Efficacy of inactive bivalent and trivalent *Streptococcus agalactiae* bacteria (biotype 1 & 2) vaccines on tilapia, O*reochromis niloticus*

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Abstract. Streptococcosis is a significant fish disease impacting tilapia culture in Indonesia, causing losses estimated up to IDR 15.0 billion annually. This study aims to assess the efficacy of bivalent and trivalent vaccines containing *Streptococcus agalactiae* bacteria on tilapia. The formula of the bivalent vaccine contains 75% of S01-196-16 and 25% of N14G isolates (v/v). Trivalent vaccine contains 30%, 35%, and 35% of N14G, NP1050, and SG01-16 isolates (v/v), respectively. A challenge test assessed the efficacy of the vaccines, and it was carried out at 30, 90, and 150 days post-vaccination by artificially infection at LD_{60}. Selected bacteria isolate to be appointed in the challenge test are N14G (biotype 2) and S01-196-16 (biotype 1). Relative Percentage of Survival (RPS) was used as the main indicator of vaccine efficacy. The results revealed that the highest RPS of a bivalent vaccine against *S. agalactiae* (S01-196-16) was achieved at the first challenge (61.84%), and trivalent vaccine against *S. agalactiae* (N14G) and *S. agalactiae* (S01-196-16) was achieved at the first challenge (61.53% and 76.20%, respectively). Bivalent and trivalent *S. agalactiae* bacteria vaccines are promising "tools" to control streptococcosis on tilapia.

1 Introduction

Tilapia (*Oreochromis niloticus*) is the second most predominant aquaculture species globally after carp, and it was known as a relatively cheaper aquatic animal protein supply for millions of families. The fish are fast-growing, tolerate a wide range of ecological zone conditions, and are more resistant to diseases compared to other cultured species [1]. The Food and Agriculture Organization [2] recorded that 72% of global tilapia was produced in Asia, and the others came from Africa and across North and South America. Furthermore, [3] noted that the largest tilapia producer in the world was China, followed by Indonesia as the second-largest producer.

In an intensive aquaculture system where the fish is stocked at high densities, the fish is under biological stress conditions and prone to various pathogens infections. The disease risk is elevated if the fish farmer fails to provide favorable conditions and disease prevention

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strategies. Fish disease outbreaks in aquaculture are mainly associated with stressful conditions due to biological, physical, and chemical stress factors such as overcrowding, malnutrition, poor water quality, and improper health management strategies [4-7].

Globally, the primary pathogenic agent causing disease on tilapia culture is *Streptococcus* spp. or "streptococcosis". In Indonesia, streptococcosis on tilapia culture mainly was caused by *S. agalactiae*, and the bacterial isolates have been collected from a wide range of geographical areas revealed two distinct biotypes, hemolytic *S. agalactiae* (biotype 1) and non-hemolytic *S. agalactiae* (biotype 2) [7]. Clinical signs of the disease were characterized by darkened body color, lethargy, loss of appetite, red discoloration of the skin, "C-shaped" body posture, erratic swimming, corneal opacity, exophthalmia (uni/bilateral), skin hemorrhages, and dropsy. Internally, congestion appeared on visceral organs (liver, spleen, and kidney), and the brain seemed to be soft [4-7]. Furthermore, it was stated that in some cases, streptococcosis did not show any obvious clinical symptoms unless there was a persistent mortality pattern up to ≈ 40% of the total population, especially in large fish (> 250 gram/fish). As a consequence, it was significantly affected the feed conversion ratio (FCR) and reduced production. So, it was considered to be one of the most pathogens impacting tilapia culture throughout the country, causing significant losses of tilapia farming with the value of losses incurred estimated at up to IDR 15.0 billion annually [8].

Vaccination is becoming a promising approach to fish disease prevention in aquaculture since it is considered a prevention method against potential and endemic pathogenic agents, cost-effective, and ensures sustainable aquaculture production [5, 6, 9-14]. The application of the "StreptoVac" vaccine containing monovalent *S. agalactiae* at laboratory and field studies, reducing losses significantly, ranging from 20-30% compared to the control group [15]. Unfortunately, in an aquaculture system, the fish can simultaneously be exposed to more than one species or biotypes pathogenic agents.

This study aims to assess the efficacy of in-active bivalent and trivalent vaccines, containing *Streptococcus agalactiae* bacteria (biotype 1 & 2) on tilapia (*Oreochromis niloticus*).

2 Material and Method

2.1 Fish

The fish used in the study are specific pathogen-free (SPF) of tilapia against *S. agalactiae* infection. The SPF population was obtained from a certified hatchery by collecting eggs from mouth brooder tilapia, and the egg was disinfected before being hatched in a biosecurity facility. The egg disinfection was taken place by immersed with iodine concentrations of 200 ppm for 15 minutes. Fifteen thousand of 4 days old fish larvae were transferred into a reared concrete pond with the size of 2 x 4 x 0.8 m^3 for two weeks, and then the fish were distributed in equal numbers into the same volume of 6 sterilized concrete ponds for two months or the fish size up to >5 g. Each pond was equipped individually with a mechanical and bio-filtration system. Water supply was totally fulfilled from deep-well; temperature and pH were monitored daily, and total ammonia concentration was monitored weekly.

A combination of live and commercial feed was given during the first month, afterward using a commercial feed only. The size of pellet diameter, protein content, and feeding management were adjusted based on weekly sampling. Daily health status monitoring of fish was carried out for behavioral, appetite, and mortality, and weekly sampling for parasite infestation and bacterial isolation, especially *Streptococcus* spp. infection.

2.2 Vaccine and vaccination

S. agalactiae isolates were used as the master seed of vaccine was based on screening of immunogenic properties, and those data have been explored and analyzed [7]. The vaccine was prepared according to the internal procedure of fish vaccine production method developed by the Research Institute for Freshwater Aquaculture and Fisheries Extension (RIFAFE), and the final stock of the vaccine solution was formulated as follows: (1). The bivalent vaccine was made of N14G (biotype 2, non-hemolytic) and S01-196-16 (biotype 1, ß-hemolytic) isolates, containing 25% of N14G and 75% of S01-196-16 (v/v). (2). Trivalent vaccine was made of N14G, NP1050 (biotype 1), and SG01-16 (biotype 1) isolates, containing 30%, 35%, and 35% of N14G, NP1050, and SG01-16 (v/v), respectively. Inactivation of the vaccine was processed by formalin killed technique (0.3% for 60 minutes). Subsequently, viability and sterility testing were performed according to the national standard for fish vaccine quality control [16].

Fish were starved for 24 hours before vaccination to ensure the intestinal tract emptying, and anesthesia was applied before immunization. Vaccine injection was carried out intraperitoneally (IP) by injecting 0.1 ML of defined vaccine solution, and the control group was injected with phosphate-buffered saline (PBS).

A challenge test assessed the efficacy of the vaccines against virulent bacterial isolates at a 60% lethal dose (LD_{60}). The relative percentage of survival (RPS) was used as the main indicator of vaccine efficacy. The RPS was calculated according to the formula developed [17]:

$$RPS = \left[1 - \frac{\%\ vaccinate\ mortality}{\%\ control\ mortality}\right] x\ 100 \quad (1)$$

2.3 Challenge test

A total of 21 plastic boxes measuring 80 L and filled with 60 L of water were used for the challenge test with a stocking density was 30 fish/box, and each treatment was done in triplicate. All of the treatment boxes were parallelly connected, operated in a single recirculation system, and individually equipped with aeration. The negative control group was placed in the same room. However, it operated in a different recirculation system.

The Challenge test was taken place at three different times; 1st, 3rd, and 5th months post-vaccination. Challenge test was conducted by injecting virulent defined *S. agalactiae* isolates at LD_{60} for 96 hours. Selected bacteria isolate to be appointed in the challenge test are N14G representing biotype 2 and S01-196-16 representing biotype 1. A standard bioassay procedure determined LD60 of bacteria, and in the study were obtained 10^6 CFU/ml for the first and second challenges; and 10^7 CFU/ml for the third challenge.

Some 0.2 ML or equal to LD_{60} of virulent bacterial suspension was injected intraperitoneally into individual fish and differentiated according to bacterial biotypes. During the challenge test period, the water temperature was monitored daily, no water exchange or other water quality parameters intervention, and feeding was given twice a day at 1.0% of total body weight.

The observation was taken place twice a day, 08.00 am and 03.00 pm, focused on behavioral abnormality, clinical signs, moribund, and fish mortality. Challenge test observation lasted for 14 days, and extending time will be designed if there was no fish mortality during a defined period. Re-isolation of targeted bacteria, random sample of moribund or fish showing obvious clinical signs was taken from each different treatment group, isolated, and identified eventually.

2.4 Analysis

The mean mortality value of vaccinated and unvaccinated fish was analyzed statistically using a one-way analysis of variance to know the differences of each treatment group. Further analysis with Tukey post-hoc was applied for multiple comparisons if needed. A value of $p < 0.05$ was considered statistically significant and denoted as $p < 0.05$. Mortality graphs of each treatment group are used to describe the mortality pattern during the defined challenge test period.

3 Results

Disinfection of tilapia's egg by using iodine solution at concentrations of 200 ppm for 15 minutes before hatched, resulting from the fish free from potentially pathogenic infection when reared in biosecurity facility. The results of fish health monitoring and regular sampling activities did not find any targeted bacterial (S. agalactiae) infection until the fish before shortly been used for the study. Based on the results, it could be assumed that the fish population used in this study was SPF against *S. agalactiae* infection.

Challenging test against *S. agalactiae* N14G bacteria (biotype 2) during the first batch (1st-month post-vaccination) showed that the lowest cumulative mortality during the observation period, which is lasted for 14 days, was obtained in group A/trivalent vaccine (24.36%), followed by B/bivalent vaccine (38.98%). The latest is a control group (63.33%). The mortality pattern during the challenge test period against *S. agalactiae* N14G bacteria can be seen in Figure 1. While the results of the challenge test against *S. agalactiae* S01-196-16 (biotype 1) showed that the lowest cumulative mortality during the observation period, which is lasted for 14 days, was obtained in group A (15.26%), followed by B (24.59%), and the latest is a control group (64.44%). The pattern of mortality during the challenge test period against *S. agalactiae* S01-196-16 bacteria can be seen in Figure 2.

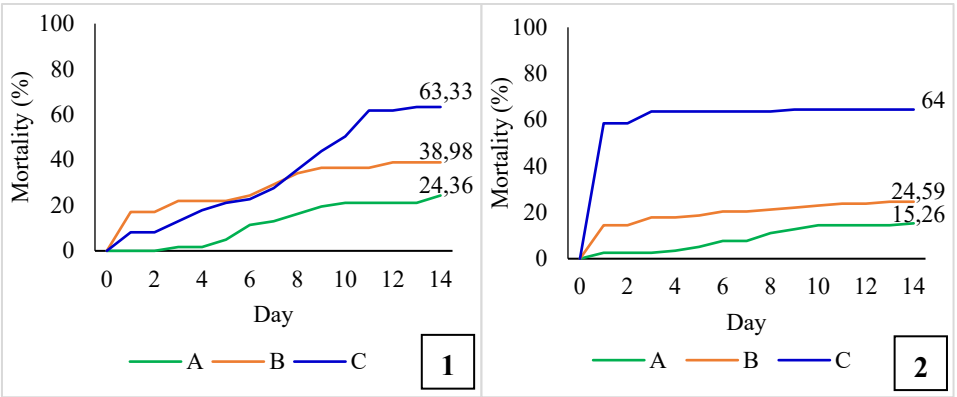

Fig. 1. & 2. Cumulative mortality of vaccinated and non-vaccinated fish at the first challenge (30 days post-vaccination) against *Streptococcus agalactiae* N14G (Fig. 1) and *S. agalactiae* S01-196-16 (Fig. 2) infections at the dose of 60% (LD_{60}). A = trivalent vaccine, B = bivalent vaccine, and C = control.

Statistical analysis on the mortality value during the batch-I challenge test against *S. agalactiae* N14G (biotype 2) and *S. agalactiae* S01-196-16 (biotype 1) bacteria showed significant differences between vaccinated fish compared to control at a 95% confidence interval ($P < 0.05$). Furthermore, the analysis value between vaccines A and B revealed significant differences between vaccines, A and B. These results indicate that vaccines A

and/or B increased the fish immunity and protection against *S. agalactiae* (biotype 1 & 2) bacterial infection.

Challenge test against *S. agalactiae* N14G bacteria in batch II (3rd-month post-vaccination) showed that the lowest cumulative mortality of test fish during the 14-day observation period was obtained in group A (34.32%), followed by B (43.53%), and the highest is a control group (61.11%). The pattern of mortality during the challenge test period against *S. agalactiae* S01-196-16 bacteria is shown in Figure 3. While the results of the challenge test against *S. agalactiae* S01-196-16 showed that the lowest cumulative mortality was obtained in group A (39.28%), followed by B (43.13%), and the highest is a control group (67.78%). The pattern of mortality during the challenge test period against *S. agalactiae* S01-196-16 bacteria is shown in Figure 4.

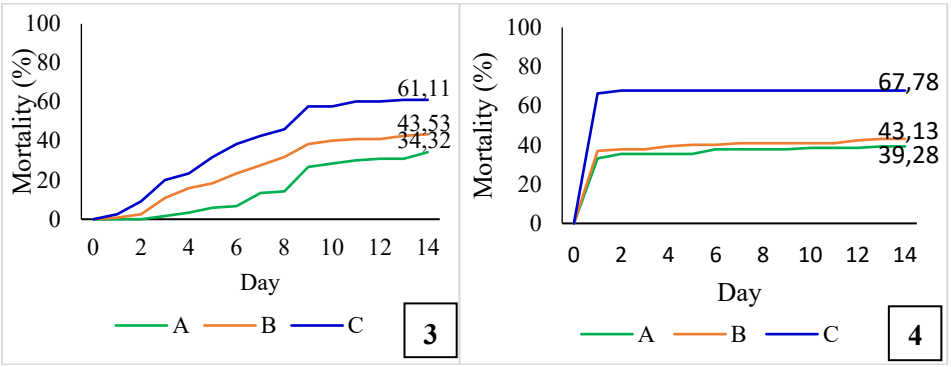

Fig. 3. & 4. Cumulative mortality of vaccinated and non-vaccinated fish at the second challenge (3rd months post-vaccination) against *Streptococcus agalactiae* N14G (Fig. 3) and *S. agalactiae* S01-196-16 (Fig. 4) infections at the dose of 60% (LD_{60}). A = trivalent vaccine, B = bivalent vaccine, and C = control.

Statistical analysis of the mortality value of the batch-II challenge test against *S. agalactiae* N14G bacteria showed a significant difference between the vaccinated fish groups compared with the control group, and further analysis indicated that there were did not show any significant difference between both of the vaccines, A and B. The same results were achieved by challenged by another isolate, *S. agalactiae* S01-196-16, which revealed a substantial difference between the vaccinated fish groups and the control group. There were also did not show any significant difference between the value of both vaccines, A and B.

The last challenge test was carried out at the 5th-month post-vaccination. The results of the test against *S. agalactiae* N14G showed that the lowest cumulative mortality was obtained in group A (42,74%), followed by B (50.43%), and the highest cumulative mortality was occurring in the control group (66.67%). The pattern of fish mortality during the challenge test for *S. agalactiae* N14G bacteria can be seen in Figure 5. While the results of the challenge test for *S. agalactiae* S01-196-16 showed that the lowest cumulative mortality was obtained in group A (41,98%), followed by B (46.48%), and the highest cumulative mortality was occurring in the control group (62.22%). The pattern of fish mortality in the latest challenge test against the *S. agalactiae* S01-196-16 bacteria is shown in Figure 6.

Statistical analysis of the cumulative mortality at the batch-III challenge test against *S. agalactiae* N14G bacteria showed a significant difference between the vaccinated fish groups and the control group. The same result was achieved against *S. agalactiae* S01-196-16 bacteria. As obtained in the batch-II challenge test, the results indicate that after 5th month of the administration, vaccines A and/or B were still able to increase the body resistance of the test fish against *S. agalactiae* bacterial infection (biotype 1 & 2).

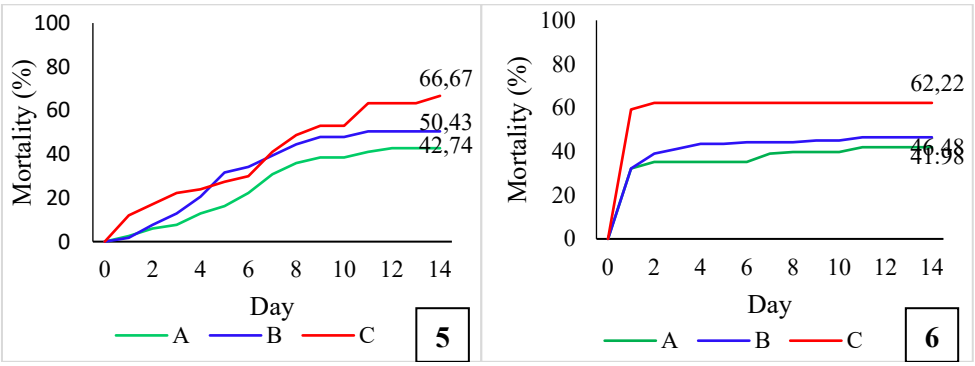

Fig. 5. & 6. Cumulative mortality of vaccinated and non-vaccinated fish at the second challenge (5th months post-vaccination) against *Streptococcus agalactiae* N14G (Fig. 5) and *S. agalactiae* S01-196-16 (Fig. 6) infections at the dose of 60% (LD_{60}). A = trivalent vaccine, B = bivalent vaccine, and C = control.

Re-isolation of bacteria were taken from randomized moribund and obvious clinical signs of fish at all of three different groups during the challenge test period, there were confirmed that the primary pathogenic agents on diseased fishes are *S. agalactiae* (biotype 1 & 2) infection, depending on the biotype bacteria were injected into the fish. Further bacterial re-isolation and identification indicate that the challenge test procedure applied in the study was properly worked and traceable.

In this study, RPS was used to evaluate the efficacy of the vaccines, calculated according to a formula developed [17]. The RPS values achievement of bivalent and trivalent vaccines against defined pathogenic *S. agalactiae* is shown in Table 1.

Table 1. Relative Percentage of Survival (RPS) of inactivated bivalent and trivalent *Streptococcus agalactiae* (biotype 1 & 2) vaccines on tilapia, *Oreochromis niloticus*

Challenge	Vaccine type	Isolate bacteria and mortality (%)		Isolate bacteria and RPS (%)	
		N14G (biotype-2)	S01-196-16 (biotype-1)	N14G (biotype-2)	S01-196-16 (biotype-1)
1st	Trivalent (A)	24.36	15.26	61.53	76.32
	Bivalent (B)	38.98	24.59	38.45	61.84
	Control (C)	63.33	64.44	-	-
2nd	Trivalent (A)	34.32	39.28	43.84	42.05
	Bivalent (B)	43.53	43.15	28.77	36.37
	Control (C)	61.11	67.78	-	-
3rd	Trivalent (A)	42.75	41.98	35.88	32.53
	Bivalent (B)	50.43	46.48	24.36	25.30
	Control (C)	66.67	62.22	-	-

At the first challenge test, the RPS value of trivalent vaccine against *S. agalactiae* biotype-1 was 76.32%, and 61.84% for a bivalent vaccine. At the same time, the RPS value of the trivalent vaccine against *S. agalactiae* biotype-2 was 61.53% and 38.45% for the bivalent vaccine. At the second challenge test, the RPS value of trivalent vaccine against *S. agalactiae* biotype-1 was 42.05%, and 36.37% for the bivalent vaccine. At the same time, the RPS value of the trivalent vaccine against *S. agalactiae* biotype-2 was 43.84% and 28.77% for the bivalent vaccine. Finally, at the third challenge test, the RPS value of trivalent vaccine against *S. agalactiae* biotype-1 was 32.53%, and 25.30% for a bivalent vaccine. Whereas the RPS value of trivalent vaccine against *S. agalactiae* biotype-2 was 35.88% and 24.36% for a bivalent vaccine.

4 Discussion

Studies on efficacious of inactive monovalent *Streptococcus* spp. (mostly *S. iniae* and *S. agalactiae*) vaccines to control streptococcosis on tilapia have been reported by many researchers with the varying achievement of success. Evaluation has been conducted on the effectiveness of *Streptococcus* spp. vaccines in tilapia (*Oreochromis niloticus*), and it was proved that there is no cross-protection property of *S. iniae* antigen against *S. agalactiae* infection at challenge test. Therefore, prevention against *S. agalactiae* infection on tilapia should be used vaccine prepared from homologous bacterin [18]. Another study on fish vaccine application by administering single and double doses of *S. agalactiae* vaccine on tilapia revealed that the RPS value of vaccinated fish was 83.6% for single doses and 96.4% for double doses [19].

The development of the monovalent *S. agalactiae* vaccine in Indonesia has been carried out intensively by research institutes and universities since the last decade. Screening for immunogenic properties of more than 50 *S. agalactiae* isolates collected from diseased tilapia in West and Central Java, and the selected isolate (*S. agalactiae* N14G) was used as an antigen source for vaccine preparation. The study indicated that the whole cell bacterial vaccine gives higher protection than broth and supernatant vaccines [4]. Improving and refining S. *agalactiae* vaccines continued [5-6, 15, 20-23] and many other researchers. Naturally, *S. agalactiae* infecting tilapia in Indonesia revealed two distinct biotypes, haemolytic *S. agalactiae* (biotype 1) and non-hemolytic *S. agalactiae* (biotype 2), and each biotype has different pathological and mortality pattern characteristics on tilapia [7].

Currently, fish vaccination has been routinely applied to control contagious and endemic diseases in commercial aquaculture operations. Many fish vaccine products utilize in-active antigens in the form of injectable adjuvanted vaccines that contain two, three or even more antigens to prevent several potential and endemic diseases [24]. The advantage of bivalent or trivalent vaccine is that a single dose containing different biotypes or antigens may be administered so that vaccines to prevent several types/variants of pathogens in aquaculture will be more effective and economically feasible for aquaculture low-value fish species.

The study results showed that inactivated bivalent and trivalent S. agalactiae vaccines containing biotype-1 & 2 have properties to work synergistically to prevent tilapia against both biotypes of *S. agalactiae* infection. The RPS value achieved by trivalent vaccines against defined pathogenic S. agalactiae biotype-1 was 76.32% and 61.84% for the bivalent vaccine. The RPS value of trivalent vaccine against *S. agalactiae* biotype-2 was 61.53% and 38.45% for a bivalent vaccine. Many studies on bivalent/multivalent fish vaccines have been reported, [25] comparing the efficacy of monovalent, bivalent, and polyvalent vaccines on tilapia. The results showed that immersion application of polyvalent vaccine gives higher effectiveness and effectively prevents more than one type of bacteria. Comparative efficacy study of 3 different preparations on rainbow trout (*Oncorhynchus mykiss*) via immersion application: lipopolysaccharide (LPS), monovalent and polyvalent vaccines. Again, it was proven that bivalent vaccine obtained higher RPS value than monovalent vaccine and LPS [26].

The protection of tilapia against pathogenic *S. agalactiae* hemolytic (biotype-1) and non-hemolytic (biotype-2) infection, after challenging by homologous isolates via IP injection at LD_{60} after 30 days post-vaccination, resulting in cumulative mortality as low as 15.26% compared to 63.33% of unvaccinated fish. At the second challenge (90 days post-vaccination), resulting from cumulative mortality as low as 34.32% compared to 61.11% of un-vaccinated fish; and at the last challenge (150 days post-vaccination), resulting in cumulative mortality in the amount of 41.98% compared to 62.22% of unvaccinated fish. [27] Studied by using the bivalent vaccine containing *S. iniae* and *Vibrio vulnificus* bacteria

on sex-reversed hybrid tilapia (*O. niloticus* x *O. aureus*), the results showed that the vaccine was working properly against both of the bacterial pathogens.

This study indicates that protective levels of the vaccine efficacy decreased in line with the addition of time, and the vaccines' immunity duration is at least five months for a single application. A specific study on the course of immunity (DoI) of *S. agalactiae* vaccine on tilapia has been carried out [10] revealed that there was a good correlation between specific antibody concentrations and survival rate after the challenge test and lasted for at least 180 days post-vaccination. Therefore, it was recommended that antibody level be used as a non-lethal monitoring tool to assess the protection level and the efficacy of vaccination. [28] using formalin killed *S. iniae* vaccine preparation to rainbow trout, *Oncorhynchus mykiss,* and the protection against homologous bacteria lasted six months post-vaccination. Specific antibody levels decreased over time, starting from an antibody titer of 1:20 to 1:1.

Relative Percent Survival (RPS) is the gold standard parameter to evaluate the efficacy of fish vaccines in aquaculture. The value is based on the calculation of the number of survivals of vaccinated fish during the defined challenge test against pathogenic agents relative to the number of the control fish [29]. The achievement of the RPS values of bivalent and trivalent vaccines in this study is relatively low. On the other hand, technical requirements of national fish drug regulation require at least 50% of RPS value for fish vaccine products to get a registration number from the competent authority. Both of the vaccines have protection against targeted pathogens. However, the duration of immunity of a single application is less than three months. Although we do not know yet, it is strongly suspected that revaccination (booster) could increase specific immunity, and the duration of protection will last longer. Furthermore, [29] noted that an ideal fish vaccine is safe for the fish and the environment, economical for large-scale production, practically administered, specific immunity protection for long-lasting, and demonstrates minimal side effects.

5 Conclusions

The summary of this study indicates that both inactive bivalent and trivalent vaccines containing different biotypes *S. agalactiae* are promising and potential to be used for “streptococcosis” prevention on tilapia. The vaccines have the properties to work correctly and synergistically against *S. agalactiae* biotypes 1 & 2. A trivalent vaccine relatively has better performance in terms of Relative Percent of Survival (RPS) than a bivalent vaccine, even though both vaccines have relatively low RPS values. Improving and refining vaccines effectively are needed to gain better protection levels and a longer duration of immunity.

6 Authors’ contribution

All authors contributed from technical research activities to writing the final manuscript. The contributions of each author are as follows, Taukhid and A.M. Lusiastuti: constructing the central concept, designing research, collecting and analyzing data, and drafting the manuscript. Septyan Andriyanto, Desy Sugiani, Tuti Sumiati, and Achmad Suhermanto: collecting and analyzing data, criticizing and revising articles. All authors discussed the results and contributed to the final manuscript.

7 Conflict of interest

The authors declare that they have no competing interests.

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