THE INTERNATIONAL JOURNAL OF
SCIENCE & TECHNOLEDGE

Efficacy of the Whole Cell Vaccine *Streptococcus agalactiae* with Vector *Artemia* sp. for Streptococcosis Prevention in Nila Fish (*Oreochromis niloticus*)

Rusli
Lecturer, Department of Budidaya Perikanan,
Politeknik Pertanian Negeri Pangkep, Indonesia
Suryati
Lecturer, Department of Budidaya Perikanan,
Politeknik Pertanian Negeri Pangkep, Indonesia
Mulyati
Lecturer, Department of Budidaya Perikanan,
Politeknik Pertanian Negeri Pangkep, Indonesia
Patang
Lecturer, Department of Pendidikan Teknologi Pertanian,
Universitas Negeri Makassar, Indonesia

Abstract:
This study aims to examine the efficacy of the oral whole-cell vaccine of S. agalactiae with Artemia sp as a vaccine vector for prevention against Streptococcosis in tilapia fish. The fish seeds used in the study were 2-3 cm in size which did not carry S. agalactiae bacteria and were reared in jars with a volume of 3 liters as many as ten birds/jar. Giving the vaccine to fish orally by first soaking the vaccine in Artemia sp. The soaking times for the vaccine were 15, 30, 60, 90, 120 and 150 minutes. Parameters observed were relative percent survival (RPS), antibody titer value, total leukocytes, phagocytic index, and leukocyte differential. The results showed that the treatment between vaccines had no significant effect, while the treatment with the control vaccine had a significant effect (P<0.05). The highest RPS value was obtained in the vaccine soaking treatment in Artemia sp. for 30 minutes and 120 minutes, with a value of 93.33%. Furthermore, the RPS was 86.67% at 15 and 150 minutes of soaking and 80% RPS at 30 minutes of soaking. This shows that the level of vaccine protection against streptococcosis in tilapia fish is very high.

Keywords: *Efficacy, tilapia fish, artemia sp, streptococcus agalactiae, soaking*

1. Introduction

The disease causing losses in tilapia fish cultivation today is streptococcosis. It is caused by *Streptococcus* bacteria. *Streptococcus* bacterial infection causes about 50% of tilapia fish mortality from the first month and increases to almost 80% until the end of the rearing period in cages in the Philippines (Clark *et al.,* 2000). Infection causes death of up to 60% in tilapia fish cultivation in South Sumatra (Yuasa *et al*., 2008). On a large scale, *Streptococcus* outbreaks continued to occur with high mortality (30-80%) in 2009-2011 (Chen *et al*., 2012). Disease cases in tilapia cultivation in several areas in West Java, Central Java, East Java, North Sulawesi and West Papua found the type of *Streptococcus* bacteria that causes Streptococcosis in tilapia fish, where 80% of streptococcosis is caused by *Streptococcus agalactiae* and 20% is caused by *Streptococcus iniae* (Taukhid & Purwaningsih, 2009). Several researchers have also reported the spread and infection of *S. agalactiae* bacteria in tilapia fish in Indonesia (Lusiastuti *et al*., 2009; Anshary *et al*., 2014).

One of the preventive efforts that can be made to overcome the problem of streptococcosis is by increasing immunity in the fish's body, including vaccination. Vaccination is an effective way to control fish diseases (Ellis, 1988). Vaccination can increase the immunity in the fish's body so that it is resistant to certain disease attacks for some time so that the death rate can be kept as small as possible. Vaccination in fish will stimulate the formation of antibodies that will protect against certain disease attacks. Hardi *et al*. (2013) stated that giving the whole cell vaccine of *S. agalactiae* by injection method provided protection to tilapia fish with an RPS value of 70%.

There are several ways to apply vaccines to fish, namely:

- Vaccine application through soaking
- Vaccine application through feed
- Vaccine application by injection

Each method of vaccination has advantages and disadvantages in its application. Vaccination of small fish (seeds) is more effective using the soaking method and the oral method. The method of giving vaccines orally by mixing feed with

vaccines has been widely used and provides a fairly good level of protection both in seeds and in fish that have been kept in cultivation ponds.

Aside from being a natural food, *Artemia* sp. can also be used as a carrier (vector) for vaccines, some essential nutrients, antibiotics, pigments, medications, and immunostimulants (Isnansetyo & Kurniastuti, 1995). The advantage of giving vaccines through *Artemia* sp. is that *Artemia* sp. is a natural food starter for fish larvae and it is hoped that the vaccine will be in the body of *Artemia* sp. and quickly enter the body of the fish (Lin *et al*., 2007). Some uses of *Artemia* sp. as a vector are as a stimulant (Hurriyani *et al*., 2012), hormone vector (Dewi *et al*., 2010) and vaccine DNA vector (Hadibowo, 2011). This study wants to know the efficacy of the *Streptococcus agalactiae* whole cell vaccine given orally with the vector *Artemia* sp.

2. Research Purposes

This study aims to examine the efficacy of the whole cell vaccine of *Streptococcus agalactiae* orally with *Artemia* sp. as a vaccine vector for preventing Streptococcosis in tilapia fish.

3. Method

3.1. Study Materials

The test animals used were tilapia seeds with a size of 2-3 cm, which had been verified as not carrying *S. agalactiae*. Before being used in the experiment, the test fish were first adapted to laboratory conditions in a temporary holding tank. Fish were given commercial feed at a dose of 3% of the weight of biomass, feed protein content of 33% and given twice a day, i.e., in the morning and evening. Water quality is guarded at optimal conditions for fish growth. Bacterial isolate *S. agalactiae* N14G was obtained from the collection of BPPBAT, Bogor. The research container used a jar with a volume of 3 liters, which was filled with 10 tilapia fish/jars.

3.2. Preparation of Vaccine

The bacterial isolate of *S. agalactiae* in a petri dish was taken as much as 1 ose, put into 10 ml of liquid BHIB aseptically and then incubated at 28°C in the incubator for 24 hours. Then 1 ml of bacterial culture was taken, put into each 9 ml of BHIB and then incubated at 28°C for 24 hours, 10 ml of the bacterial culture was then put into each 90 ml of BHIB and incubated for 72 hours, assuming a concentration of 4 x 109 cfu/mL (Evans *et al*., 2006). Bacterial culture with a volume of 100 ml added neutral buffer formaline of 3% of the culture volume (Hardi *et al*., 2013; Amrullah, 2014) and again incubated for 24 hours. An inactivated whole cell vaccine of bacterial culture was made and then centrifuged at 12,000 RPM for 30 minutes with a temperature of 4°C. The supernatant solution and the pellet precipitate formed were then separated. The separated bacterial pellet precipitate was then washed by adding 100 ml of Phosphate Buffer Saline (PBS), then centrifuged at 5,000 rpm for 15 minutes. Bacterial cell washing activities were carried out three times with PBS, pellet precipitate, then added PBS back up to 100 ml and stored in the refrigerator for later use in fish vaccination.

3.3. Vaccination

Before vaccinating tilapia fish, artemia enrichment is done first with whole cell vaccine by immersing *Artemia* sp. in the vaccine solution at different times as follows:

- Treatment E: Soaking of *Artemia* sp. with whole cell vaccine for 2 hours
- Treatment E: Soaking of *Artemia* sp. with whole cell vaccine for 1,5 hours
- Treatment C: Soaking of *Artemia* sp. with whole cell vaccine for 1 hour
- Treatment B: Soaking of *Artemia* sp. with whole cell vaccine for 0.5 hours
- Treatment A: Soaking of *Artemia* sp. with whole cell vaccine for 0.25 hours
- Treatment F: *Artemia* sp. Without Soaking the vaccine (control)

Artemia sp., the brand used is Supreme Plus which is produced by Golden Mark®, USA, with a hatching rate of around 80-90%. Cist *Artemia* sp. hatched in a mineral water bottle upside down with a dark wall and equipped with an aeration system. *Artemia* sp. incubated with a salinity of 29 ppt for 18-24 hours. *Artemia* sp., which have hatched, separated using a filter with a size of 150 mesh and then weighed according to the dose to be given to tilapia fish. One *Artemia* sp. is able to eat bacteria as much as 105 cfu/mL (Lin *et al*., 2007), so it takes 40 *Artemia* sp. for one tilapia fish to be vaccinated with a bacterial dose of 109 cfu/mL.

3.4. Challenge Test

The challenge test of fish that have been vaccinated with *S. agalactiae* bacteria at a dose according to the Lethal Dose 50% (LD50) was done by soaking fish for 30 minutes in water containing virulent *S. agalactiae* bacteria. Fish mortality is recorded and the relative level of vaccine protection is calculated using the Relative Percent Survival (RPS) (Ellis, 1988) formula:

$$\mathbf{RPS} = 1 - \left(\frac{\textbf{Percent of immunized mortality}}{\textbf{Percent of control mortality}}\right) \mathbf{x\ 100\%}$$

3.5. Antibody Titer Test

Antibody titers were calculated by taking fish blood on the 14th day. After the fish were vaccinated, the blood was centrifuged at 5,000 rpm for 5 minutes. After the serum was separated from the blood cells, the serum was transferred to

Eppendorf and incubated at 44°C for 20 minutes to inactivate the complement. The agglutination test was carried out in microplate titers by injecting 25 µl of PBS solution into each well, then putting 50 µl of serum into the first well. Serum and PBS solution were stirred to become homogeneous, then transferred to the second well as much as 25 µl and so on until serial dilution occurs up to the 11th well.

Bacteria as much as 25 µl were inoculated into each well up to well 12. The microplate was shaken gently to homogenize the mixture in the well. Furthermore, the serum and bacteria mixture was incubated at 37°C for 2 hours and then stored in the refrigerator at 4°C overnight until lumps (fog) formed. The occurrence of clumps of small particles at the base of the microplate is an indicator of the presence of antibodies in the serum, where the last well-containing lumps are the agglutination titer value. Table 1 shows the antibody titer values.

Observation Hole Number (n)	Serum Dilution	Antibody Titers (-log2)
1	1: 4	2
2	1: 8	3
:	:	:
:	:	:
11	1: 4096	12
12		Kontrol

Table 1: Readings of Antibody Titer Values

3.6. Calculation of Haematological Parameters

Observation of haematological parameters was carried out 3 times during the study, namely:

- Before the vaccine treatment,
- After the vaccine treatment,
- After the challenge test

This activity was carried out by taking blood samples from the test fish and then observing the number of leukocytes, phagocytic activity, and differential leukocytes. Blood draw using a sterile syringe that has been rinsed using sodium citrate (Na-citrate) 3.8% as an anticoagulant. Blood was taken from the caudal vein and then placed in a microtube which had also been rinsed with 3.8% Nasitrate for further observation. Haematological parameters measured included white blood cell count, phagocytosis activity measurement, and leukocyte differential.

3.6.1. White Blood Cell Count (Blaxhall Dan Daisley, 1973)

The blood sample was sucked using a white pipette to a scale of 0.5 and then the Turk solution was sucked up to a scale of 5-11. The pipette is then shaken in a figure-eight shape for 3-5 minutes to homogenize the blood with Turk's solution. The first two drops from the pipette are discarded and the next drop is dropped on the hemacytometer to count the number of white blood cells. Observations were made using a microscope by counting the number of blood cells in five large hemacytometer boxes. Calculation of the number of white blood cells using the following formula (Blaxhall dan Daisley, 1973):

$$\Sigma \text{ SDP} = \text{Average of calculated cell x } \frac{1}{Large\ Box\ Volume} \text{ x Dilution factor}$$

3.6.2. Measurement of Phagocytosis Activity (Anderson Dan Siwicki, 1993)

Blood samples were taken as much as 50 µL and placed in a sterile microtube. The blood was then mixed with 50 µL of *Staphylococcus aureus* bacteria at a density of 108cfu/mL and homogenized. The mixture was then incubated for 20 minutes. After that, 5 µL of the mixture was taken and dripped onto a glass preparation to be used as a smear preparation. After drying, the preparations were soaked in methanol for 5-10 minutes and then dried. After drying, the preparations were soaked in Giemsa solution for 10-15 minutes and then dried again. After drying, the preparations can be observed using a microscope and the percentage of cells that are actively carrying out the phagocytosis process is calculated from the 100 observed phagocytic cells. Determination of the value of phagocytosis activity using the following formula:

$$\text{AF (\%)} = \frac{\Sigma Phagocytic\ Active\ Cells}{\Sigma Phagocytic\ cells} \text{ x 100\%}$$

3.6.3. Differential Leukosit (Blaxhall Dan Daisley, 1973)

Leukocyte differential observation was carried out before the fish were vaccinated and after the fish have been vaccinated and after the fish have been challenge tested. Observation of differential leukocytes begins with the preparation of smear preparations. The smear preparation was made by dropping fish blood on an object glass and then air-dried. Furthermore, the smear preparations were fixed in methanol solution for 5 minutes, and after that, they were soaked in Giemsa solution for 15 minutes. Then the preparations were rinsed with running water, dried and then covered with a cover glass. Leukocyte differential was observed under a microscope, the percentage of leukocyte cells was calculated by observing 10 visual fields and each counted leukocyte cell is grouped and presented according to its type.

3.7. Analysis of Data

Relative Percent Survival (RPS) data and fish mortality were analyzed by analysis of variance to determine the effect of the treatment being tried. If the results of the analysis are significantly different ($P < 0.05$), then proceed with

Duncan's test. Data on total leukocytes, phagocytic activity, differential leukocytes and antibody titer values were analyzed descriptively.

4. Results and Discussion

4.1. Mortality and Relative Percent Survival (RPS)

The death of vaccinated and unvaccinated fish occurred 24 hours after the challenge test. Fish mortality with vaccine treatment was significantly different from control (unvaccinated fish) (P<0.05), while the treatment between each vaccine treatment was not significantly different (P> 0.05). The mortality rate of vaccinated fish was lower than that of control fish, where the highest mortality of vaccinated fish was in treatment C, namely 10%, while the average mortality of control treatment fish was 50% (Table 2). This indicates that the vaccine treatment is able to increase the body's immunity in fish. The RPS value is used to determine the effectiveness of the vaccine to protect fish after being challenged with virulent bacteria that cause disease. The calculation results show that the vaccine treatment had no significant effect, while the treatment of vaccinated fish with a control (without giving the vaccine) had a significant effect.

The five types of vaccine treatments were able to provide protection against test fish from post-challenge virulent bacterial infections with RPS values of 80-93%. This is indicated by the lower mortality rate of vaccinated fish compared to the mortality of non-vaccinated fish (control). The treatment of giving vaccines to fish can stimulate the fish's immune system against the inducing virulent bacterial infection so that the mortality rate of vaccinated fish is lower. Vaccines are antigenic substances used to produce active immunity against a disease to prevent or reduce the effects of infection (Alifuddin, 2002).

Treatments	Mortality (%)	RPS (%)
A	6,67[a]	86,67 [a]
B	3,33 [a]	93,33 [a]
C	10 [a]	80 [a]
D	3,33 [a]	93,33 [a]
E	6,67 [a]	86.67 [a]
K	50[b]	0 [b]

Table 2: Mortality Values and Fish RPS of Fish of Post-Vaccination

4.2. Description of Fish Blood

Fish leukocytes are also a non-specific defense system. Leukocytes are divided into three groups, namely: lymphocytes, monocytes and polymorphonuclear granulocytes, depending on the absence or presence of fine granules in the cytoplasm (Alifuddin, 2002). The success of vaccination can be seen from the resulting RPS value. However, why there is a success or failure of vaccination can be seen from several supporting parameters such as total leukocytes, leukocyte differential, phagocytic index, and antibody titers (Table 3).

Leukocytes are blood cells that play a role in the immune system. Leukocytes help rid the body of foreign bodies, including invading pathogens through the immune response system and other responses. Fish blood leukocyte levels after being vaccinated increased, both in the treatment of vaccinated and non-vaccinated fish (control). This shows that specific and non-specific immunity work together after vaccination. Rastogi (1977) stated that normal fish blood counts ranged from 20,000-150,000 cells/mm^3. Vaccine treatment was higher than control. This shows that vaccine treatment can increase the ability of immune system cells (leukocytes) to proliferate and differentiate the consequence of bacterial infection. Sakai *et al*. (1995) stated that the leukocyte population increased due to an infection.

Treatments	Total Leukocytes (10^5 cells/mm)	Index of Phagocytic (%)	Neutrophils (%)	Lymphocytes (%)	Monocytes (%)	Value Titer of Antibody (-log2)
Control	71	42,86	23	45	32	3
A	82,4	52	25	50	20	6
B	76,8	60,7	29	46	35	7
C	76,8	56	29	45	26	7
D	82,6	55	22	46	32	7
E	82	55	24	48	28	8

Table 3: Some Parameters of Post-Vaccination Tilapia Blood

The types of leukocytes observed post-vaccination were lymphocytes. Lymphocytes act as memory cells that form antibodies. The increase in lymphocytes was also in line with the formation of antibodies in fish after 5-10 days after vaccination. Monocytes and neutrophils play a role in the process of phagocytosis. Monocytes are more likely to phagocytize large particles, whereas neutrophils are more likely to phagocytize small particles. Leukocytes are the main cells of the body's defense system, so it is very important to know about changes in the number or appearance of two groups of leukocytes in the blood circulation, namely agranulocytes and polymorphonuclear granulocytes. Tilapia fish has a fairly complete type of leukocyte, consisting of: agranulocytes, including lymphocytes and monocytes and polymorphonuclear granulocytes, including neutrophils, eosinophils and basophils.

This is in accordance with what was revealed by Clem *et al*. (1985) and Chinabut *et al*. (1991) that fish leukocytes consist of three types, including lymphocytes, monocytes and neutrophils. The leukocyte differential values of vaccinated fish and control fish were relatively the same. This is a sign that the fish is fighting the infection. The overall leukocyte differential data showed that the vaccine components given were able to increase fish-specific and non-specific defense cells.

Phagocytosis is a defense mechanism in an organism's body by ingesting foreign objects and then destroying them (Kamiso, 2001). Phagocytic cells consist of monocytes, macrophages and granulocytes. Phagocytic cells will recognize and engulf antigenic particles, including bacteria and damaged host cells, through three stages of the process, namely: attachment, phagocytosis, and digestion (Irianto, 2005). The phagocytosis activity of vaccinated fish was higher than that of non-vaccinated fish. This also proves that vaccination can boost the immune system in fish through the phagocytosis mechanism.

Antibodies are protein molecules produced by plasma cells as a result of interactions between antigen-sensitive B lymphocytes and antigens, where antibodies have a special ability to bind to antigens and accelerate their destruction and removal. New antibodies are discovered about a week after the first injection and their serum levels rise to a peak after 10-14 days (Tizard, 1982). The antibody titer value indicates the formation of antibodies in fish after the giving of the vaccine.

The results of the antibody titer agglutination test analysis showed that the treatment of vaccine administration to fish through the vector *Artemia* sp. can form protective antibodies in the test fish. Based on table 1, it can be seen that the antibody titer value of fish during the study showed a value of 6 in treatment A. In treatments B, C, and D, the antibody titer value was 7. The highest titer value was shown in treatment E, with a value of 8. This shows that the vaccine given is able to increase the immune system in the test fish to form specific antibodies. Differences in antibody titers between treated and control fish, Where the antibody titers in the fish given the treatment were relatively higher than the control fish. This indicates that the vaccine is able to stimulate immunity in the body of the test fish. Tizard (1982) said that several factors that affect antibody responses are vaccine dose, the timing of vaccine administration, the antigenicity of bacteria, and the immunogenic response of vaccinated fish. New antibodies are discovered about a week after the first injection and their serum levels rise to a peak after 10-14 days. The lowest titer value was shown in the control treatment (without giving of vaccine). This shows that the fish immune system is weak in protecting fish, which is characterized by high fish mortality, which is an average of 50% (Table 2). In the control treatment, antibody titers were still found, although the amount was small. This indicates that, naturally, tilapia already has an immune system. Vaccination will stimulate the natural immune system resulting in an increase in antibody titers.

5. Conclusion

The conclusion from testing the efficacy of whole cell vaccines administered through Artemia sp. is a vaccine treatment capable of providing protection against test fish from post-challenge virulent bacterial infections with an RPS value of 80-93%, with a low mortality rate of vaccinated fish, namely 3.33-10% compared to the mortality of non-vaccinated fish (control), which is an average of 50%.

6. References

i. Alifuddin, M. (2002). Immunostimulation in aquatic animals. *Jurnal Akuakultur Indonesia*, *1*(2), 87–92.
ii. Amrullah. (2014). Immunoprotection of Protein Toxoid Vaccine of Bakteri Streptococcus agalactiae In Tilapia fish (*Oreochromis niloticus*). Dissertation. Sekolah Pascasarjana. Institut Pertanian Bogor. Bogor.
iii. Anderson, D. P., & Siwicki, A. K. (1993). Basic Hematology and Serology for Fish Health Programs. Paper Presented in Second Symposium on Diseases in Asian Aquaculture. *Aquatic Animal Health and the Environment*. Phuket, Thailand. 25–29th October 1993.
iv. Anshary, H., Kurniawan, R. A., Sriwulan, Ramli, & Baxa, D. V. (2014). Isolation and Molecular Identification of the Etiological Agents of streptococcosis in Nile Tilapia (*Oreochromis niloticus*) Cultured in Net Cages in Lake Sentani, Papua, Indonesia. *National Institutes of Health. US National Library of Medicine*.
v. Blaxhall, P. C., & Daisley, K. W. (1973). Routine Haematological Methods for Use with Fish Blood. *Fish Biology, 5*, 577–581.
vi. Chen, M., Wang, R., Li-Ping Li, Liang, W. W., Jian, L., Huang, Y., Lei, A. Y., & Wei-Yi Huang. (2012). Screening Vaccine Candidate Strains Against Streptococcus Agalactiae of Tilapia Based on PFGE genotype. *Volume 30, Issue 42, 14 September 2012, Pages 6088–6092*. Elsivier.
vii. Chinabut, S., Limsuwan, C., & Kitsawat, P. (1991). Histology of the Walking Catfish, *Clarias batrachus*. Thailand: International Development Research Centre (IDRC).
viii. Clark, J. S., Pallers, B., & Smith, P. D. (2000). Prevention of Streptococcus in Tilapia by Vaccination in the Philippine Experiences. *5th Int. Symposium on tilapia aquaculture in the 21st century*, 2, 545–552.
ix. Clem, L. W., Faulman, E., Miller, N. W., Ellsaesser, C., Lobb, C. J., & Cuchens, M. A. (1985). Monosytes as Accessory Cells in Fish Immune Response. *J Development and Comparative Immunology, 9*, 803–809.
x. Dewi, R. R. S. P. S., Alimuddin, A. O. Sudradjat, & Sumantadinata, K. (2010). Transfer Effectiveness and PhGH Gene Expression in Patin Siam Fish (*Pangasionodon hypophthalamus*). Final Report of Research Results 2010. LRPTBPAT, Sukamandi.
xi. Ellis, A. E. (1988). *Fish Vaccination*. Academic Press.
xii. Evans, J. J, Klesius PH, & Shoemaker CA. (2006). An overview of Streptococcus in warmwater fish. *Aquaculture Health International, 7*, p10–14.

xiii. Hadibowo,S.S. (2011). Potential Test of *Artemia sp.* as a DNA Vaccine Carrier Vector for Goldfish Seeds *Cyprinus Carpio*. Thesis. Intitut Pertanian Bogor.

xiv. Hardi, E.H, Sukenda, Harris, E., & Lusiastuti, A.M. (2013). Potential Vaccine Candidates of *Streptococcus agalactiae* for Prevention of Streptococcosis on Tilapia Fish (*Oreochromis niloticus*). *Jurnal Veteriner*, *Vol 14*(4).

xv. Hurriyani, Y., Sukenda, & Widanarni. (2012). Improved larval resistance of Patin Fish (*Pangasius hypophthalmus*) through *Artemia*, which is enriched with *paci-paci* extract (*Leucas lavandulaefolia*) against *Aeromonas hydrophila* infection, Thesis. Institut Pertanian Bogor.

xvi. Irianto, A. (2005). *Pathology of Teleost Fish*. Yogyakarta: Gadjah Mada University Press.

xvii. Isnansetyo, & Kurniastuty, A. (1995). Techniques Culture of Phytoplankton and Zooplankton; Natural Food for Hatchery of Marine Organisms. Yogyakarta: Kanisius.

xviii. Kamiso, H.N. (2001). Immunology and Vaccination in Fish. DUE Project. Faculty of Fisheries, Riau University, Pekanbaru.

xix. Lin, C.C., Jhon, H.Y.L., Ming, S.C., & Huey, L.Y. (2007). An Oral Nervous Necrosis Virus Vaccine that Induced Protective Immunity in Larvae of Grouper *Epinephelus coioides*. *Aquaculture, 268*, 265–273.

xx. Lusiastuti, A.M., Taukhid, Kusrini, E., & Hadie, W. (2009). Sequens Analysis of *S. agalactiae*: A Pathogen Causing Streptococcosis in Tilapia (*Oreochromis niloticus*). *Indonesia Aquaculture Journal, 4(2)*, 87–92.

xxi. Rastogi, S.C. (1977). *Essentials of Animal Physiology*. Willey Eastern Limited. India, p. 204–223.

xxii. Sakai, M., Atsuta, S., & Kobayashi, M. (1995). The activation of leucocytes in rainbow trout (*Onchorhynchus mykis*) by oral administration of *Clostridium butyricum* bacterin. In M. Sharif, J. R. Arthur, & R. P. Subangsihe (Eds.), *Diseases in Asian Aquaculture II. Proceedings of Second Symposium on Diseases in Asian Aquaculture* (pp. 427–432). Manila: Asian Fisheries Society.

xxiii. Taukhid, & Purwaningsih, U. (2009). Screening of *Streptococcus spp.* Isolates as Candidate Antigens in Vaccine Manufacturing, and its Efficacy in Preventing Streptococciasis in Tilapia Fish *Oreochromis niloticus*. BRPBAT. Bogor.

xxiv. Tizard, I. (1982). *An Introduction to Veterinary Immunology*. Terjemahan Pengantar Imunologi Veteriner, Publisher of Universitas Airlangga.

xxv. Yuasa, K., Kamaishi, T., Hatai, K., Bahnnan, M.,and Borisuthpeth, P. (2008). Two cases of streptococcal infection of cultured tilapia in Asia, pp. 259-268. In M.G. Bondad-Reantaso, C.V. Mohan, M. Crumlish, & R.P. Subasinghe (Eds.), *Diseases in Asian Aquaculture VI*. Fish Health Section, Asian Fisheries Society, Manila, Philippines (505 pp.).