International Journal of Sciences: Basic and Applied Research (IJSBAR)

ISSN 2307-4531
(Print & Online)

http://gssrr.org/index.php?journal=JournalOfBasicAndApplied

Administration of *Bacillus* NP5 and Oligosaccharide to Enhance the Immune Response in Tilapia *Oreochromis niloticus* towards Streptococcosis

Tanbiyaskur[a], Widanarni[b]*, Angela Mariana Lusiastuti[c]

[a]*Aquaculture Program, Faculty of Agriculture, Sriwijaya University, Palembang, Indonesia*

[b]*Department of Aquaculture, Faculty of Fisheries and Marine Sciences, Bogor Agricultural University, Jalan Agatis, Dramaga Campus, Bogor 16680, West Java, Indonesia*

[c]*Freshwater Aquaculture Research Station, Ministry of Marine Affairs and Fisheries, Bogor, Indonesia*

[b]*Email: widanarni@yahoo.com*

Abstract

Streptococcus agalactiae is a pathogen that causes streptococcosis which is becoming one of the major problems in tilapia culture. Application of probiotics, prebiotics, and synbiotics are expected to be an alternative in solving this problem by improving the immune response in tilapia. The aim of this study was to assess the effectiveness of *Bacillus* NP5 probiotic, oligosaccharide prebiotic and a combination of the two to stimulate the immune system for controlling *S. agalactiae* infection. The study consisted of five treatments with four replications, which were positive control P0 (+); negative control P0 (-); P1 (*Bacillus* sp NP5, probiotic 1%); P2 (oligosaccharide prebiotic 2%), and P3 (synbiotic: *Bacillus* sp NP5 probiotic 1% and oligosaccharide prebiotic 2%). The 15-20 g tilapia BEST strain was reared at a density of 10 fish per aquarium (60cm x 30cm x 40 cm). The administration of probiotic, prebiotic and synbiotic was applied for 14 days. On day 15, the fish were challenged by injecting of 0.1 ml *S. agalactiae* per fish at a concentration of 10^5 CFU ml^{-1}, and then the fish were reared for 14 days and fed the control feed. The administration of probiotic, prebiotic, and synbiotic enhanced the immune responses and resistance to *S. agalactiae* bacterial infection in tilapia.

---

* Corresponding author.

E-mail address: widanarni@yahoo.com.

The results showed that the fish were given synbiotic treatment had the highest survival rate (83.34%) and better immune responses which was showed by a better erythrocytes, hemoglobin, hematocrit, total leukocyte, and phagocytic activity, than the positive control.

Keywords: *Bacillus* NP5; oligosaccharide; *Oreochromis niloticus*; streptococcosis

1. Introduction

Tilapia (*Oreochromis niloticus*) is a freshwater fish which has great potential to be developed. This species has a fast growth rate, rapid reproduction, has plenty of flesh, and easy to be cultivated [1]. Those characteristics have made the demand for tilapia increases. Consequently, the intensification of aquaculture can be avoided. However, the intensification of aquaculture without cares to the environmental condition may cause negative effects such as diseases. One of the diseases that are a main problem in tilapia cultivation is streptococcosis which is caused by *Streptococcus agalactiae. S. agalactiae* can cause high mortality and a high economic loss in tilapia culture. Based on the study by [2], *S. agalactiae* could cause up to 90% mortality in tilapia 6 days post-injection. This infection is septicaemic and the bacteria spread to several organs, such as the brain, eyes, and kidney [3].

The control of *S. agalactiae* in tilapia could be done through the probiotics application. Probiotics do not accumulate in the fish body and do not cause resistance of pathogenic organisms likewise the use of antibiotics [4]. According to [5], probiotic can stimulate the immune system and suppress pathogenic bacteria in culture of Nile tilapia. This is also supported by [6] who stated that the application of *Enteroccus faecium* as a probiotic could increase growth and improve immune response in tilapia. However, the application of probiotics has several weaknesses such as the competition for nutrients, viability, and colonization in the digestive tract which naturally contains hundreds of other bacteria. If the probiotic bacteria do not get the adequate amounts of nutrients for its survival, and exacerbated by the extreme changes in intestinal environment, the probiotic bacteria will quickly wash out [7]. Another approach which could be attempted to overcome these weaknesses is by supplying the nutrients needed by the probiotic to survive in the digestive tract. The nutrients or materials needed by probiotics are known as prebiotics [8]. A synbiotic is a combination of a probiotic and a prebiotic in a balanced composition for supporting the survival and growth of beneficial bacteria in a living organism's digestive tract [7]. The supply of nutrients or specific substrates needed by the probiotic bacteria is expected to improve the survival rate of probiotic. The increasing of the probiotic survival and activity are believed to improve the function role of the probiotic in the digestive tract which will stimulate the fish immune system. The aim of this study was to assess the effectiveness of the administration of *Bacillus* NP5, oligosaccharide, and the combination of them to improve the immune response of tilapia towards streptococcosis.

2. Materials and Methods

2.1 Experimental Design

This study used the completely randomized design consisting of five treatments with four replications, including the feeding without the administration of the probiotic, prebiotic, or synbiotic and challenged by *S. agalactiae*

(P0(+)); the feeding without the administration of probiotic, prebiotic, or synbiotic and not challenged by *S. agalactiae* (P0(-)); the feeding with the administration of probiotic 1% (1g $100g^{-1}$ feed: [9]) and challenged by *S. agalactiae* (P1); the administration of 2% (2g $100g^{-1}$ feed: [10]) prebiotic and challenged by *S. agalactiae* (P2); the feeding with the administration of synbiotic (1% probiotic + 2% prebiotic) and challenged by *S. agalactiae* (P3).

2.2 The Preparation of Probiotic, Prebiotic, and Synbiotic

The probiotic used in this study was *Bacillus* NP5 which was isolated from the digestive tract of tilapia and had been tested *in vitro* for its antagonistic activity towards *S. agalactiae* [9] using the Kirby-Bauer method [11]. The prebiotic used was oligosaccharide extracted from sweet potato var. sukuh using ethanol 70% [12]. The Total Dissolved Solids (TDS) of the prebiotic was measured using the method developed by [13]. The synbiotic was made by mixing the probiotic and prebiotic according to the respective treatments.

2.3 The Experimental Fish, The Feeding Trial and The Challenge Test

The 15-20 g tilapia BEST strain was reared at a density of 10 fish per aquarium (60cm x 30cm x 40 cm). The *in vivo* assay was conducted by mixing the probiotic, prebiotic, and synbiotic with 2% egg yolk as a binder then sprayed to the feed [9].

The probiotic which was administered in P1 and P3 was 10^6 CFU ml^{-1}, whereas the prebiotic doses in P2 and P3 was 2% with 5% TDS [14]. The fish were fed commercial feed with a protein content of 38% three times a day by *at satiation*. The administration of probiotic, prebiotic and synbiotic was applied for 14 days. On day 15, the fish were challenged by injecting of 0.1 ml *S. agalactiae* per fish at a concentration of 10^5 CFU ml^{-1} which is the LD_{50} [15], then the fish were reared for 14 days and fed the control feed. In order to maintain water quality in the rearing tanks, 10% of the total water volume in the tanks was siphoned daily.

2.4 Enumeration of Total Viable Bacteria Count

Total viable bacteria count was enumerated on day 14 after probiotic, prebiotic, or synbiotic treatments by the spread plate technique using Trypticase Soy Agar (TSA) medium, and then incubating at 28-29ºC for 24-48 hours. The number of colonies were counted and multiplied by the dilution factor [11].

2.5 Measurement of The Fish Immune Responses

The fish immune responses were observed on day 0, 7, and 14 after probiotic, prebiotic, or synbiotic treatment and also on day 7 and day 14 post-challenge test. The immune responses observed were erythrocyte count (EC) [16], hemoglobin level (Hb) [17], hematocrit (Ht) [18], leukocyte count (LC) [16], and phagocytic index (PI) [18].

3. Results

Observation results showed that the survival rates of tilapia after treatment with probiotic, prebiotic, or synbiotic were not significantly different among the treatments which ranged about 95%-100%. Otherwise, after the challenge test with *S. agalactiae*, the survival rates of P1 (80.56%), P2 (72.23%), and P3 (83.34%) were significantly higher than P0(+) (13.89%) (Figure 1). In addition, the results of TVBC in tilapia intestines showed that TVBC in the controls (P0(+) and P0(-)) were lower than P1, P2 and P3, while between P1, P2 and P3, the numbers were not significantly different (Figure 2).

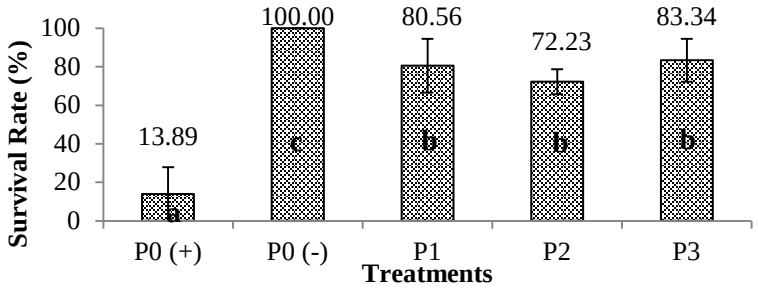

Figure 1: The survival rate of tilapia after the challenge test with *S. agalactiae*; P0(+), positive control; P0(-), negative control; P1, probiotic; P2, prebiotic; P3, synbiotic. Different superscript letters show results which are significantly different (P<0.05).

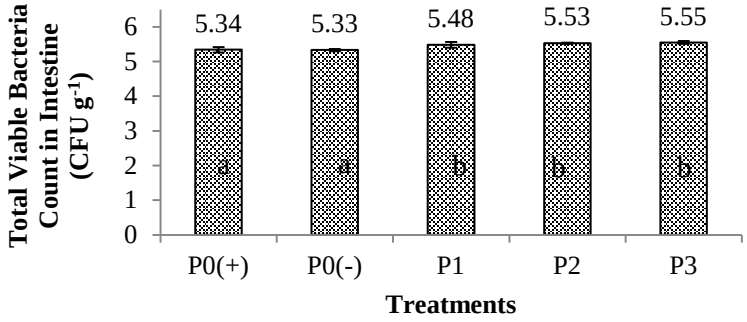

Figure 2: The total viable bacteria count in the intestine of tilapia on day 14 post-treatment with probiotic, prebiotic, and synbiotic; P0(+), positive control; P0(-), negative control; P1, probiotic; P2, prebiotic; P3, synbiotic. Different superscript letters show results which are significantly different (P<0.05).

The microscopic blood parameters as indicators of the immune response which were measured in this study were EC, Hb, Ht, LC, and PI. In the 1st and 2nd week post-administration of the probiotic (P1), prebiotic (P2), and synbiotic (P3), EC (Figure 3), Hb (Figure 4), and Ht (Figure 5) of tilapia increased. However, in the 3rd week after the challenge test by *S. agalactiae*, EC, Hb, and Ht decreased and increased again in the 4th week.

The EC increased since week 1 of the treatment with the highest EC shown by P3, was $15.16 \pm 0.29 \times 10^5$ cells mm^{-3} (Figure 3). The EC continued to rise in the 2nd week after the administration of probiotic, prebiotic, and synbiotic which were showed similar pattern as 1st week. The EC in tilapia started to decrease in the 3rd week

after the challenge test, with the lowest count at 7.69±0.30x10^5 cells mm^{-3} in P0(+). Treatment P1, P2, and P3 also showed a decreasing in EC that were significantly different (P<0.05) from P0(+). The EC increased in the end of the challenge test (14 days post-infection).

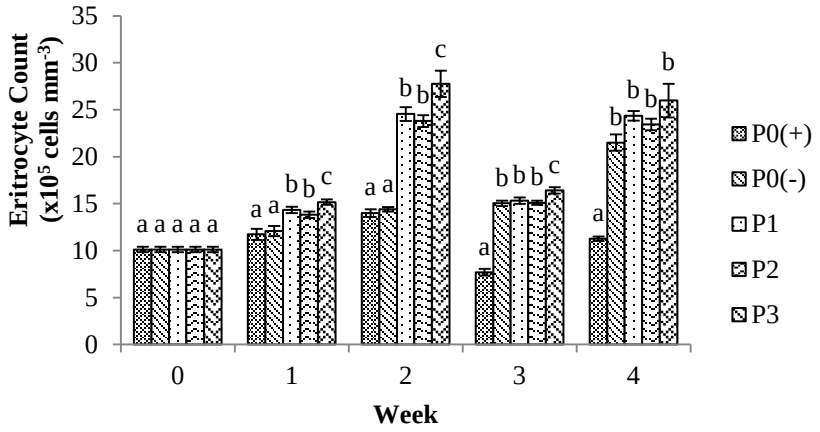

Figure 3: The erythrocyte counts of tilapia during the treatment with probiotic, prebiotic and synbiotic (week 1 and 2) and after the challenge test with *S. agalactiae* (week 3 and 4). P0(+), positive control; P0(-), negative control; P1, probiotic; P2, prebiotic; P3, synbiotic. Different superscript letters between treatments in the same observation period show significantly different results (P<0.05)

The Hb in the blood of tilapia had a positive correlation to EC (Figure 4). The Hb increased during the 1st week and continued to rise during the 2nd week, but declined in the 3rd week (7 days after the challenge test). During the 3rd week, the lowest Hb was found in P0(+) was 4.2 g%, while in the 4th week, the Hb level showed a tendency to rise.

The Ht increased from week 0 to week 2. In the 2nd week, the highest Ht was found in P3 (36.38±1.33%) followed by P1 (33.63±2.5%) and P2 (31.98±1.8%) (Figure 5). In week 3, Ht in each treatments decreased, but the Ht of P1, P2, and P3 higher than P0(+). The lowest Ht in this study was showed in the 4th week, in P0(+) at 10.63±1.38%.

The LC in treatments with probiotic (P1), prebiotic (P2), and synbiotic (P3), showed a slight increase (Figure 6). The LC during the study showed an increase in week 1 and week 2. The highest increase in LC occurred in week 3 or after the challenge test with *S. agalactiae*, and then declined in the 4th week. The LC in P1, P2, and P3 were significantly different (P<0.05) from P0(+) and P0(-). After the challenge test with *S. agalactiae*, LC in the synbiotic treatment (5.73±0.81x10^5 cells mm^{-3}) was higher than positive control (4.75±0.21x10^5 cells mm^{-3}).

In Figure 7, it can be seen that PI of tilapia in the 1st and 2nd week increased. The higher PI were found in P3 in the 2nd and 3rd week (35.15±1.49; 40.86±1.6%). After the challenge test with *S. agalactiae*, PI increased in the 3rd week then decreased again in the 4th week. The highest post-challenge test PI was also found in P3 (40.86±1.60%). The PIs in P1, P2, and P3 tended to be higher than P0 (+) and P0 (-).

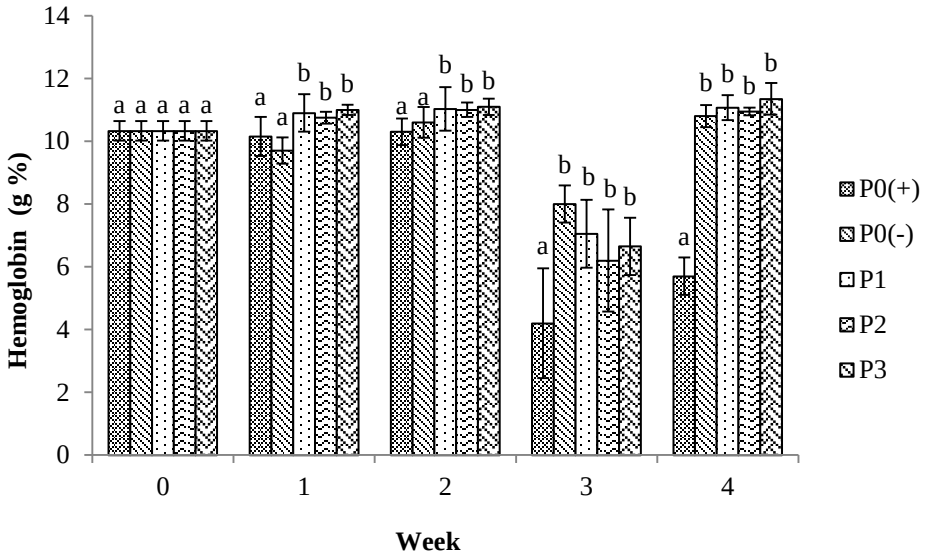

Figure 4: The hemoglobin levels of tilapia during the treatment with probiotic, prebiotic and synbiotic (week 1 and 2) and after the challenge test with *S. agalactiae* (week 3 and 4). P0(+), positive control; P0(-), negative control; P1, probiotic; P2, prebiotic; P3, synbiotic. Different superscript letters between treatments in the same observation period show significantly different results (P<0.05)

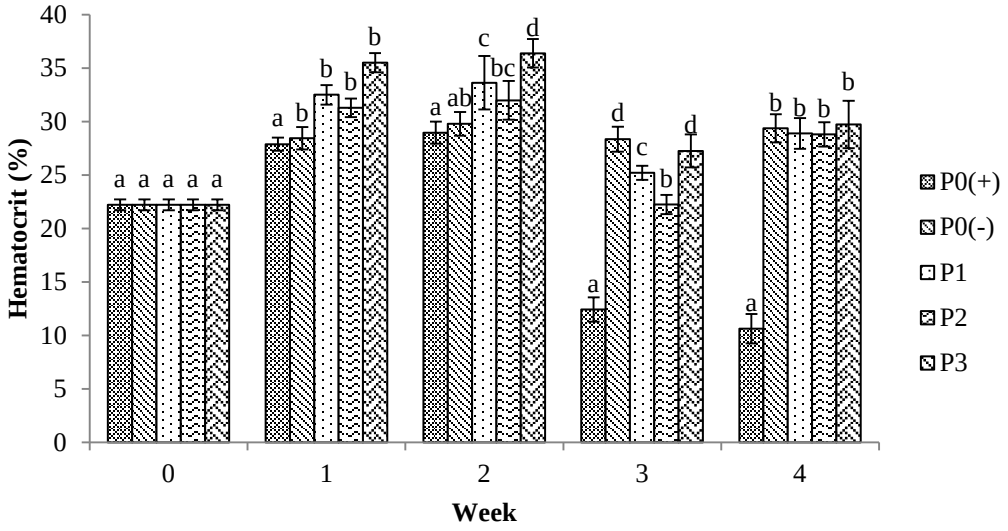

Figure 5: The hematocrit levels of tilapia during the treatment with probiotic, prebiotic and synbiotic (week 1 and 2) and after the challenge test with *S. agalactiae* (week 3 and 4). P0(+), positive control; P0(-), negative control; P1, probiotic; P2, prebiotic; P3, synbiotic. Different superscript letters between treatments in the same observation period show significantly different results (P<0.05)

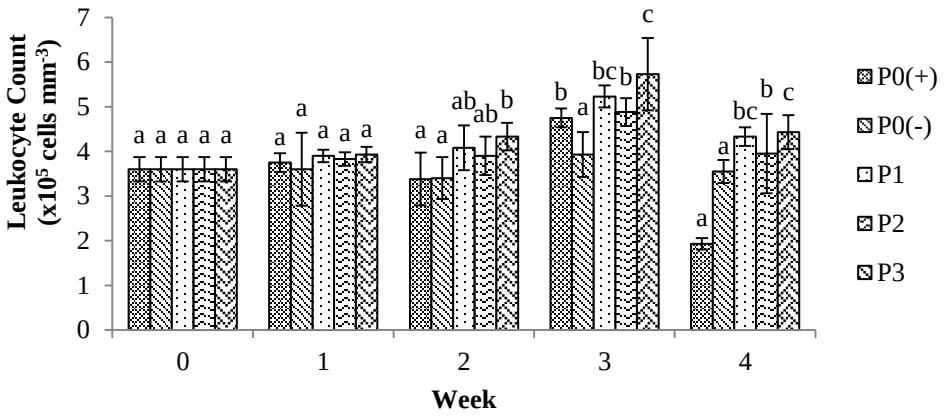

Figure 6: The leukocyte counts of tilapia during the treatment with probiotic, prebiotic and synbiotic (week 1 and 2) and after the challenge test with *S. agalactiae* (week 3 and 4). P0(+), positive control; P0(-), negative control; P1, probiotic; P2, prebiotic; P3, synbiotic. Different superscript letters between treatments in the same observation period show significantly different results (P<0.05)

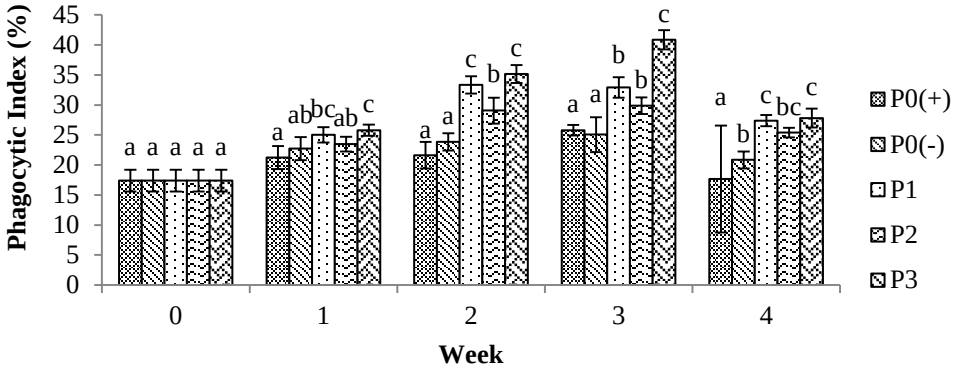

Figure 7: The phagocytic indices of tilapia during the treatment with probiotic, prebiotic and synbiotic (week 1 and 2) and after the challenge test with *S. agalactiae* (week 3 and 4). P0(+), positive control; P0(-), negative control; P1, probiotic; P2, prebiotic; P3, synbiotic. Different superscript letters between treatments in the same observation period show significantly different results (P<0.05)

4. Discussion

This study showed that the administration of probiotic, prebiotic, and synbiotic was able to improve the resistance of tilapia towards *S. agalactiae* infection. These results were in line with the study by [19] who found that the application of a combination between *B. subtilis* and *L. acidophilus* to tilapia could improve the fish ability against pathogens infection. Moreover, [20] stated that the addition of a combination between *B. coagulans* and chitosan oligosaccharide showed the better survival rate than control.

The results indicated that the administration of probiotic, prebiotic, and synbiotic in feed was able to stimulate beneficial bacteria growth, activity, and dominance in the intestine of tilapia. These were in line with the study by [21] who showed that the addition of *Pediococcus acidilactici* could increase the number of beneficial bacteria in the form of lactic acid bacteria in the intestines of tilapia compared to control.

The results of this study also indicated that probiotic bacteria were capable to utilize prebiotic to stimulate their growth in the fish intestines, where prebiotic will be fermented by probiotic to produce short chain fatty acids (SCFA), i.e. lactic acid, acetic acid, propionic acid, and butyric acid [22]. Large amounts of these fermentation products in the non-dissociated form could disturb the internal cellular pH balance of the pathogenic bacteria or non-beneficial bacteria. Therefore, the number of pathogenic bacteria would decrease whereas the number of beneficial bacteria or bacteria which can promote the fish health would increase.

The increasing of beneficial bacteria number in the digestive tract would improve the fish immune response. The mechanism of probiotics to improve the fish immune response is related to the number of cells which play roles in the immune system that are found in the digestive tract, i.e. acidophilic granulocytes (AGs), Ig^+ cells, T cells, macrophages, granulocytes and IgM [23]. The interaction of probiotic bacteria in the digestive tract could increase and activate the immune system cells in the fish body, in which [24] stated that the addition of probiotic could improve the non-specific immune system such as lysozyme activity, neutrophilic migration, and the plasma's bactericidal activity, increase the resistance of tilapia towards *Edwardsiella tarda*.

The normal fish usually have EC of $10\text{-}30x10^5$ cells mm^{-3} [25], in which EC during the administration of probiotic, prebiotic, and synbiotic in this study was still in the normal range. In addition, [26] explained that the application of *Lactobacillus acidophilus* and β-glucan for 8 weeks could significantly increase the number of erythrocytes in snakehead, but after infection with *A. hydrophila*, the erythrocyte count decreased due to erythrocyte lysis.

The drop of EC was believed caused by the disorders in the kidneys of tilapia due to *S. agalactiae* infection. According to [27], one of the toxins secreted by *S. agalactiae* is hyaluronidase. This toxin is an enzyme which could function as a spreading factor, facilitating the spread of other toxins in the host body. Furthermore, [28] stated that the other toxins secreted by *S. agalactiae* are superoxide dismutase and polysaccharide capsules. Superoxide dismutase is a toxin which enables *S. agalactiae* to pass through phagocytes when opsonin does not happen, whereas the polysaccharide capsule is a toxin which can suppress the activity of complements so that the elimination of *S. agalactiae* by macrophages is delayed. These toxins are the cause of the kidneys disorders in the fish which cause EC decrease. The rising of EC in the end of this study indicated that the fish body did homeostatic effort after an infection by pathogenic bacteria.

There is a strong correlation between Hb, red blood cells, and Ht, in which the lower of the number of red blood cells will show the lower of the hemoglobin content in the blood. A similar pattern was demonstrated by [26] who found a significant increasing in the hemoglobin level in treatments with *L.acidophilus* and β-glucan, but after infection with *A. hydrophila*, the hemoglobin level decreased. The low Hb after the challenge test signified that tilapia infected with *S. agalactiae* faced a problem in its erythrocyte.

The rise of Ht in this study after the administration of probiotic, prebiotic, and synbiotic was in line with the results of several other studies, such as the supplementation of 0.2% MOS in snakehead, 1% FOS in juvenile stellate sturgeon, and the combination of *B. subtilis* and *L. acidophilus* in tilapia [19, 26, 29]. In addition, the decline in Ht of P0(+) after the challenge test is suggested caused by the spread of tissue and organ damage due to the extracellular product virulence of *S. agalactiae*. *S. agalactiae* has septicaemia characteristic and can spread through the blood, so it can reach the target organs quickly and develop its virulence. According to [30], the low hematocrit level could be used as an indicator of anemia in the fish. This might be happened because the fish lose their appetite due to stress and the disease.

Leukocytes are a part of the non-specific immune system [31]. These results demonstrated that the administration of the synbiotic could raise LC higher than positive control. According to [30], leukocytes are the main defense against pathogens and could increase quickly during infections. In addition, [32] explained that the increase in leukocyte numbers could happen because of the increased cell division as a response to an infection by the pathogenic bacteria. This statement was supported by [26], who found that after infection with *A. hydrophila,* the total leukocyte in snakehead increased significantly compared to before infection. On the other hand, the decreasing of LC in the end of this study indicated that the infected fish were displaying signs of recovery.

One of the immune response mechanisms developed by the fish body in defending itself from infection is phagocytosis. Effective phagocytosis in early pathogenic invasions could prevent infection. The result of this study were in line with the results of the study by [33] who found that *Lactobacillus rhamnosus* supplementation in feed for 2 weeks could increase phagocytic activity in tilapia. The increase in PI indicated that there was an increasing in the immune response in the form of increasing leukocytic activity against the pathogen. The high PI demonstrated that the fish had an ability to produce phagocytic cells which found in blood in the form of the higher levels of monocytes and neutrophils and when there is a pathogenic microorganism exposure, then the blood cells are ready to perform phagocytosis. This is in line with the results of the study by [20] who stated that the combination between chitosan oligosaccharide (COS) and *B. coagulans* could significantly increase phagocytic activity compared to the control and produce a synergistic effect on the improvement of the immune system of koi and resistance towards *Aeromonas veronii.*

5. Conclusion

The administration of probiotic, prebiotic, and synbiotic could improve the immune response of tilapia against *S. agalactiae.* The best results were demonstrated by the synbiotic treatment with a survival rate of 83.34% and the best immune response with the erythrocyte count, hemoglobin level, hematocrit level, leukocyte count, and phagocytic index of 27.75 x 10^5 cells mm^{-3}, 11.1g %, 36.38%, 5.73 x 10^5 cells mm^{-3}, and 40.86%, respectively. The further study about application of synbiotic in long duration and observes its effects on growth performances of tilapia will be interesting to be studied.

References

[1] Apún-Molina, J.P., A. Santamaría-Miranda, A. Luna-González, S.F. Martínez-Díaz and M. Rojas-Contreras. "Effect of potential probiotic bacteria on growth and survival of tilapia *Oreochromis niloticus* L. cultured in the laboratory under high density and suboptimum temperature". *Aquaculture Research*, 40: 887-894, 2009.

[2] Evans, J.J., D.J. Pasnik, P.H. Klesius and S. Al-Ablani." First report of *Streptococcus agalactiae* and *Lactococcus garvieae* from a wild bottlenose dolphin (*Tursiops truncatus*)". *J. Wildl. Dis.*, 42: 561-569, 2006.

[3] Sheehan, B., Y.S. Lee, F.S. Wong, J. Chan, L. Labrie, C. Komar, N. Wendover and L. Grisez. "AquaVac Strep Sa: A novel vaccine for control of *Streptococcus agalctiae* biotype 2 infections in farmed tilapia", Proceeding Managing *Streptococcus* in Warm Water Fish, Veracruz, Mexico, 2009.

[4] Guo, J., K. Liu, S. Cheng, C. Chang, J. Lay, Y. Hsu, J. Yang and T. Chen. "Selection of probiotic bacteria for use in shrimp larviculture". *Aquaculture Research*, 40: 609-618, 2009.

[5] El-Haroun, E.R., A.M.A.S. Goda and M.A.K. Chowdhury. "Effect of the dietary probiotic biogen® supplementation as a growth promoter on growth performance and feed utilization of nile tilapia *Oreochromis niloticus* (L.)". *Aquaculture Research*, 37: 1473-1480, 2006.

[6] Wang, Y., Z. Tian, J. Yao and W. Li. "Effect of probiotics, *Enteroccus faecium*, on tilapia (*Oreochromis niloticus*) growth performance and immune response". *Aquaculture*, 277: 203-207, 2008.

[7] Lisal, J.S. "[Probiotic and prebiotic concept to modulate colon microbiota]". *Medical Nusantara*, 26: 256-262, 2005 (In Indonesian).

[8] Roberfroid, M.B. "Prebiotics and probiotics: are they functional foods?". *Am. J. Clin. Nutr.*, 71: 1682-1687, 2000.

[9] Putra, A.N. "[Study of probiotic, prebiotic and synbiotic to increase growth performance of tilapia (*Oreochromis niloticus*)]". Master Thesis, Bogor Agricultural University, Indonesia, 2010 (In Indonesian).

[10] Mahious, A.S., F.J. Gatesoupe, M. Hervi, R. Metailler and F. Ollevier. "Effect of dietary inulin and oligosaccharides as prebiotics for weaning turbot, *Psetta maxima* (Linnaeus, C. 1758)". *Aquaculture International*, 14: 219-229, 2006.

[11] Madigan, M.T., J.M. Martinko and J. Parker. *Brock Biology of Microorganisms. Tenth Edition*. New Jersey: Prentice-Hall Inc., 2003, pp: 146-147.

[12] Muchtadi, D. [*Evalution of Food Nutritional Value*]. Bogor: General Directorat of High Education Inter University Center of Bogor Agricultural University, 1989, (In Indonesian).

[13] Apriyantono, A., D. Fardiaz, N.L. Puspitasari, Sedarnawati and Budiyanti. [*Guidelines for Food Assesment Laboratory*]. Bogor: IPB Press, 1989, (In Indonesian).

[14] Marlis, A. "[Isolation of oligosaccharides from sweet potato (*Ipomoea batatas* L.) and the effect of processing on their prebiotic potency]". Master Thesis, Bogor Agricultural University, Indonesia, 2008 (In Indonesian).

[15] Taukhid. *Efectivity of Using of Streptococcus spp. Vaccine in Tilapia (Oreochromis niloticus) Juvenile by Immersion Technique to Prevent Streptococcosis Disease. Research Report of Research Grant for Researchers and Engineers of Marine Affairs and Fisheries*. Bogor: Freshwater Aquaculture Research Station, Ministry of Marine Affairs and Fisheries, 2009, (In Indonesian).

[16] Blaxhall, P.C. and K.W. Daisley. "Routine haematological methods for use with fish blood". *J. Fish Biol.*, 5:771-781, 1973.

[17] Wedemeyer, G.A. and W.T. Yasutake. *Clinical Methods for The Assessement of The Effect Environmental on Fish Health*. California: U.S. Fish and Wildlife Service, 1977, pp: 1-17.

[18] Anderson, D.P. and A.K. Siwicki. "Basic hematology and serology for fish health programs", Proceeding of the Second Symposium on Diseases in Asian Aquaculture "Aquatic Animal Health and the Environment", Phuket, Thailand, 1993.

[19] Aly, S.M., Y.A. Ahmed, A.A. Ghareeb and M.F. Mohamed. "Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of Tilapia nilotica (*Oreochromis niloticus*) to challenge infections". *Fish & Shellfish Immunology*, 25: 128-136, 2008.

[20] Lin, S., S. Mao, Y. Guan, L. Luo and Y. Pan. "Effects of dietary chitosan oligosaccharides and *Bacillus coagulans* on the growth, innate immunity and resistance of koi (C*yprinus carpio koi*)". *Aquaculture*, 342–343: 36–41, 2012.

[21] Standen, B.T., M.D. Rawling, S.J. Davies, M. Castex, A. Foey, G. Gioacchini, O. Carnevali and D.L. Merrifield. "Probiotic *Pediococcus acidilactici* modulates both localised intestinal- and peripheral-immunity in tilapia (*Oreochromis niloticus*)". *Fish & Shellfish Immunology*, 35: 1097-1104, 2013.

[22] Merrifield, D.L., A. Dimitroglou, A. Foey, S.J. Davies, R.T.M. Baker, J. Bøgwald, M. Castex and E. Ringø. "The current status and future focus of probiotic and prebiotic applications for salmonids". *Aquaculture*, 302: 1-18, 2010.

[23] Nayak, S.K. "Probiotics and immunity: a fish perspective". *Fish & Shellfish Immunology*, 29: 2-14, 2010.

[24] Taoka, Y., H. Maeda, J. Jo, S. Kim, S. Park, T. Yoshikawa and T. Sakata. "Use of live and dead probiotic cells in tilapia *Oreochromis niloticus*". *Fisheries Science*, 72: 755-766, 2006.

[25] Takashima, F. and T. Hibiya, 1996. *An Atlas of Fish Histology. Normal and Pathological Features, Second Ed.* Tokyo: Kondansha Ltd., 1996, 195p.

[26] Talpur, A.D., M.B. Munir, A. Mary and R. Hashim. "Dietary probiotics and prebiotics improved food acceptability, growth performance, haematology and immunological parameters and disease resistance against *Aeromonas hydrophila* in snakehead (*Channa striata*) fingerlings". *Aquaculture*, 426–427: 14–20, 2014.

[27] Palacios, G.C., M.N. Gonzalez, M. Beltran, J.L. Arredondo, J. Torres and F. Solorzano. "High-virulence clone of group B streptococci unable to grow at high temperatures is present in serotypes other than type III". *Current Microbiology*, 54: 42-47, 2007.

[28] Segura, M. and M. Gottschalk. "Extracellular virulence factors of streptococci associated with animal diseases". *Front. Biosci.*, 9: 1157-1188, 2004.

[29] Akrami, R., Y. Iri, H.K. Rostami and M.R. Mansour. "Effect of dietary supplementation of fructooligosaccharide (FOS) on growth performance, survival, *Lactobacillus* bacterial population and hemato-immunological parameters of stellate sturgeon (*Acipenser stellatus*) juvenile". *Fish & Shellfish Immunology*, 35: 1235-1239, 2013.

[30] Talpur, A.D. and M. Ikhwanuddin. "*Azadirachta indica* (neem) leaf dietary effects on the immunity response and disease resistance of Asian seabass, *Lates calcarifer* challenged with *Vibrio harveyi*". *Fish & Shellfish Immunology*, 34: 254–264, 2013.

[31] Secombes, C.J. "The Non-specific Immune System: Cellular Defenses", in *The Fish Immune System: Organism, Pathogens and Environment*. Iwama, G., T. Nakanishi (eds.). London: Academic Press, 1996, pp: 63-103.

[32] Martins, M.L., J.L.P Mourino, G.V. Amaral, F.N. Vieira, G. Dotta, A.M.B Jatoba, F.S. Pedrotti, G.T. Jeronimo, C.C. Buglione-Neto and G. Pereira-Jr. "Haematological changes in Nile tilapia experimentally infected with *Enterococcus* sp.". *Braz. J. Biol.*, 68: 657-661, 2008.

[33] Pirarat, N., T. Kobayashi, T. Katagiri, M. Maita and M. Endo. "Protective effects and mechanisms of a probiotic bacterium *Lactobacillus rhamnosus* against experimental *Edwardsiella tarda* infection in tilapia (*Oreochromis niloticus*)". *Vet. Immunol. Immunopathol.*, 113: 339-347, 2006.