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Application of Probiotic that are in Isolation From Giant Prawns and Black Tiger Shrimps on the Leukocytes of Nile Tilapia (*Oreocromis niloticus*) Infected by *Streptococcus. Iniae*

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Abstract

Streptococcosis is a common disease found in the cultivation of Nile tilapia, mainly caused by *Streptococcus iniae*. It is necessary to find relatively safer means of protection, one of which is using probiotics. Probiotics given to fish may improve the non-specific immune system so as to improve the fish health. This study aims to determine the effects of probiotics derived from the digestive tract of giant prawns (*Macrobranchiumrosenbergii* De MAN) and black tiger shrimps (*Penaeusmonodon*) sprayed on the feed on the leukocytes and the survival rates of Nile tilapia.

This study employed experimental method by using a completely randomized design (CRD) with 5 treatment levels and 3 repetitions. The treatments

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included P0 (nile tilapia not given probiotics), P1 (giving *Bacillus sp* probiotics G4 isolate), P2 (giving *Bacillus sp* isolate combined from giant prawns G1+G2+G3+G4+G5), P3 (giving *Bacillus sp* probiotics W9 isolate) and P4 (giving *Bacillus sp* probiotics, a combined isolate of black tiger shrimps tiger shrimps W1+W2+W8+W9+W10). As much as 105 CFU/ml probiotics was sprayed on every 15ml/kg feed during the treatments.

The results showed that the provision of *Bacillus sp* probiotics from both giant prawns and black tiger shrimps improved the health of Nile tilapia as seen from the differentiation of leukocytes and survival rates. The best treatment was found in P3 (isolate W9) after being infected with *Streptococcus iniae* with leukocytes reaching 83.00 x 103 cell/mm3, lymphocytes 89.67%, monocytes 10.33%, neutrophils 10% and survival rates 80.00%.

Conclusion: The addition of probiotics derived from the digestive tract of giant prawns (*M. rosenbergii* De Man) and black tiger shrimps (*P. monodon*) on feed given for 30 days manages to suppress the pathogenicity of *Streptococcus iniae*, increase the immune response of Nile tilapia (*O. niloticus*) and reduce fish mortality due to *Streptococcus iniae* infection.

Keywords: Leukocytes, Probiotic, *Bacillus* sp, *Oreochromisniloticus, Streptococcus iniae*.

INTRODUCTION

Tilapias have become the world's second most important cultured fish after carps. The market for tilapia is associated with strong demand, driven by continued increases in population (Siddiki *et al*, 2018). Nile tilapia ((*Oreochromis niloticus* (Linnaeus, 1757)) is a worldwide important species in aquaculture because of its fast growth, firm and tasty flesh, resistance against harsh conditions and ease production of fingerlings under captivity (Gómez-Márquez *et al*. 2003). It is one of freshwater commodities that has a high economic value, easy to culture and has a thick body flesh, making it suitable to be used as refined products, one of them is fillet. Tilapia fillet has very wide export market, especially in the United States (Yustiati *et al*, 2018). Tilapias are among the most important farmed fishes, and their production continue to expand exponentially across the globe (Mathew *et al*, 2017)

Nile tilapia is cultured in more than 100 countries, and its production was estimated at 2,790,350 metric tonnes in 2011, and valued at 4.52 billion USD (FAO, 2014). This species is favoured by fish farmers due to its fast growth rate and its ability to tolerate a wide range of environmental conditions (Grammer *et al*, 2012). Resistance to diseases and good consumer acceptance have further promoted its culture worldwide (Chakraborty and Banerjee, 2009)

A major problem associated with intensive fish culture operations is the increased susceptibility of fish to infectious diseases. The total losses from disease outbreaks in aquaculture worldwide have reached billions of dollars annually and have been identifed as a major threat to the sustainability of aquaculture industry. Fish disease outbreaks adversely affect aquaculture production. The risk of losing profits due to diseases and parasites is already manifesting (Akoll and Mwanja, 2012). Cases of aquatic disease incidences leading to mortality rates of 60% have been reported in hatcheries and grow-out systems in Uganda. Infectious parasites and bacteria are reported to affect private and public fish farms with profound effects (Steigen *et al*, 2013).

Efforts to improve the fish immunity without any side effects have recently been developed. Several studies on the use of probiotics in fish and crustaceans have exhibited promising results (Kesarcodi-Watson *et al*, 2008; El-Rhman *et al*, 2009; Zhou *et al*, 2009), allowing probiotics to substitute the antibiotic as growth promoters. Providing probiotics in fish is useful to strengthen the nonspecific cellular immune system, noted by an increase in the number of renal macrophages and the improved phagocytic activity. Probiotics can also be used as a therapy in fish culture and a biological control strategy to suppress the growth of resistant bacterial diseases (Hassaan and Ghonemy, 2014). The probiotics of live microbes have shown their effectiveness to mitigate the effects of stress, resulting in greater production of Nile tilapia (Ghazalah et al, 2010). Olvera *et al*, (2001) concludes that yeast has a positive effect on fish performance when cultured under stress conditions by means of lowered dietary protein, leading to improved growth and feed efficiency.

Probiotics from *Bacillus sp* group have been widely applied for biotechnology purposes, including the types of enzymes and amino acids produced, as well as the production of antibiotics to control pathogens (Surokulova *et al*, 2007). Probiotics *Bacillus sp.* have also been employed in the cultivation using aquarium. The usage of about 104 CFU/ml probiotics of this type was shown to inhibit the growth of *Aeromonas hydrophilic* in giant cat fish (*Clarias gariepinus*) (Ulkhaq *et al*, 2014). Several studies reported that probiotics can improve growth and immune response of Nile tilapia (Rusdani *et al*, 2016). Moreover, Essa *et al* (2010) showed that all diets containing different probiotic groups significantly ($P < 0.05$) improved Nile tilapia growth and feed utilization compared to the control diet. This study was conducted to evaluate the effect of dietary supplementation on commercial probiotics on the growth and production of Nile tilapia (*Oreochromis niloticus*) in brackish water environment.

The usage of *Bacillus sp.* probiotics from the giant prawns and black tiger shrimp from Riau is still unpopular. For this reason, the researchers are interested in investigating the effect of *Bacillus sp* isolated from giant prawns and black tiger shrimps on improving the health of Nile tilapia (*Oreochromis niloticus sp*) by looking at the leukocytes rates of those infected by *S. iniae*.

MATERIALS AND METHODS

Experimental design

The study was conducted from March to June 2018 in the Laboratory of Parasites and

Fish Diseases, Riau University, Pekanbaru, Indonesia. The Completely Randomized Design (CRD) method with 5 treatment levels and three replications were used in this study. What differentiated one treatment from another was the probiotic sources isolated from freshwater prawns (*Macrobranchium rossenbergii*) and black tiger shrimps (*Penaeus monodon*) as follows:

P0: Control (without probiotics)

P1: Provision of probiotic *Bacillus* sp isolate G4 (the best isolate originated from freshwater prawn)

P2: Provision of probiotic *Bacillus* sp, a joint isolate of freshwater prawn (G1+G2+G3+ G4+G5)

P3: Probiotics of *Bacillus* sp isolate W9 (the best isolate originated from black tiger shrimps)

P4: Provision of probiotic *Bacillus* sp is a joint isolate of black tiger shrimps (W1+W2+W8+W9+W10)

Probiotic Bacteria isolate

The given probiotics from freshwater prawns and black tiger shrimps with a density of 10^5 CFU/ml was sprayed according to dose on commercial F-999 feed given *at satiation* three times a day during the study. On the 32nd day, the treatment was infected with *S. iniae* with a density of 10^5 CFU/ml by intramuscular injection with a dose 0,1 ml/fish. Fish blood was taken after treatment (day 30th) and after the infection (day 44th). The measured parameters included total leukocytes, leukocyte differentiation (lymphocytes, monocytes and neutrophils) and survival rates.

Samples of *S. iniae* bacteria used were collected from Fish Pest Inspection Laboratory of Station I of Fish Class Quarantine of Sultan Syarif Kasim II, Pekanbaru. 150 Nile tilapia samples were obtained from Fish Corner Hall (BBI) Sipungguk Kampar District (each aquarium contains 10 fish).

Probiotic isolates used from freshwater prawn and black tiger shrimps have been sequenced 16S rDNA, then recycled using Nutrient Agar (NA) and Nutrient Broth (NB) media, a collection of Marine Microbiology laboratory, Riau University, which have been published by Feliatra *et al* (2016). The used fish feed was F-999 commercial pellets.

The maintenance container used was a 40x30x30 cm aquarium with 15 fish filled with water as high as 25 cm (30 L) and aerated. Nile tilapia with varying sizes (8-12 cm) were put into the aquarium with a density of 10 fish/container and acclimatized for 3 days.

Probiotics derived from freshwater prawn and black tiger shrimps were re-grown on NA agar medium, and incubated at 27-28°C for 24 hours. Then, they were harvested by taking probiotics that grow on NA agar as 1 ose, which were then put into medium NB (Nutrient Broth) with volume 50 ml (as starter bacteria) before being incubated for 24 hours.

The starter bacteria that have grown in NB media were then mixed into 250 ml NB medium, so the volume of probiotics amounted to 300 ml and was incubated again. The density of probiotic bacteria (up to 10^5 CFU/ml) was measured using a spectrophotometer. For treatment of P1 and P3, 15 ml probiotics that have been crushed with a density of 10^5 CFU/ml were used. Then, about 250 ml aquades was added together with 2,25 ml of NaCl. Once homogenized, the probiotic solution was sprayed into 1 kg of commercial feed, then air dried.

To prepare the combined probiotics of the gastrointestinal tract from freshwater prawn (G1+G2+G3+G4+G5) and the black tiger shrimps (W1+W2+W8+W9+W10), each isolate was taken with the same density and amount (3ml) and then made with the same procedure as above (Setiawati *et al,* 2013).

S. iniae cultures have been tested at Fish Quarantine Station and Quality Control and Fishery Product Safety (SKIPM) Class I of Sultan Syarif Kasim Airport, Pekanbaru. The *S. iniae* bacteria were used for the challenge test with a density of 10^5 CFU/ml. The fish were treated with infectious doses of 0,1 ml/fish on day 32. Furthermore, the fish were kept for 12 days, during which the fish were still fed in the treatment.

Blood collection was done by using syringe with a size of 1 ml and dampened with 3.8% Na Citrate in caudal section to prevent blood clotting. Then, the blood in the syringe was inserted into the microtube. The measured parameters include: total leukocytes, differentiation of leukocytes (lymphocytes, monocytes, neutrophils) according to Blaxhall and Daischel (1992), and level of survival rate using that of Effendie (2002).

The data obtained from the results of this study include total leukocytes, leukocyte differentiation, and survival rate. All statistical comparisons used one-way analysis of Variance (ANOVA) and Duncan test. The differences were considered to be statistically significant at $p<0.05$

RESULTS

Total leukocytes of Nile tilapia (*Oreochromis niloticus*)

The examination of the amount of leukocyte aimed to see the health level of fish. The given probiotics have a close relationship with changes in the average amount of leukocytes. An increase in the number of leukocytes can be used as a sign of infection, stress or leukemia. The total range of leukocytes of Nile tilapia during pre-infected studies ranged from 58.83 to 74.67×10^3 cells/mm^3 (figure 1). The largest amount of leukocytes were found in P3 treatment, while the smallest was obtained in P1.

After the challenge test with *S. iniae* bacteria, the total figure of leukocytes increased in all treatments, 75-95 cells/mm^3. The largest figure was found in the control treatment (P1), while the smallest was obtained in P4. This suggests that giving probiotics has an effect on the total amount of leukocytes.

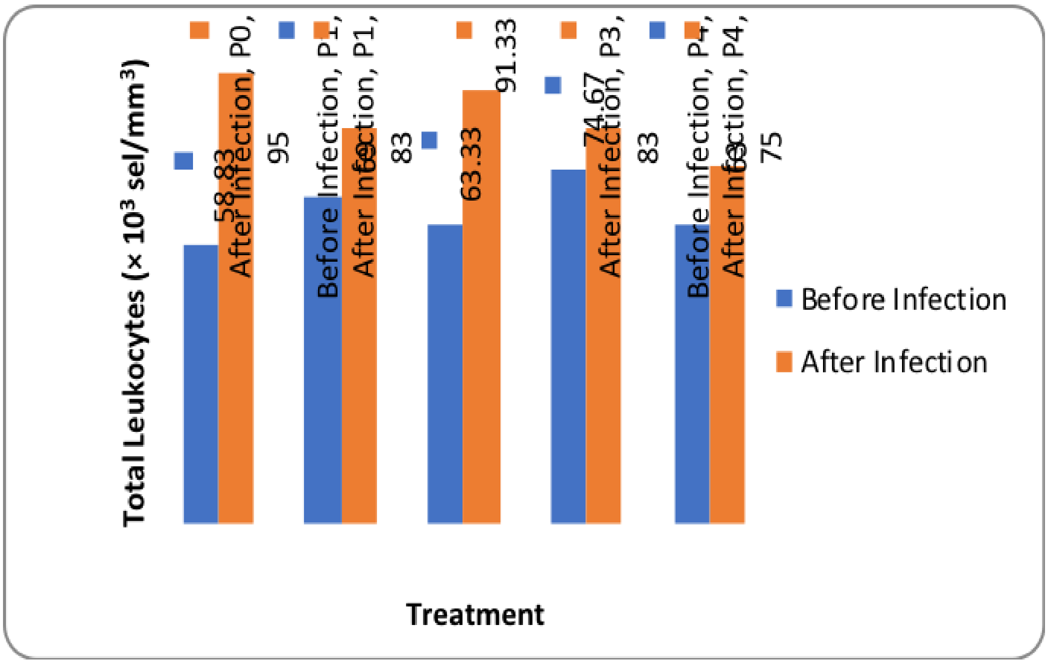

Figure 1: The amount of leukocytes of Nile tilapia (*Oreochromis niloticus*) before and after being infected by *S. iniae*

P0: Control (without probiotics)

P1: Provision of probiotic *Bacillus* sp isolate G4 (the best isolate originated from freshwater prawn)

P2: Provision of probiotic *Bacillus* sp, a joint isolate of freshwater prawn (G1+G2+G3+G4+G5)

P3: Probiotics of *Bacillus* sp isolate W9 (the best isolate originated from black tiger shrimps)

P4: Provision of probiotic *Bacillus* sp is a joint isolate of black tiger shrimps (W1+W2+W8+W9+W10)

Leukocytes Differentiation

The examination and identification of the blood test of the fish showed that fish blood cells consist of both erythrocytes and leukocytes. The leukocytes on its own consist of lymphocytes, monocytes, and neutrophils/heterophils. The observation of leukocyte differentiation was performed to see changes in the types of leukocytes that occurred after the addition of probiotics and being infected with *S. iniae*.

Lymphocytes of Nile tilapia (*Oreochromis niloticus*)

After observation, the lymphocytes were found to exhibit a higher proportion, ranging between 85,33 - 90,33%, while the percentage of post infection with *S. iniae* of all treatments decreased (about 79,67 – 89,67%) (see figure 2). The figure for lymphocytes before infection was bigger than that of after infection for all treatments.

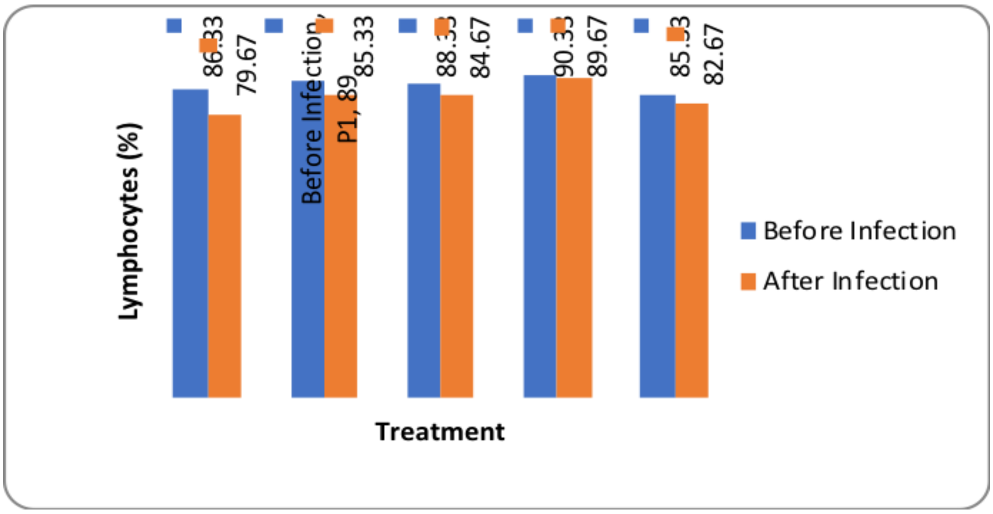

Figure 2: The percentage of lymphocytes of Nile tilapia (*Oreochromis niloticus*) before and after being infected by *S. iniae*

Monocytes of Nile tilapia (*Oreochromis niloticus*)

The results showed that the amount of monocytes of controlled fish and those treated with *Bacillus* sp probiotics ranged from 4,67 to 7,33%, while its post-infection figure increased from 8,67 to 10,33% (figure 3). While the largest amount of monocytes was obtained in P3, the smallest figure was observed in P2. Before treatment, P3 had the smallest figure of monocytes. This suggests that giving probiotics has an effect on the amount of monocytes.

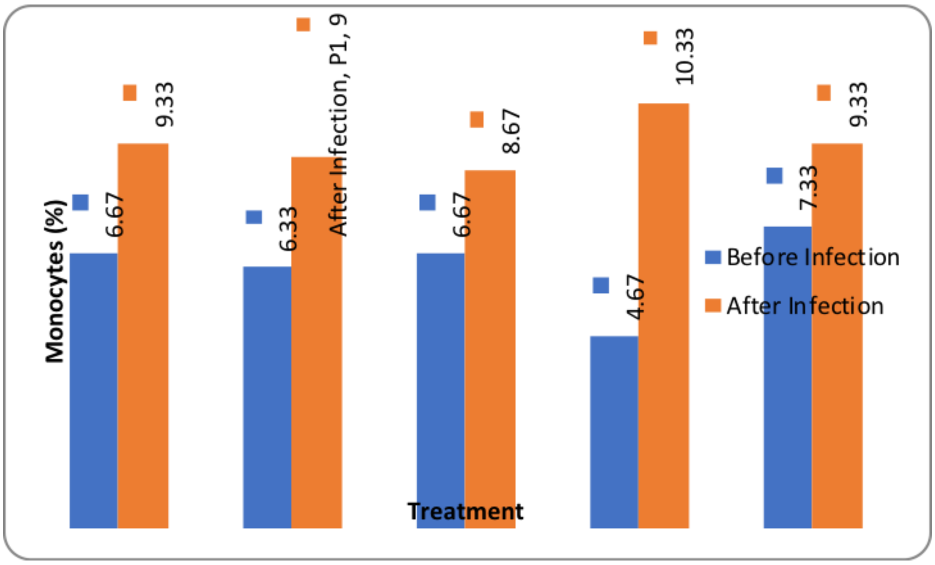

Figure 3: The percentage of monocytes in Nile tilapia (*Oreochromis niloticus*) before and after being infected by *S. iniae*

Neutrophils of Nile tilapia (*Oreochromis niloticus*)

Neutrophil cells are leukocytes capable of leaving blood vessels. After investigation, it was found that the amount of neutrophil of controlled fish after being fed with *Bacillus* sp probiotics ranged from 4,65 to 7,33% (figure 4). The fluctuation in the number of neutrophil and monocyte cells in the blood is closely related to their role as a fish defense line. Neutrophil cells have limited energy reserves, although they have rapid, but not lasting, activity as seen in post-infected fish. The percentage of neutrophils then increased between 5,98 - 10%. The largest figure of neutrophils was obtained in P3, while the smallest was observed in control (P0). This suggests that giving probiotics has an effect on the percentage of neutrophils.

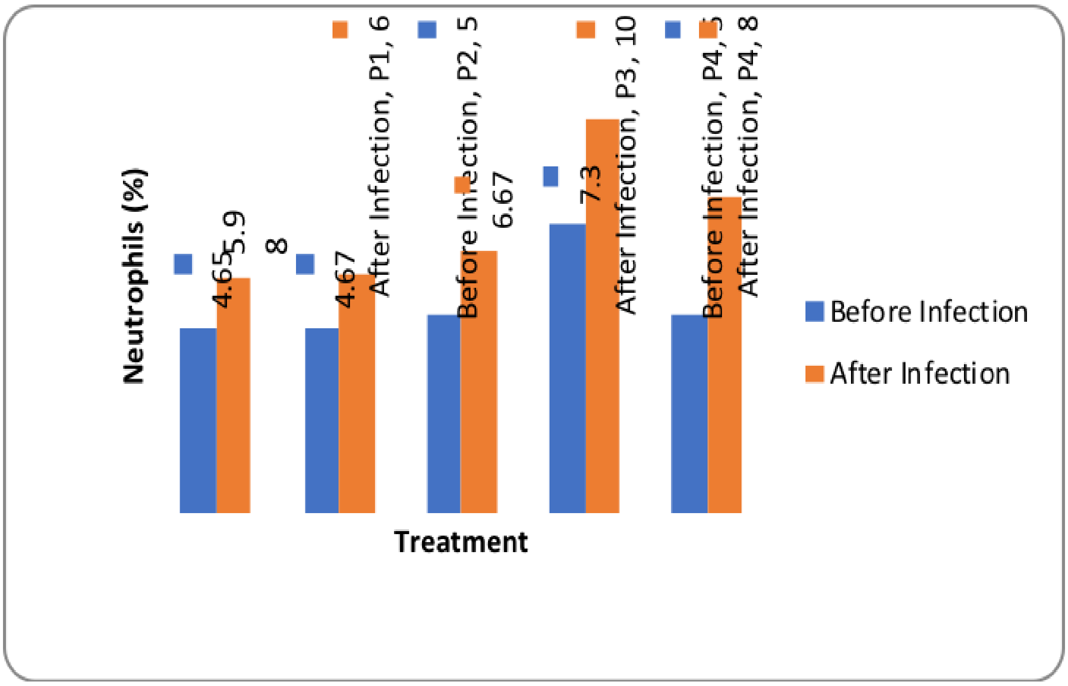

Figure 4: The percentage of Neutrophils of Nile tilapia (*Oreochromis niloticus*) before and after being infected by *S. iniae*

Survival rate of Nile tilapia (*Oreochromis niloticus*)

The survival rate (SR) is the percentage of animals living at the end of the study in comparison with those tested at the beginning of the study. The average SR of Nile tilapia fed with *Bacillus sp* probiotics for 30 days and infected with *S. iniae* bacteria whose survival rate was higher than that of control (P0) was 36,67%, while the SR of Nile tilapia treated with probiotics ranged from 63,33 - 80% (figure 5).

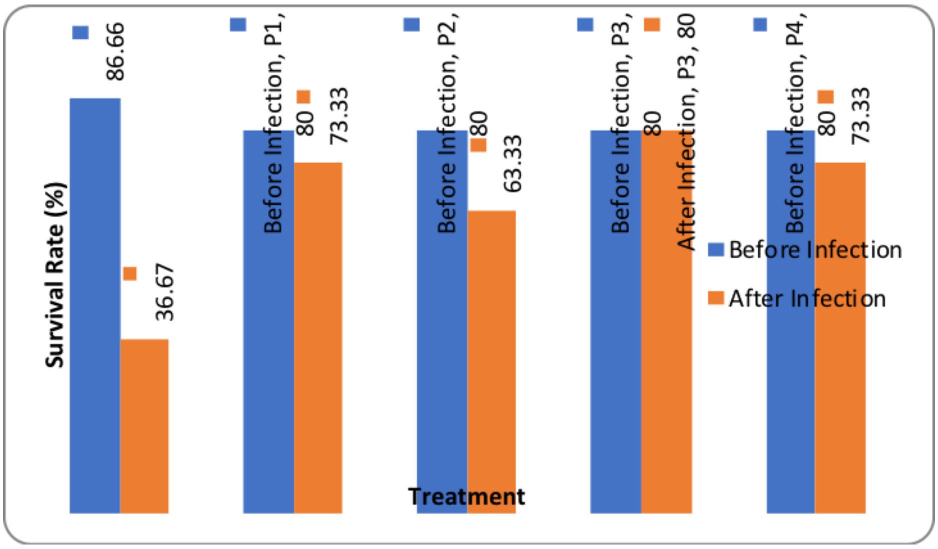

Figure 5: The average survival rate of Nile tilapia (*Oreochromis niloticus*) before and after being infected by *S. iniae*

DISCUSSION

The total amount of leukocytes of Nile tilapia during pre-infected stage ranged from 58.83 to 74.67x10^3 cells/mm^3. The presence of infections such as inflammation was a characteristic of non-specific immune responsiveness. Similar responses would also be likely to occur due to factors such as trauma, chemicals, toxins, parasites, bacteria and viruses (Anderson and Siwicki, 1995). This figure is considered normal as the range of leucocytes for healthy Nile tilapia is between 20,00 to 150,00 x 10^3 cells/mm^3 (Roslina, 2015).

The increased figure of leukocytes indicates a protective response to the presence of foreign entity, in this case, the *S. iniae* bacteria entering the fish body. The infection of *S. iniae* causes the fish to send more leukocyte cells to the infection area as a defensive act, increasing the amount of leukocyte cells associated with the performance of fish immune system in reducing pathogen attack. The higher the number of pathogen attacks, the higher the production of leukocytes in the blood (Martin *et al*, 2004). Giving probotics of *L acidophilus* and *B subtilis* on Nile tilapia can improve non-specific defense. The amount of Nile tilapia’s leukocytes before being infected ranged from 58.83 to 74.67 x 10^3 cells/mm^3, and it grew after infection of *Vibrio harveyi* bacteria. Probiotics can suppress the growth of Gram-positive and Gram-negative bacteria because they can damage the bonds of ß-1, 4 glycosidic, N-acetyl muramic acid and N-acetyl glucosamine in the bacterial cell wall of peptidoglycan layer (Akter *et al*, 2016).

The results of ANOVA test showed that probiotic feeding had a significant effect on the total amount of post-infection leukocytes ($p < 0.05$). Further tests showed that the results generated from P1, P3 and P4 were significantly different from that of control (P0). It was further found that the provision of probiotics isolated from freshwater prawn and black tiger shrimps could improve the fish's immune system, which is related to the presence of bacteriocin contained in probiotics *Bacillus* sp and to the fact that antibacterial compounds are able to inhibit the growth of pathogen bacteria. The previous test results of invitro demonstrated that the probiotic *Bacillus* sp isolated from freshwater prawn and black tiger shrimps are sensitive to pathogenic bacteria *Aeromonas hydrophila, Vibrio alginolyticus* and *Pseudomonas stutzeri* (Feliatra *et al*, 2018).

Lymphocytes are the most dominant type of leukocyte cells found in leukocyte populations. The main function of this entity is to form memory cells against antigens, thus generating antibodies (Anderson, 1974). The amount of fish lymphocytes range from 71,12% to 82,88% of the total leukocytes (Blaxhal, 1972), whereas the percentage of lymphocytes in Nile tilapia is normal, ranging from 68% to 86% (Hardi, 2011).

Feed containing probiotic *Bacillus* sp had a significant effect on percentage of lymphocyte cell as shown in the significant difference of results between P0 treatment and P1, P2, P3 P4 ($p < 0.05$). The percentage of lymphocyte cells was influenced by the presence of foreign antigens, so the immune substances were disturbed by the entry of infections that caused the number of lymphocytes to decrease. Lymphocyte deficiency can decrease the concentration of antibodies and lead to increasing potential of disease infection.

Lymphocytes include white blood cell components responsible for the body's defense, especially the formation of antibodies after stimulated antige. In addition to cellular defense, humoral defenses also occur in the form of antibody. The presence of pathogen in the fish body will be responded by B cells assisted by helper T cells to stimulate antibody formation. Moreover, the defense system to destroy pathogens also activates the memory system so that a return attack by the same pathogens will be responded faster (Lukistyowati, 2011).

The amount of monocyte cells is less when compared with that of lymphocytes. Monocytes are able to penetrate capillary blood vessels and enter the tissues and transform into macrophages (Abbas *et al.,* 2005). The amount of monocytes in the leukocyte population is about 1% of the total leukocyte, and this will increase when there is a foreign entity entering the tissue or bloodstream (Nabib and Pasaribu, 1989).

The results showed that the amount of controlled fish monocytes and those treated with *Bacillus* sp probiotics ranged from 4,67 to 7,33%, while the figure in the post-infection stage increased from 8,67 to 10,33%. The results of ANOVA test showed that the feed containing *Bacillus sp* probiotics had minimum effect on the percentage of monocyte cells in Nile tilapia during maintenance and post-infection with *S. iniae* ($p>0.05$).

The difference in the amout of monocytes in the blood of the treated fish is due to the different ability of each type of probiotic in enhancing the non-specific immunity. The increased post-operative monocyte cells in all treatments were thought to be due to monocyte cells performing phagocytic activity when the fish were infected with *S. iniae*. Monocytes in fish function like macrophages and are sometimes referred as blood macrophages that play an important role in phagocytes of pathogenic microorganisms. Fish macrophages can also produce nitric oxide (NO), which in mammals can become potent bactericidal agents such as peroxinitrite and OH^+ (Secombes, 1996).

The amount of monocytes will increase rapidly (±48 hours) after infection (Nitimulyo, 2004). Inflammatory processes during tissue damage by infection and antigen-antibody reactions will lead to double monocytes production. The monocytes in the blood become shorter; monocyte maturation becomes faster, and shortly results in damaged tissue (Maftuch, 2007). Increased monocyte cells are used as an indicator of increased immune system response, in which monocytes are the major phagocyte cells in fish, circulating in the blood, migrating into the tissues and becoming macrophages (Abbas *et al.,* 2010).

Neutrophil cells are leukocytes capable of leaving blood vessels. In the blood of fish, they amount up to 6 - 8% of the total leukocyte, containing enzymes useful for destroying the organism they eat (Roberts, 2001). The results showed the amount of neutrophil of controlled fish after being fed with *Bacillus* sp probiotics ranged from 4,65 to 7,33%. The results of ANOVA test showed that the provision of probiotic *Bacillus sp* managed to make a difference in the percentage of neutrophil cells in Nile tilapia during the maintenance between treatment P0 and those of P3 and P4 ($p<0.05$). Fluctuations in the figure of neutrophil and monocyte cells in the blood are closely related to their role as a fish defense line. Neutrophil cells have limited energy reserves, although they have a rapid, but not lasting, activity as seen in post-infected fish. The percentage of neutrophils then increased between 5,98-10%.

The results of ANOVA test showed that the feed containing probiotic *Bacillus sp* was able to make a difference in the percentages of neutrophil cells in Nile tilapia between P0 and P3 and P4 ($p <0.05$) in the post-infection stage. The increasing amount of neutrophil cells in the treatment demonstrated an immune response to the presence of an antigen or a foreign protein.

The survival rate (SR) is the number of animals living at the end of the study compared with that tested at the beginning of the study measured in one study unit. The average SR of Nile tilapia fed with *Bacillus sp* probiotics for 30 days and infected with *S. iniae* bacteria whose survival rate was higher than that of control (P0) was 36,67%, while the SR of Nile tilapia treated with probiotics ranged from 63,33 - 80%.

The result of ANOVA test showed that the feed containing probiotic *Bacillus sp* was able to make a difference in the survival rate of Nile tilapia in the post-infection stage between treatment P0 and P1, P2, P3, P4 ($P <0.05$). This indicates that the probiotic *Bacillus sp* can increase the resistance of Tilapia fish to *S. iniae* infection.

Bacillus sp probiotics can suppress the pathogenity of *S. iniae* by more than 50%. One method for knowing its effectiveness is by looking at the number of virulent organisms that can kill 50% of test animals (Ellis, 1988). The probiotic ability of *Bacillus* sp in inhibiting *S. iniae* infection is due to the fact that it produces substances that can kill or inhibit the development of *S. iniae,* one of which is bacteriocin, where the highest content is found in the W9 isolate used in this study as P4 treatment

(Utamy, 2015). Lactic acid bacteria (probiotics) can produce organic acids peroksida and bacteriocin that are antibacterial (Daeschel, 1992). How probiotics truly work is not yet fully known, but in principle it can be differentiated into three ways: suppressing microbial populations through competition of antimicrobial compounds, altering microbial metabolism by increasing or decreasing enzyme activity, and stimulating immunity through increased levels of antibodies or macrophage activity (Austin *et al*., 2000). The benefits of probiotics have been studied in fish and other aquatic organisms, including improvements in intestinal balance and feed use, aids digestion, inhibits pathogenic microorganisms and promotes immune responses (Kesarkodi-Watson *et al.*, 2008; Nayak, 2010). The large number of BALs that are already functioning as probiotics can be beneficial to host health when administered in sufficient quantities (Buntin *et al.*, 2008).

CONCLUSION

Probiotics from freshwater prawn and black tiger shrimps improve the fish health and affect the leukocyte differentiation in Nile tilapia after *S. iniae* infection. The best treatment is in P3 (W9 Isolate) from black tiger shrimps with total leukocyte of 83,00 x 10^3 sel/mm^3, lymphocyte 89,67%, monocytes 10,33%, 10% neutrophils, and survival rate 80,00%. The addition of probiotics isolated from freshwater prawn (*M. rosenbergii* De Man) and black tiger shrimps (*P. monodon*) in feed given for 30 days can suppress the pathogenicity of *S. iniae,* increase the immune response in nile tilapia (*O. niloticus*) and may reduce the mortality rate of fish because of *S. iniae* infection.

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