http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 18 (2): 59-66, 2015
ISSN 1028-8880

ansinet
Asian Network for Scientific Information

RESEARCH ARTICLE OPEN ACCESS

DOI: 10.3923/pjbs.2015.59.66

Application of Probiotic, Prebiotic and Synbiotic for the Control of Streptococcosis in Tilapia *Oreochromis niloticus*

[1]Widanarni and [2]Tanbiyaskur
[1]Department of Aquaculture, Bogor Agricultural University, Indonesia
[2]Aquaculture Program, Faculty of Agriculture, Sriwijaya University, Palembang, Indonesia

ARTICLE INFO
Article History:
Received: January 08, 2015
Accepted: February 14, 2015

Corresponding Author:
Widanarni,
Department of Aquaculture,
Faculty of Fisheries and Marine Sciences,
Bogor Agricultural University,
Jalan Agatis, Dramaga Campus,
Bogor 16680, West Java, Indonesia

ABSTRACT
One of the fish diseases that is becoming the main problem in tilapia culture is streptococcosis caused by *Streptococcus agalactiae*. Application of probiotic, prebiotic and synbiotic are expected to be an alternative for controlling the disease. The purpose of this study was to examine the effectiveness of the administration of probiotic, prebiotic and synbiotic through artificial feed to control streptococcosis in tilapia. This study consisted of five treatments with three replications, namely positive control, negative control; 1% probiotic treatment; 2% prebiotic treatment and synbiotic treatment (1% probiotic and 2% prebiotic). Results showed that fish survival rate before the challenge test for all treatments was between 95 and 100%. Growth and feed conversion ratios in probiotic, prebiotic and synbiotic treatments were better than that of the controls. After the challenge test, the fish survival rate in probiotic, prebiotic and synbiotic treatments were 74.08, 74.08 and 85.19%, respectively; whereas, in the positive control it was only 18.52%. Results showed that *S. agalactiae* bacteria could be found in the brain, kidney, liver and eyes. The number of *S. agalactiae* bacteria and the damage level of various target organs in probiotic, prebiotic and synbiotic treatments were lower than that of positive control.

Key words: Probiotic, prebiotic, synbiotic, *S. agalactiae*, *Oreochromis niloticus*

INTRODUCTION

Streptococcus agalactiae is a species of pathogenic bacteria which causes one of the major problems in tilapia cultivation, causes a high mortality rate and a huge economic loss. *Streptococcus agalactiae* can cause disease with chronic or acute effects, depending on the degree of the infection. Chronic clinical signs are lesions on the body surface, red spots on the fins, lethargy and appetite loss. The acute signs which are lethal are assumed to be due to the loss of fluids from the distal digestive tract. Before the fish dies, it exhibits lethargy and the tendency to stay at the bottom of the aquarium, shows low interest in feeding, exhibits whirling, curling its body into a "C", changes in body color and the operculum opens more quickly (Evans *et al*., 2006). *Streptococcus agalactiae* usually attacks the brain, eyes and other organs which have high water content (Evans *et al*., 2002).

The conventional control of the disease involves chemicals such as drugs, antimicrobials and disinfectants (Gomez-Gil *et al*., 2000). Uncontrolled use of antibiotics for treating diseases cause imbalances in the natural dynamics of the microorganisms involved in fish cultivation. Therefore, the use of the chemicals above is not recommended. One of the alternatives to control the disease is the application of probiotics, prebiotics and synbiotics (the combination between probiotics and prebiotics). Verschuere *et al*. (2000) defined probiotics as live microbial agents which have beneficial effects on the host by balancing the host's intestinal micro flora. Probiotics also give benefit to the host by strengthening the host's immune system, improving the quality of the host's living environment and increasing the nutritional value of the feed. The success of the application of probiotics has become the foundation of other concepts such as prebiotics and synbiotics (Nayak, 2010). Prebiotics are feed ingredients which cannot be digested by the host but give benefit to the

host by selectively improving the metabolic activity and growth of one or more of the bacteria in the intestines (Roberfroid, 2000; Schrezenmeir and de Vrese, 2001), whereas synbiotics are a combination between probiotics and prebiotics in the effort of supporting the survival and growth of beneficial bacteria in live organism's digestive tract (Schrezenmeir and de Vrese, 2001). Various studies have demonstrated the benefits of applying probiotics and prebiotics in aquatic animal cultivation (Merrifield *et al*., 2010; Nayak, 2010; Ringo *et al*., 2010). In several studies, the application of synbiotics has produced better results compared to the applications of probiotics and prebiotics separately (Li *et al*., 2009; Rodriguez-Estrada *et al*., 2009; Zhang *et al*., 2010). This study was aimed to evaluate the effectiveness of the administration of probiotic, prebiotic and synbiotic through feed in controlling *S. agalactiae* infection in tilapia.

MATERIALS AND METHODS

This study was conducted using the Completely Randomized Design (CRD) which consisted of 5 treatments, i.e., the administration of feed without the addition of any probiotic, prebiotic or synbiotic and then challenged by *S. agalactiae* (positive control); the administration of feed without the addition of any probiotic, prebiotic or synbiotic and not challenged by *S. agalactiae* (negative control); the administration of feed with the addition of 1% probiotic and then challenged by *S. agalactiae* (Pro); the administration of feed with the addition of 2% prebiotic and then challenged by *S. agalactiae* (Pre); the administration of feed with the addition of 1% probiotic+2% prebiotic and then challenged by *S. agalactiae* (Syn).

The probiotic used in this study was *Bacillus* sp. NP5 which had been isolated from the digestive tract of tilapia and had been tested for its antagonistic activity against *S. agalactiae in vitro* (Putra, 2010). Before being used in the challenge test, the Koch postulate was applied to increase the *S. agalactiae*'s virulence. The prebiotic used was oligosaccharides extracted from sweet potatoes var. sukuh using 70% ethanol (Muchtadi, 1989). The prebiotic's Total Dissolved Solids (TDS) were measured using the method developed by Apriyantono *et al*. (1989) to measure the prebiotic's dissolved solids concentration.

Tilapia BEST strain weighing 15-20 g were reared in 15 aquariums (60×30×40 cm^3) at a density of 10 individuals per aquarium. The *in vivo* assay was conducted by mixing the probiotic, prebiotic and synbiotic with egg yolk amounting to 2% of the feed weight and spraying the mixture thoroughly onto the fish feed. One percent of *Bacillus* sp. NP5 (1 g per 100 g feed) (Putra, 2010) at a concentration of 10^6 CFU mL^{-1} was administered in the probiotic treatment. Two percent of the prebiotic (2 g per 100 g feed) (Mahious *et al*., 2006) with a TDS of 5% (Marlis, 2008) was added.

Tilapia were fed commercial feed three times a day by ad satiation. Probiotic, prebiotic and synbiotic were administered to the feed and fed to the fish once a day for 14 days. On the 15th day, tilapia were challenged by injecting them with *S. agalactiae* at a dose of 0.1 mL per individual at a concentration of 10^5 CFU mL^{-1} which is the LD_{50} dose (Taukhid, 2009). After being injected with *S. agalactiae*, the fish were reared for 14 days and fed the control feed. In order to maintain water quality in the aquarium, 10% of the volume of water in the aquarium was siphoned every day.

The parameters observed during the study were survival rate (Effendie, 1979), Daily Growth Rate (DGR) (Huisman, 1987), Feed Conversion Ratio (FCR) (Zonneveld *et al*., 1991), clinical signs, total *S. agalactiae* count on target organs and the histopathology. The tilapia's survival rate was calculated at the end of the probiotic, prebiotic and synbiotic treatment and after the challenge test using *S. agalactiae*. The FCR and DGR were calculated after 14 days of probiotic, prebiotic and synbiotic treatment. Clinical signs, histopathology and the total *S. agalactiae* count on target organs were observed after the challenge test using *S. agalactiae*.

Statistical analysis: The data obtained was analyzed using ANOVA with the SPSS 14 program and then followed by the Duncan test.

RESULTS

The survival rate of tilapia after 14 days of probiotic, prebiotic and synbiotic administration was 100%, not significantly different from the controls (95.0-97.5%). However, after the challenge test with *S. agalactiae*, there was a quite a lot of deaths in the positive control, resulting in the lowest survival rate, i.e., 18.52% (Table 1). The treatment using probiotic, prebiotic and synbiotic resulted in the higher survival rates, i.e., 74.08, 74.08 and 85.19%, respectively.

The daily growth rate in the synbiotic treatment was higher than the other treatments (Table 1). Moreover, FCR in the positive control, negative control, probiotic, prebiotic and synbiotic treatments were 2.28, 2.18, 1.82, 1.78 and 1.77, respectively (Table 1).

Table 1: Tilapia's Survival Rate (SR), Daily Growth Rate (DGR) and Feed Conversion Ratio (FCR) after treatment with probiotic, prebiotic and synbiotic

	Treatments				
Parameters	Control (+)	Control (-)	Pro	Pre	Syn
SR (%)	18.52±12.83^a	100.00±0.00^c	74.08±6.4^b	74.08±6.4^b	85.19±12.8^b
DGR (%)	1.41±0.07^a	1.40±0.13^a	1.99±0.12^b	1.83±0.25^b	2.09±0.20^b
FCR	2.28±0.15^a	2.18±0.22^a	1.82±0.09^b	1.78±0.18^b	1.77±0.14^a

Different superscript letters in the same row signify significantly different results ($p<0.05$)

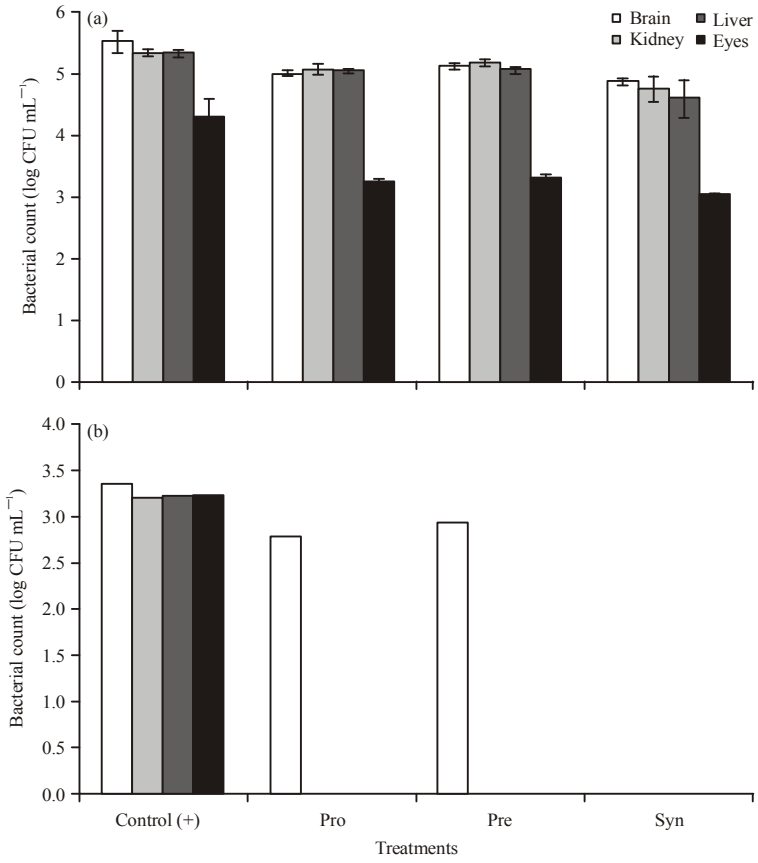

Fig. 1(a-b): Bacterial count of *Streptococcus agalactiae* in target organs in (a) Week 3 and (b) Week 4

Observations of clinical signs were done to note the development of the *S. agalactiae* infection in tilapia. After the challenge test with *S. agalactiae*, there were macroscopic changes in the external organ anatomy such as the external part of the operculum, eyes and body of tilapia. On the first day of the *S. agalactiae* infection, tilapia exhibited changes in color, becoming paler and vertical black stripes started to show on the fish's body and the pupils shrank. The next day, tilapia exhibited clear operculum; the operculum first became yellowish then translucent. The next level of damage was the eyes became cloudy or purulent then swollen and finally they detached from the eye socket. Prior to death, tilapia started whirling and their bodies curled into a "C" shape.

The *Bacillus* sp. NP5 probiotic bacteria's ability to suppress the growth of *S. agalactiae* in tilapia could be seen from the number of *S. agalactiae* in target organs, i.e., the brain, kidney, liver and eyes. The results of this study demonstrate that in the third week (7 days post-challenge test) *S. agalactiae* was found in the brain, kidney, liver and eyes in all treatments (Fig. 1). The results of histopathological examinations of the fish infected by *S. agalactiae* showed that 71.2% of the damage was found in the brain and the rest were found in the kidney, liver and eyes. In the fourth week (14 days post-challenge test), *S. agalactiae* was no longer found in all the target organs in the fish treated with synbiotic while in the fish treated with probiotic or prebiotic separately the bacteria was only found in the brain. However, in the positive control, *S. agalactiae* was still found in all target organs.

The number of *S. agalactiae* in target organs caused some changes that could be observed by histopathology slides. In the brains of infected tilapia there were signs of encephalitis, i.e., congestion, hypertrophy and vacuolization, necrosis and degeneration in the positive controls, whereas, the fish in other treatments only experienced hyperplasia and hypertrophy (Fig. 2). Moreover, the positive control kidney of tilapia which had been infected with *S. agalactiae* underwent pathological changes in the form of hypertrophy, hemorrhage, degeneration, congestion, exhibiting the presence of inflammation cells and necrosis. In treatment with probiotic, prebiotic and synbiotic, there was only hemorrhage and congestion (Fig. 3). In the liver of the positive control (Fig. 4), there were signs of atrophy, fatty degeneration, congestion and hemorrhage. In the fish treated with probiotic and prebiotic there was congestion, hemorrhage and hypertrophy, whereas, in those treated with synbiotic there was only hemorrhage and congestion. The pathological changes were also obtained in the eyes of the positive control in the form of hypertrophy, hyperplasia, vacuolization and necrosis (Fig. 5).

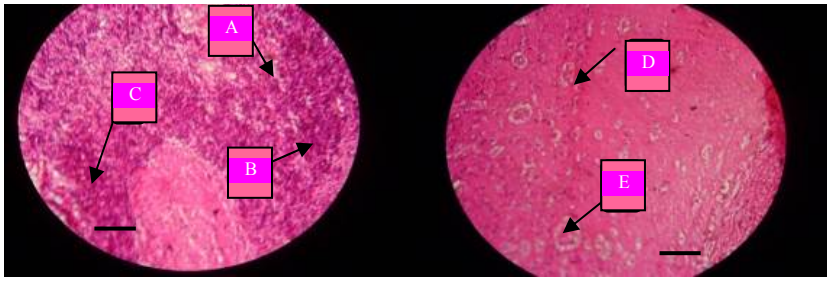

Fig. 2: Histopathology of the brain of tilapia injected with *Streptococcus agalactiae*, A: Hyperplasia, B: Hypertrophy, C: Necrosis, degeneration, D: Congestion, E: Vacuolization (1 bar = 50 μm)

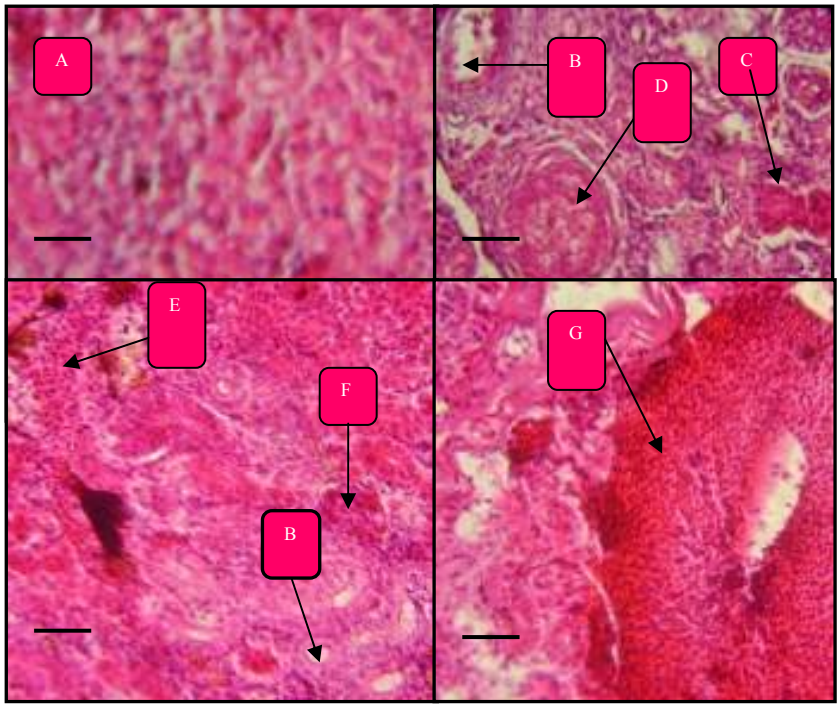

Fig. 3: Histopathology of the kidney of tilapia injected with *Streptococcus agalactiae*, A: Normal kidney, B: Vacuolization, C: Inflammation cells, D: Deposition of hyaline, E: Congestion, F: Degeneration, necrosis, G: Haemorrhage (1 bar = 50) μm

DISCUSSION

The treatment using probiotic, prebiotic and synbiotic resulted the higher survival rates. This was because the probiotic, prebiotic and synbiotic administered were able to improve the fish's immune response, enabling them to suppress the growth of *S. agalactiae*. The highest death rate occurred on the 4th and 5th day post-challenge test in all treatments. This is because the peak of *S. agalactiae*'s virulence factors are assumed to be on those days. According to Evans *et al.* (2004), most of the tilapia deaths post *S. agalactiae* infection happened between day 4-7. The study by Taukhid (2009) also showed that the highest tilapia mortality in the *S. agalactiae* LD_{50} test happened between day 4-5.

Synbiotic treatment showed the better growth performance in this study. The assumption is that it is because the activity and growth of the probiotic bacteria increased with the addition of the prebiotic which was then able to improve feed utilization in tilapia. The study by Putra (2010) showed that the addition of synbiotic to feed resulted in the highest growth rate because of the higher bacterial population and digestive tract enzyme activity compared to the control. In this study, the growth in the individual probiotic, prebiotic and synbiotic

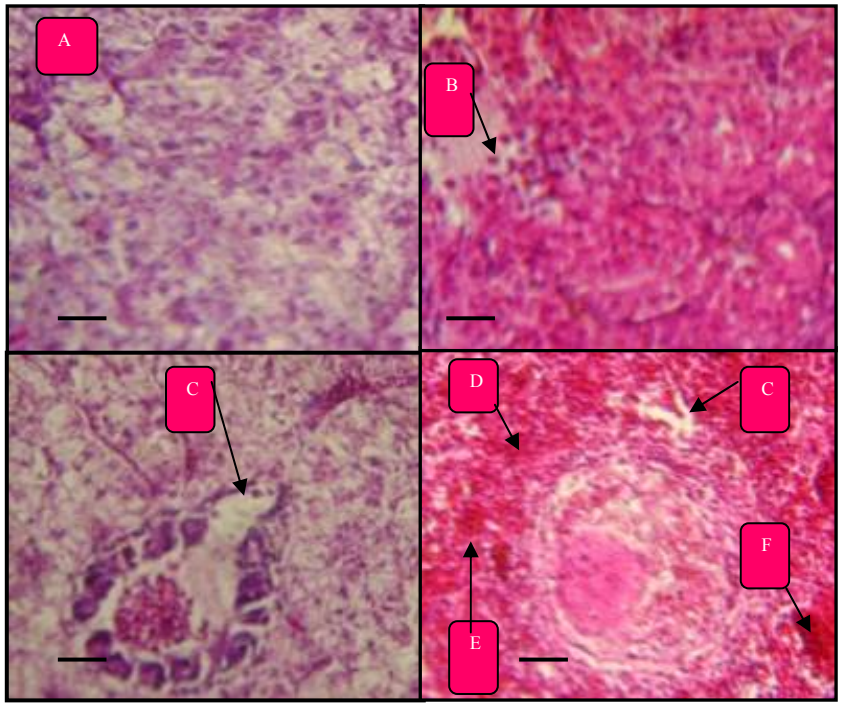

Fig. 4: Histopathology of the liver of tilapia injected with *Streptococcus agalactiae*, A: Normal liver, B: Atrophy, C: Fatty degeneration, D: Hypertrophy, E: Congestion, F: Hemorrhage (1 bar = 50 μm)

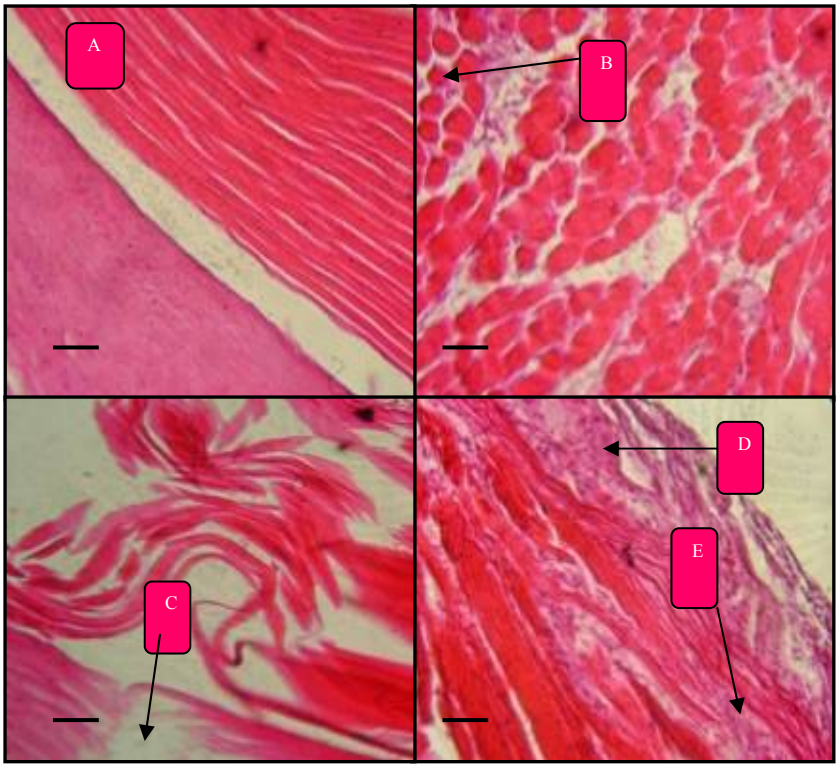

Fig. 5: Histopathology of the eye of tilapia injected with *Streptococcus agalactiae*, A: Normal eye, B: Hypertrophy, C: Vacuolization, D: Hyperplasia, E: Necrosis (1 bar = 50 μm)

treatments were not significantly different among them but were significantly different from the controls. This indicates that the addition of probiotic, prebiotic and synbiotic could improve the tilapia's growth performance due to better nutrient utilization compared to the controls. The synergistic effect in the synbiotic treatment is not significant; probably because the 1% probiotic and 2% prebiotic combination is not yet an optimum combination. The low FCR values in the probiotic, prebiotic and synbiotic treatments showed that the feed utilization was better than the controls. The FCR value has a positive correlation to the daily growth rate. The addition of probiotic, prebiotic and synbiotic in feed were able to improve the fish's ability to utilize feed due to the rise in the number of beneficial bacteria in the fish's digestive tract. The study by Putra (2010) demonstrated that *Bacillus* sp. NP5 is an amylolytic bacteria strain which could secrete amylase which has an important role in the fish's digestion process, i.e., hydrolyzing the carbohydrate in feed in the fish's digestive tract. This bacteria ability to produce amylase can optimize feed utilization in the tilapia's digestive tract. The administration of a prebiotic could facilitate the growth of beneficial micro flora in the intestines and in turn improve feed utilization. In the study by Putra (2010), the administration of probiotic, prebiotic and synbiotic individually could increase the activity of amylase and protease in tilapia's digestive tract. This increases the digestibility of protein and carbohydrate in feed, increasing the amounts of protein and energy from the feed that could be absorbed by the intestines and be utilized by the fish which in the end optimizes feed utilization. The more protein and energy stored by the fish's body will show the higher protein retention and the better growth rate.

The experimental fish in this study showed some external anatomy changes after the challenge test with *S. agalactiae*, in which those signs were consistent with the study by Evans *et al.* (2006). *Streptococcus agalactiae* injected into the fish would enter blood vessels and would be carried by the blood stream to the brain. Hernandez *et al.* (2009) stated that the brain is the main target of *S. agalactiae* after entering the blood flow. On the other hand, the results demonstrated that the probiotic, prebiotic and synbiotic administered were able to suppress the number of *S. agalactiae*. This is postulated to be due to tilapia's increased immune response in the form of the increased number and activity of macrophages in tissues, causing an increased phagocytosis and an increased number of bacteria removed.

Besides changes external organs, there were also changes in internal organs such as the tilapia's liver, kidney and brain. The changes were apparent in the tilapia's histopathology. According to Cheville (1999), focal necrosis could be in the form of liquidization of tissues as a result of an enzymatic reaction due to the introduction of toxins. Based on the microscopic observation, *S. agalactiae* infection caused degeneration of the brain in the positive control. According to Roberts (2001), bacterial infections could cause disturbances in cell metabolism (degeneration) which is signified by intercellular accumulation which can be seen microscopically as numerous cells packed together, distended cells, paler color and the discovery of vacuoles and necrosis. The degeneration of the brain cells is the cause of the fish's loss of balance, whirling and their tendency to swim to the surface. According to Hardi (2011), fish which exhibit signs of whirling show signs of degeneration and necrosis of the cranial cerebellum at histopathological examination. Microscopically, vacuolization is seen as empty spaces in the brain which occurs as a result of cell damage which led to cell destruction. Vacuolization is thought to be caused by infections carried through the blood flowing to the brain, causing the damage to the organ's tissues. The kidney plays a role as an excretory organ which filters waste materials from the blood. The kidney also actively fights the entry of foreign microorganisms (pathogens) through the presence of macrophages and lymphocytes in the kidney. If there is an infection, the kidney will demonstrate resistance mechanisms such as the formation of white blood cells such as monocytes, lymphocytes and granulocytes. Rombout *et al.* (2005) stated that in teleostei fish, the kidney plays a role in the formation of various white blood cell groups such as the monocytes and granulocytes (neutrophils, basophils and eosinophils). High intensity attacks from pathogenic bacteria causes the kidney to work in overdrive, causing cell damage. In addition, the bacteria which succeed in attacking the kidney will secrete exotoxins which have the ability to cause hemorrhages in the epithelial cell of the tubules.

Inflammation of the liver was indicated by infiltration of inflammation cells which showed that the pathogens had infected liver cells. The migration of inflammation cells is the indication of a defense reaction toward toxic materials entering the body in order to destroy the infectious agent. According to Ressang (1984), inflammation could be triggered by bacteria which could potentially secrete toxins. Liver cell damage was found in all treatments in week 3 (7 days post *S. agalactiae* infection). In week 4, damage was still found in the positive control, but no longer found in treatments with probiotic, prebiotic and synbiotic. This showed that the fish in these treatments have already recovered, approaching the normal condition. This was also shown by the results of the bacterial count of the *S. agalactiae* in the kidneys; in fish treated with probiotic, prebiotic and synbiotic, there were no *S. agalactiae* found.

Hypertrophy is the increased volume of a certain organ or tissue due to increased cell size. Hyperplasia is the increased number of cells but the cells are normal sized. Even though hypertrophy and hyperplasia are two different processes, but they often coincide. According to Hardi (2011), hypertrophy and hyperplasia in the choroidal zone cause the fish to experience exophtalmia (the eyes bulge both laterally and bilaterally). Hypertrophy and hyperplasia were observed in

positive control in week 3 and 4 but were not found in fish treated with probiotic, prebiotic and synbiotic. This is probably because the probiotic, prebiotic and synbiotic were able to increase the tilapia's immune response, making the treated fish more resistant compared to positive control. *S. agalactiae* which proliferate in the eye enter through the blood stream and secrete exotoxins which damage the choroidal zone causing the changes found there. In this study, hemorrhage was found in the positive control, signifying that *S. agalactiae* is septicaemic, capable of spreading its virulence factor through blood vessels and reach the eye. Macroscopic observations showed damage to the eyes (exophtalmia) between day 4-7 and this was in line with the findings of the study by Evans *et al.* (2006).

CONCLUSION

The administration of probiotic, prebiotic and synbiotic through feed could control streptococcosis in tilapia by suppressing the number of *S. agalactiae* and the level of damage on the target organs, i.e., the brain, kidney, liver and eyes.

REFERENCES

Apriyantono, A., D. Fardiaz, N.L. Puspitasari and S.S. Budiyanto, 1989. [Guidelines for Food Assessment Laboratory]. IPB Press, Bogor, (In Indonesian).

Cheville, N.F., 1999. Introduction to Veterinary Pathology. 2nd Edn., John Wiley and Sons, New York, ISBN: 9780813824963, Pages: 352.

Effendie, M.I., 1979. [Fisheries Biology Method]. Yayasan Dewi Sri Bogor, Bogor, (In Indonesian).

Evans, J.J., P.H. Klesius, P.M. Gilbert, C.A. Shoemaker and M.A. Al Sarawi *et al.*, 2002. Characterization of β-haemolytic group B *Streptococcus agalactiae* in cultured seabream, *Sparus auratus* L. and wild mullet, *Liza klunzingeri* (Day), in Kuwait. J. Fish Dis., 25: 505-513.

Evans, J.J., P.H. Klesius and C.A. Shoemaker, 2004. Efficacy of *Streptococcus agalactiae* (Group B) vaccine in tilapia (*Oreochromis niloticus*) by intraperitoneal and bath immersion administration. Vaccine, 22: 3769-3773.

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

Gomez-Gil, B., A. Roque and J.F. Turnbull, 2000. The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms. Aquaculture, 191: 259-270.

Hardi, E.H., 2011. [Potential vaccine candidate of *Streptococcus agalactiae* for prevent streptococcosis on Nila tilapia (*Oreochromis niloticus*)]. PhD Thesis Bogor Agriculture University, Bogor, (In Indonesian).

Hernandez, E., J. Figueroa and C. Iregui, 2009. Streptococcosis on a red tilapia, *Oreochromis* sp., farm: A case study. J. Fish Dis., 32: 247-252.

Huisman, E.A., 1987. Principles of Fish Production. Department of Fish Culture and Fisheries, Wageningen Agriculture University, Wageningen.

Li, J., B. Tan and K. Mai, 2009. Dietary probiotic *Bacillus* OJ and isomaltooligosaccharides influence the intestine microbial populations, immune responses and resistance to white spot syndrome virus in shrimp (*Litopenaeus vannamei*). Aquaculture, 291: 35-40.

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

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

Merrifield, D.L., A. Dimitroglou, A. Foey, S.J. Davies and R.T.M. Baker *et al.*, 2010. The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture, 302: 1-18.

Muchtadi, D., 1989. [Evaluation of Food Nutritional Value]. Directorate-General of High Education, Inter-University Center for Biotechnology, Bogor Agricultural University, Bogor, (In Indonesian).

Nayak, S.K., 2010. Probiotics and immunity: A fish perspective. Fish Shellfish Immunol., 29: 2-14.

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

Ressang, A.A., 1984. [Specific Veterinary Pathology]. 2nd Edn., N.V. Publisher, Denpasar, (In Indonesian).

Ringo, E., R.E. Olsen, T.O. Gifstad, R.A. Dalmo, H. Amlund, G.I. Hemre and A.M. Bakke, 2010. Prebiotics in aquaculture: A review. Aquacult. Nutr., 16: 117-136.

Roberfroid, M.B., 2000. Prebiotics and probiotics: Are they functional foods? Am. J. Clin. Nutr., 71: 1682S-1687S.

Roberts, R.J., 2001. Fish Pathology. 3rd Edn., Churchill Livingstone, London, ISBN-13: 978-0702025631, Pages: 492.

Rodriguez-Estrada, U., S. Satoh, Y. Haga, H. Fushimi and J. Sweetman, 2009. Effect of single and combined supplementation of *Enterococcus faecalis*, mannan oligosaccharide and polyhydrobutyrate acid on growth performance and immune response of rainbow trout *Oncorhynchus mykiss*. Aquacult. Sci., 57: 609-614.

Rombout, J.H.W.M., H.B.T. Huttenhuis, S. Picchietti and G. Scapigliati, 2005. Phylogeny and ontogeny of fish leucocytes. Fish Shellfish Immunol., 19: 441-455.

Schrezenmeir, J. and M. de Vrese, 2001. Probiotics, prebiotics and synbiotics-approaching a definition. Am. J. Clin. Nutr., 73: 361S-364S.

Taukhid, 2009. [Effectivity of using of *Streptococcus* spp. vaccine in tilapia (*Oreochromis niloticus*) juvenile by immersion technique to prevent streptococcosis disease]. Ministry of Marine Affairs and Fisheries, Research Report of Research Grant for Researchers and Engineers of Marine Affairs and Fisheries Freshwater Aquaculture Research Station, Bogor, (In Indonesian).

Verschuere, L., G. Rombaut, P. Sorgeloos and W. Verstraete, 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol. Mol. Biol. Rev., 64: 655-671.

Zhang, Q., H. Ma, K. Mai, W. Zhang, Z. Liufu and W. Xu, 2010. Interaction of dietary *Bacillus subtilis* and fructooligosaccharide on the growth performance, non-specific immunity of sea cucumber, *Apostichopus japonicus*. Fish Shellfish Immunol., 29: 204-211.

Zonneveld, N., E.A. Huisman and J.H. Boon, 1991. [Principles of Fish Cultivation]. Gramedia Pustaka Utama, Jakarta, (In Indonesian).