blood sample with 0.5 scale pipette (special pipette for leucocytes gauge). It was continued by as well absorbing the Turk solution to 11 scale, and homogenized by shaking to form an 8 figure. The first drop was thrown away and the next drops were put into the hemocytometer and covered with coverglass [6]. This gauge was done in 4 hemocytometer big boxes and counted by using the formulation below:

\[
Total \ leucocytes = total \ of \ counted \ leucocytes \ cell \times 50 \ cell/mm^3
\]

The parameters of water quality are temperature, pH and dissolved oxygen (DO). The water quality measurement was carried out twice at the beginning and at the end of the research.

3. Results and Discussions

3.1. Total erythrocytes

The research result of probiotic provision by immersion to Nile tilapia taken care for 30 days and infected with _S. Iniae_ bacteria is about 152.33±5.13±x10^4 cell/mm³-182.67±7.637 x10^4 cell/mm³. This result is different from Maryadi [7] research using _Bacillus_ probiotic and is implemented to media
treatment that the number of erythrocytes of Nile tilapia is going around 1.05-3.0x10^6 sel/mm³, which is deemed as the normal amount.

After the infection with *S. Iniae*, the number of erythrocytes within Nile tilapia blood is decreased. The *Bacillus* probiotic provision proves to be capable of preserving the quantity of erythrocytes in the blood of Nile tilapia as an influence of *S. Iniae* infection, though that a reduction occurred. The decline of the erythrocytes is assumingly occurred for the extracellular product generated by the *S. Iniae*, that is hemolicin; an extra cellular enzime having the ability to lyseerythrocytes by producing a toxin in a form of hemolcin [8].

The erythrocytes total reduction after the infection is also believed to appear because of the entrance of phagocytosis bacteria. The process needs oxigen resulting a decrease in erythrocytes. The bacteria entering the body would undergo phagosytosis process where the phagosite cells will recognize and digest the requiring oxigen bacteria particles so that erythrocytes decline is happening [9]. The complete explanation can be seen in Figure 1.

Based on the statistical test (ANOVA), the erythrocytes amount of Nile tilapia given probiotic treatment, which is isolated from the digestive track of giant freshwater prawn and giant tiger prawn, has an effect on the number of erythrocytes after infection with *S.iniae* bacteria, observed on the P3 treatment compared to the control of P0 (p<0.01). It designates that the *Bacillus* sp. probiotic provision indeed has a tangible impact towards the change of the erythrocytes number to Nile tilapia. Specifically, the number of erythrocytes of Nile tilapia provides crucial information as to the physiology condition and demonstrates its health status [10].

![Figure 1. Nile tilapia total erythrocytes treated with probiotic from the digestive track of giant freshwater prawn and giant tiger prawn.](image)

3.2. *The level of hemoglobin*

The observation result towards the level of hemoglobin of Nile tilapia given *Bacillus sp* probiotic treatment by an immersion and protection for 30 days reveals that there is an improvement on the P0 as 6.20 g/dL, P1 for 7.00 g/dL, P2 as 6.80 g/dL, P3 for 7.80 g/dL and P4 as 7.00 g/dL. These numbers are identical with a research project conducted by Salasia *et al.*, [11] where the level of hemoglobin of Nile tilapia was going on for 5.05-8.33 g/dL. This condition indicates that the *Bacillus sp* probiotic
provision can increase the number of hemoglobin in the blood contrasted to the control treatment of P0. The detailed information can be viewed Figure 2.

![Figure 2. Hemoglobin quantity of Nile tilapia given probiotic treatment taken from digestive track of giant freshwater prawn and giant tiger prawn.](image)

Once the infection has finished, a decrease on the hemoglobin level in the blood of Nile tilapia is happening. The P0 is 4.87 g/dL, P1 is 5.60 g/dL, P2 is going around 5.13 g/dL, P3 as 7.47 g/dL and P4 is 6.60 g/dL. Phagocytic activity appears to be the main cause of the decline of hemoglobin for its activity requires more oxygen in resisting pathogenic bacteria [12]. Jeong [13] mentions that several Streptococcus sp. yield hemolisin as a result of extra cellular protein secretion of lipid membrane possessing ability to lyse hemolysis, reducing the amount of hemoglobin and red blood cells.

On the basis of a statistical test (ANOVA), the Nile tilapia level of hemoglobin given probiotic treatment isolated from the digestive track of giant freshwater prawn and giant tiger prawn after the infection with S.iniae bacteria describes a significant distinctive influence (p<0.05) compared to the control treatment (P0). The low level of hemoglobin denotes that the S.iniae infected Nile tilapia undergoes an erythrocytes disorder. The existence of Streptococcus sp toxin affects the hemoglobin stability. The possibility of kidney infection engenders a low red blood cells production, causing anemia and limited number of hemoglobin to the fish[14]. Saputra et al. [15] add that the quantity of hemoglobin is linear with the number of erythrocytes. The higher the total of hemoglobin, the greater the number of erythrocytes.

### 3.3 The Level of Hematocrit

The observation result of hematocrit level of Nile tilapia for 30 days of culture with probiotic immersion shows that there is an increase of hematocrit on the P0 treatment for 30.67%, P1 for 33.33%, P2 for 29.67%, P3 for 35%, and P4 for 30%. The detailed information can be seen in Figure 3.

Hardi [8] contends that the normal level of hematocrit of Nile tilapia is around 27.3-37.8%. This signifies that the probiotic Bacillus provision can improve the capacity of hematocrit in Nile tilapia blood. Nevertheless, hematocrit decline occurs after the infection with S. Iniae bacteria. This hematocrit intensity drop depicts a defect on the red blood cells as a result of bacteria infection [16].

Based on a statistical test (ANOVA), the Control (P0) has no considerable difference with the P1,P2,P3 and P4 at the post-infection by using S.iniae bacteria (p>0.05). The total of hematocrit of Nile tilapia after an infection with the S.iniae bacteria is still normal. The fish are able to maintain the hematocrit amount in the blood because an antibacterial substance within the probiotic is presumably existing. As stated by Sumardi et al. [17] Bacillus sp. bacteria generate such immunity and antimicrobial as bacteriocin. The whole bacteriocin within the Bacillus bacteria may possibly control
the pathogenic bacteria by inhibiting its growth and attacking the bacteria cellulose. Bacteriocin will hence give resistance to each pathogenic bacterium entering the body.

**Figure 3.** Hematocrit level of Nile tilapia given treatment of probiotic from digestive track of giant freshwater prawn and giant tiger prawn.

3.4. *Total Leucocytes*

The scrutiny result of the leucocytes total of Nile tilapia taken care for 30 days with probiotic indicates a rise on the leucocytes within the blood on the 60.33x10^3 cell/mm^3 P0, 68.00x10^3 cell/mm^3 P1, 62.33x10^3 cell/mm^3 P2, 76.00x10^3 cell/mm^3 P3 and 62.00x10^3 cell/mm^3 P4. Those numbers are rather similar to a study conducted by Moyle and Cech [18], declaring that the amount of leucocytes of Nile tilapia is generally between 20-150x10^3 cell/mm^3.

The leucocytes increase happens after the infection with *S. Iniae* bacteria. The leucocytes increase is caused by the appearance of and an infection initiated by *S. Iniae* bacteria. Additionally, Matofani *et al.*, [19] also says that the leucocytes increase occurs for the increase coming on the cellular defense. A clearer depiction is presented in Figure 4.

**Figure 4.** Total leucocytes of Nile tilapia (*O. niloticus*) given probiotic provision from digestive track of giant freshwater prawn (*M. rosenbergii* De Man) and giant tiger prawn (*P. monodon*)
A statistical test (ANOVA) points out that the total of leucocytes of Nile tilapia given *Bacillus* probiotic treatment, which is isolated from the digestive track of both giant freshwater prawn and giant tiger prawn after an infection with *S. iniae* bacteria (P1, P2, P3 and P4) is truly dissimilar to the Control (P0) (p<0.01). Obviously, it designates that the *Bacillus* probiotic provision could suppress the *S. iniae* infection. Todor[20]; Torkar and Martijasic [21] explain that apart from yielding bacteriocin antimicrobial, *Bacillus* also has other antimicrobial chemical compound such as bacitracin, pumulin, gramicidin, laterosporin and tyrocidine, effectively oppose the Gram positive bacteria, as well as colistin and polymyxin, functioned to well-resist the Gram negative bacteria.

Immunity can be improved by affording probiotic in three different ways [22]. First, improving the macrophage activity that can be seen from the microorganism increase, undergoing phagocytosis or carbon particles. Second, improving a systematic antibody production like immunoglobulin and interferon, and lastly improving local antibody on the mucus surface such as to the intestinal wall.

Matofani et al., [19] declares that the number of leucocytes is multiplied, occurring as a result of the cellular resistance system from within the body. In addition, probiotic also contains bacteriocin having its own mechanism in hampering the pathogenic bacteria growth and even causing death to the bacterial cell, sensitive to bacteriocin [23]. Bacteriocins from probiotic bacteria produced from the LAB isolates may be useful as a food biopreservative for controlling microbial deterioration, enhancing the hygienic quality, and extending the self-life of fish and seafood products [24].

### 3.5 Water Quality of Treatment Media

The measurement result of water quality during the research shows in Table 1. All parameters analyzed did not show a real difference between one treatment and another.

**Table 1.** Water quality of treatment media of Nile tilapia given probiotic taken from digestive track of giant freshwater prawn and giant tiger prawn

| Parameter          | P0    | P1    | P2    | P3    | P4    | Kordi (2010) |
|--------------------|-------|-------|-------|-------|-------|--------------|
| Temperature (°C)   | 27.8  | 28    | 27.8  | 28    | 28    | 25 – 33      |
| DO (mg/L)          | 3.9-4.3 | 3.9-4.6 | 4.0-4.2 | 3.9-4.6 | 4.0-4.2 | >3           |
| pH                 | 6.8-7.0 | 6.4-7.1 | 6.2-7.0 | 6.4-7.1 | 6.2-7.0 | 6.0-8.5      |

Abdelhamid et al., [25] mention that these drastic effects related with the presence of some pollutants from agricultural and urban drainages whether in the rearing water or in the feeding Dried Sewage Sludge (DSS) that can negatively affect fish health, production and quality, as well as could be inter the food chain and threat human health.

### 4. Conclusion

The probiotic provision, either isolated from giant freshwater prawn (*M. rosenbergii*) or giant tiger prawn (*P. monodon*), shows a positive indication in affecting and improving the health of Nile tilapia (*O. niloticus*). The research results *Bacillus* sp type of probiotic P3 isolates (TP4) as the best isolate, isolated from digestive track of giant tiger prawn (*P. monodon*). The blood parameters after infection with *S. iniae* bacteria demonstrate outcome as the followings: 174.67x10⁴ cell/mm³ total of erythrocytes, 6.65 g/dL hemoglobin, 33.67 % hematocrit, 80.00x10³ cell/mm³ leucocytes, and 80% survival rate. The water quality measurement result during the research was around 27-28°C, 4.0-4.6 ppm dissolved oxygen, and 6.2-7.1 pH.

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