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Research Article
Simultaneous Administration of *Boesenbergia pandurata* Extract and Vaccination to Stimulate Immune Response in Tilapia, *Oreochromis niloticus*

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Abstract

Background and Objective: The use of adjuvants or immunostimulants is often necessary to increase vaccine efficacy, in this study we evaluated the improvement of the immune response in tilapia treated by either oral and immersion administration with vaccine and *Boesenbergia pandurata* extract (BPE). **Materials and Methods:** The initial concentration of BPE and the cell density of vaccine were 900 mg L^{-1} and 10^4 CFU mL^{-1} for oral administration while 10^6 CFU mL^{-1} for immersion, respectively. The extract and vaccine were mixed homogeneously in a ratio of 1:1. Further, the mixture was supplemented to feed at 1 mL g^{-1} feed. Tilapia with average initial body weight of 15 g were fed containing vaccine and BPE 3 times a day. The other group of fish was immersed with vaccine and BPE for 20 min. After 7th (d7), 14th (d14) and21th (d21) days of treatment, a challenge test was conducted by intramuscularly injection of 0.1 mL of *Aeromonas hydrophila* and *Pseudomonas fluorescens* mixture (1:1) at a density of 10^5 CFU mL^{-1}. Antibody levels, total white blood cell (WBC) and phagocytic activity (PA) were evaluated to determine the immune improvement of the fish. Furthermore, relative percent survival (RPS) and the survival rate (SR) were evaluated at week 2 and 4 after challenge test. **Result:** Results indicated that the all parameters of tilapia immune system were increased ($p<0.05$) after 2-4 weeks of both administration methods. Meanwhile, the efficacy of the vaccine has increased by combining BPE treatment using immersion method better than oral method. The RPS of vaccination plus extract by immersion was 83-100% and by oral administration was 83-87%. **Conclusion:** The present results implied that *B. pandurata* extract boost the efficacy of the *Pseudomonas* sp. vaccine by increasing the immune system and diseases resistance in tilapia.

Key words: *Boesenbergia pandurata*, immunostimulant, vaccine, non-specific immune

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

The need of eco-friendly countermeasure methods in diseases control is a crucial key for the development of sustainable aquaculture. Sustainable and intensive aquaculture systems have been faced by the risk of disease incidence and the spread of disease[1,2]. Some of the biological approaches to tackle the disease as preventive measures are needed when vaccination is not feasible in juvenile fish, crustaceans and molluscs. Vaccination as a method used for preventing disease infection in fish has been widely used in fish farming and proved with impressive results[3].

The vaccines which stimulating the adaptive immune system might be accomplished through injection[4], immersion[5,6] or oral[7,8], with the advantages and disadvantages in each method. According to Hardi *et al.*[9], traditionally, vaccines consist of attenuated pathogens, inactive pathogens or antigenic subunits. The use of attenuated vaccines or antigenic subunit vaccines has weak immunogenic properties in fish. Thus, an adjuvant and immunostimulant are needed to enhance the immune response and to optimize protection against pathogens. Adjuvants are defined as substances helping to improve the adaptive and protective responses of vaccine[10-12]. Some research report the efficiency vaccine that's combining with the adjuvant and immunostimulant. Hardi *et al.*[13] explain about increasing the *Edwardsiella tarda* formalin-killed vaccine efficiency with oral administration of immunostimulants, Aucouturier *et al.*[14] research about effectives of co-injection of interleukin 8 with the glycoprotein gene from viral haemorrhagic septicemia virus (VHSV) in rainbow trout (*Oncorhynchus mykiss*), Bogwald and Dalm[15] explained about the adjuvant effect of mushroom glucan and bovine lactoferrin to increase the *Aeromonas hydrophila* vaccination effect in catla fish.

Previous research has proved that injection of *Boesenbergia pandurate* mixed with *Pseudomonas* sp. vaccine increases RPS up to 100% in tilapia[16]. Extract of BP was immunostimulant that can improve the non-specific immunity of tilapia to against *Aeromonas hydrophila* and *Pseudomonas fluorescens*[17,18] and the concoction of BPE with *S. ferox* extract effective to prophylaxis and treatment the *Aeromonas hydrophila* and *Pseudomonas fluorescens* infection in tilapia (*Oreochromis niloticus*)[16].

Though the study regarding vaccine have been carried out in a great number publication, the method of vaccine greatly influences its efficacy. Some vaccines on the market are produced experimentally and only working out when injected (either intraperitoneally or intramuscularly). Therefore, the searching for alternative methods of vaccination (feed and immersion) in fish should be a major concern because it is technically more practical method.

MATERIALS AND METHODS

This research was done at the Laboratory of Environmental Microbiology, Faculty of Fisheries and Marine Science and Forest Products Chemistry, Faculty of Forestry, Mulawarman University, East Kalimantan, Indonesia. The research begun from January-June, 2018.

Preparation of the fish and bacterial strains (*A. hydrophila* and *P. fluorescens*): Tilapia (*Oreochromis niloticus*) which was at the age of 30 days (average total length of 5±0.5 cm) was obtained from the Freshwater Fish Seed Center, Perjiwa, Kutai Kartanegara. Before being used, the fish was quarantined in the laboratory for 7 days, fed with commercial pellets (781-1N) (CP Prima Indonesia). Fish spleen, gills and heart samples were collected to ensure that the fish was not infected by *Aeromonas* and *Pseudomonas* by streaking the samples on a specific medium of GSP agar (Merck®). If no bacterial growth on the medium after 24 h incubation at 30°C, the fish population was used for the experiment. However, when the growth of bacteria was observed on the medium, the fish was immersed in 3% formalin for 5 min and quarantined longer[19].

Aeromonas hydrophila (EA-01) and *P. fluorescens* (EP-01) for challenge test were obtained from the collection of the Microbiology Laboratory, Faculty of Fisheries and Marine Science, Mulawarman University, Indonesia. The bacteria were grown in the brain heart infusion broth medium (BHIB DIFCO®) for 24 h at 30°C. The bacteria concentration for challenge test was 10^5 CFU mL^{-1} and injected 0.1 mL each fish intramuscularly. The bacteria density for vaccine using oral administration method was 10^4 CFU mL^{-1}, while by immersion method was 10^6 CFU mL^{-1}.

Sample preparation and extraction of *Boesenbergia pandurata*: *Boesenbergia pandurata* which was collected from traditional markets in Samarinda was washed and air dried, then sliced in a.p. 0.5 cm. The sample was dried in oven at 40°C for 24-48 h. The dried pieces of *B. pandurata* were chopped using a blender. The chopped sample was then macerated in 96% ethanol (w/v, 1:1) at room temperature for 48 h[8]. the macerated result was filtrated through a filter paper and the extract was evaporated to dryness at 40°C. The obtained extracts were kept in the refrigerator at -4°C until used. The concentration[16,17] of BPE was 900 mg L^{-1}.

Vaccine preparation: Vaccine was prepared following the method previously used by Dalmo and Bogwald[11] as follow: The *P. fluorescens* was cultured in BHIB medium (BD BactoTM) for 24 h at 30°C until bacterial concentration reached 10^6 CFU mL^{-1}. The bacterium was in activated with 3% of neutral buffered formalin for 24 h at 30°C. The vaccine viability was tested by inoculating the vaccines on GSP agar medium and incubated for 24 h at 30°C. If there was no bacterial growth on the medium, the vaccine was used for further experiment. Before being used, the suspension was centrifuged at 7,000 g for 30 min at room temperature, the pellet was washed twice with phosphate buffer saline (PBS) and the vaccine was stored at refrigerator until used. The density of bacterial cells was 10^4 CFU mL^{-1} for oral vaccination and 10^6 CFU mL^{-1} for immersion vaccination.

Research design: This experiment consisted of 4 groups in triplicates to evaluated the effect of BPE with different methods administration to increasing the vaccine efficiency in tilapia.

Group A : Oral administration fish with combination of *Pseudomonas* vaccine and BPE (1:1)

Group B : Immersion fish with combination of *Pseudomonas* vaccine and BPE (1:1)

Group C : Immersion fish with *Pseudomonas* vaccine without BPE

Group D : Control group (fish are given a normal feed without vaccine and BPE)

Oral administration method was performed by mixing vaccine and BPE (ratio 1:1) to the commercial feed at rate 0.1 mL g^{-1} feed. Fish were fed with the mixture three times a day in the morning, afternoon and evening. Meanwhile, immersion administration was done by immersing the fish in the vaccine and extract solution for 20 min.

Each group was challenged with the bacteria by intramuscular injection of 0.1 mL *P. fluorescence* and *A. hydrophila* mixture (1:1) at day 7 (d7), 14 (d14) and21 (D21) post vaccination. The density of each bacterium was 10^5 CFU mL^{-1}. Blood sampling was carried out to evaluate non-specific immune parameters after 2 and 4 weeks of the challenge test. The blood was withdrawn through the caudal vein using 1 mL syringe with 3% EDTA as anticoagulant.

White blood cells count: To examine total white blood cells, fish blood was withdrawn, put into micro tube and the blood sample was sucked with a leukocyte pipette up to 0.5. The blood sample was added with Turk's solution into 11 scales, wiggling the pipette to homogeneously. The first droplet of blood mixture was removed and inserted into the hemocytometer, covered with a cover glass, put on the microscope and accounted the cells. The number of white blood cell was determined on the five large boxes of hemocytometer and calculated by using the formula as follow Kent *et al.*[20]:

$$\text{Total leucocyte} = \Sigma \text{ leucocyte cells} \times 50 \text{ cell mm}^{-3}$$

Antibody levels: Antibody levels were measured with ELISA[21]. The reading of the measurement used Microplate Reader (Kayto RT-2100C). The number of specific antibodies was stated in the OD value with a wavelength of 450 nm.

Phagocytic index: Phagocytic index was measured according the method published previously[19,20,22]. A 50 µL of blood was put into the micro tube and added with 50 µL of a *Staphylococcus* aqueous suspension in PBS (10^7 cells mL^{-1}). The mixture was homogenized and incubated at room temperature for 20 min. Following the incubation, the mixture was placed on the glass object, dried it out and fixed with methanol for 5 min and continued to air-dry. After completely dried, the sample was stained with Giemsa for 15 min, washed with running water and dried with tissue. Finally, the sample was observed for 100 phagocyte cells.

Survival rate (SR) and RPS (Relative percent survival): The effectiveness of BPE and vaccine was measured by observing the fish mortality after challenges test with *A. hydrophila* and *P. fluorescens*. The survival rate (SR) and relative percent survival (RPS) were measured using Ellis[23] method:

$$\text{Survival rate}(\%) = \frac{\sum \text{alive fish at the end of the research}}{\sum \text{alive fish at the start of the research}} \times 100$$

$$\text{RPS}(\%) = 1 - \left[\frac{\text{Percent of fish treatment mortality}}{\text{Percent of control mortality}}\right] \times 100$$

Statistical analysis: All data were presented in the average and standard deviation of three independent measurements. All parameters were analyzed using nonparametric two-way ANOVA (SPSS 22 Inc., USA). A significant difference at ($p<0.05$) was used to determine the significance difference between control and vaccine groups.

RESULTS

White blood cells and phagocytic activity: The total WBC in all groups had increased since week 2 after challenges. However, in 2 and 4 weeks at d14 after challenge, the WBC of fish in group B showed significantly different ($p<0.05$) with control. The vaccination was also able to increase the WBC and resulted significantly different ($p<0.05$) with controls in groups A, B and C in the d21 after challenge. This study discovered, only the administration of *B. pandurata* extract mixed with vaccines through immersion (B) showed significantly increase and different with the other treatments (Table 1).

Similar results can also be seen on the tilapia phagocytic activity (PA) in the groups A, B and C. The tilapia in the groups A and B showed significantly different phagocytic index ($p<0.05$) compared to control D at d14 and d21 challenges (Table 2). Meanwhile, the significantly increase in PA in group B occurred at week 2 through immersion and at week 4 through oral administration. The increasing vaccine efficacy was indicated by the increase in PA value that was also higher found than vaccine without extract group C since d14 challenges. The significantly increasing of PA in group C occurred at d21 challenges. In addition, the administration of BPE. in the A and B groups was able to enhance vaccine efficacy indicated by high PA value comparing to the C group, at d14 and d21.

Antibody levels: Antibodies are Immunoglobulin (Ig) molecules secreted by plasma B cells and are the final step of their response to the specific phatogen[24].The antibody level of fish in the groups A, B and C showed significantly higher than that group D at d14 and d21 (Table 3). Either oral and immersion administration) were found no significantly different ($p>0.05$) in the antibody level. This study discovered that oral or immersion methods administration of vaccine and extract is able to increase significantly fish antibodies since d14 (week 2) challenges. While vaccine administration without extract (group C) increasing significantly occurred in d14 (week 4). Thus a new theory on improvement of antibody levels in fish, is vaccine with extract combination.

Relative percent survival (RPS): Vaccination in tilapia can decrease the mortality after infection and mortality in group A and B, lower than group C in d7 challenges. Even, no fish mortality in group B after challenges with bacteria in d21. The data of mortality of fish, explain about the SR too, the highest SR up to 100% in immersion methods in d21 challenges. Table 4 results indicated that the combination treatment of vaccine and BPE provided a level of protection to bacterial infections, starting from d7 of the challenge test. The vaccination mix with BPE (group A and B) give higher RPS than vaccination (group C) in all days challenges and the RPS significantly different between group (A and B) to group C.

Table 1: Total white blood cells (cells mm^{-3}) of tilapia after vaccination and challenge tests with *A. hydrophila* and *P. fluorescens*

	D7		D14		D21	
Vaccine treatments	Week 2	Week 4	Week 2	Week 4	Week 2	Week 4
A	10436.7±0.003^a	14026.7±0.002^a	13926.7±0.002^a	25300.0±5.9^b	25910.0±0.001^b	26380.0±0.30^b
B	11210.0±0.002^a	18916.7±0.002^a	20693.3±1.300^b	30450.0±0.4^c	31350.0±0.100^c	32143.3±0.00^c
C	10700.0±0.60^a	13206.7±0.200^a	12316.7±0.001^a	15356.7±0.3^a	23140.0±0.100^b	23543.3±0.60^b
D	3805.4±5.40^a	10110.0±0.001^a	10766.7±0.600^a	14166.7±0.8^a	13426.7±0.003^a	12790.0±0.40^a

Means in the same column followed by the same superscript letters are not significantly different, as determined by Tukey's test ($p>0.05$), D7: Challenges time in days 7 after vaccination, D14: Challenges time in days 14 after vaccination, D21: Challenges time in days 21 after vaccination, Week 2: Observation time in day 14 after challenges, Week 4: Observation time in day 28 after challenges, A: Treatment of vaccination+oral administration of BPE, B: Treatment of vaccination+immersion administration of BPE, C: Vaccination treatment, D: Control group

Table 2: Phagocytic activity (%) of tilapia after vaccination and challenge test with *A. hydrophila* and *P. fluorescens*

	D7		D14		D21	
Vaccine treatments	Week 2	Week 4	Week 2	Week 4	Week 2	Week 4
A	30.33±0.58^a	31.00±0.00^a	41.67±0.58^c	56.83±0.76^c	57.67±0.58^c	58.67±0.58^c
B	31.00±0.00^a	32.33±0.29^a	40.33±0.58^c	55.00±0.50^c	57.00±0.00^c	58.00±0.00^c
C	25.67±1.15^a	26.33±1.04^a	32.00±0.00^a	38.50±0.50^c	45.00±0.00^c	49.00±1.00^c
D	20.50±0.50^b	21.00±0.00^b	21.33±0.58^b	22.97±0.45^b	22.33±0.58^b	22.33±0.58^b

Means in the same column followed by the same superscript letters are not significantly different, as determined by Tukey's test ($p>0.05$), D7: Challenges time in days 7 after vaccination, D14: Challenges time in days 14 after vaccination, D21: Challenges time in days 21 after vaccination, Week 2: Observation time in day 14 after challenges, Week 4: Observation time in day 28 after challenges, A: Treatment of vaccination+oral administration of BPE, B: Treatment of vaccination+immersion administration of BPE, C: Vaccination treatment, D: Control group

Table 3: Antibody levels in tilapia after vaccination and challenge test with bacteria of *A. hydrophila* and *P. fluorescens*

Vaccine	D7		D14		D21	
treatments	Week 2	Week 4	Week 2	Week 4	Week 2	Week 4
A	0.07±0.00[a]	0.10±0.00[a]	0,16±0.00[b]	0.17±0.01[b]	0.18±0.00[b]	0.18±0.00[b]
B	0.07±0.00[a]	0.09±0.00[a]	0.16±0.00[b]	0.15±0.01[b]	0.17±0.00[b]	0.17±0.00[b]
C	0.07±0.00[a]	0.07±0.00[a]	0.08±0.00[a]	0.12±0.01[b]	0.12±0.00[b]	0.15±0.00[b]
D	0.07±0.00[a]	0.06±0.00[a]	0.07±0.00[a]	0.07±0.00[a]	0.07±0.00[a]	0.07±0.00[a]

Means in the same column followed by the same superscript letters are not significantly different, as determined by Tukey's test (p>0.05), D7: Challenges time in days 7 after vaccination, D14: Challenges time in days 14 after vaccination, D21: Challenges time in days 21 after vaccination, Week 2: Observation time in day 14 after challenges, Week 4: Observation time in day 28 after challenges, A: Treatment of vaccination+ oral administration of BPE, B: Treatment of vaccination+immersion administration of BPE, C: Vaccination treatment, D: Control group

Table 4: Relative percent survival (RPS) of tilapia after challenging on d7, d14 and d21 with *A. hydrophila* and *P. fluorescens*

	Mortality (%)			SR (%)			RPS (%)		
Groups	D7	D14	D21	D7	D14	D21	D7	D14	D21
A	13±0.06[a]	13±0.06[a]	10±0.00[a]	86.67±5.77[a]	86.67±5.77[a]	90±0.00[a]	83±0.07[a]	83±0.07[a]	87±0.01[a]
B	13±0.06[a]	10±0.00[a]	0.00[d]	87.00±5.77[a]	90.00±0.00[a]	100±0.00[a]	83±0.07[a]	87±0.01[a]	100±0.00[a]
C	23±0.06[b]	20±0.00[b]	20±0.00[b]	76.70±5.77[a]	80.00±0.00[a]	80±0.00[a]	70±0.06[b]	74±0.02[b]	74±0.02[b]
D		77±0.06[c]			23.00±5.77[b]				

Means in the same column followed by the same superscript letters are not significantly different, as determined by Tukey's test (p>0.05), D7: Challenges time in days 7 after vaccination, D14: Challenges time in days 14 after vaccination, D21: Challenges time in days 21 after vaccination, Week 2: Observation time in day 14 after challenges, Week 4: Observation time in day 28 after challenges, A: Treatment of vaccination+oral administration of BPE, B: Treatment of vaccination+immersion administration of BPE, C: Vaccination treatment, D: Control group

Data of mortality, SR and RPS (Table 4) descript the level of infection protection in tilapia. Both administration of vaccine mix extract can reduce the mortality than vaccine and control group. However, between immersion and oral does not show significantly differences (p<0.05) in RPS.

DISCUSSION

Vaccines are chosen to prevent bacterial infections because they are safe and effective. Unfortunately, sometimes vaccines cannot provide their own protection, especially vaccines that are made from recombinant of antigens or non-virulent pathogens[4]. Therefore, the use of adjuvants or immunostimulants is often needed to improve the efficacy of these vaccines. The use of adjuvants in vaccination for humans[25,26] and fish[27,28] has been done for a long time, because their capability in increasing of immunogenicity from the given vaccines. Interestingly, some ingredients of adjuvants derive from aluminum, water-in-oil emulsions (Freund's adjuvants), cell microorganisms components and plant extracts[26,29].

Past study on different vaccination methods (bath, cohabitation, intramuscular and intraperitoneal) in the challenge test with furunculosis affection show the different level of protection (RPS) in *Salmo salar*[30,31]. In addition, Poornima *et al.*[32] based on their research in the vaccination of furunculosis in *Oncorhynchus clarkia*, found that intramuscular injection of vaccine is more effective than oral vaccination and immersion method show the more effective than oral administration. The results are in line with the present finding confirmed that the injection of combination BPE. and *Pseudomonas* sp. vaccine provided 90% protection in the challenged tests at d7 and d14 and 100% at d21 Hardi *et al.*[19]. Moreover, in this research RPS of group B (Immersion method) reached 83, 87 and 100% when be challenged at d7, d14 and d21, respectively. The RPS was lower when the vaccine was administered orally with RPS of 83-87%. Current result also found that the most effective method on the use of BPE as an adjuvant for *Pseudomonas* sp. vaccine is through immersion.

Plant extracts from *Quillaja saponaria* Molina and *Gypsophila paniculata*[5], *B. pandurata*, *S. ferox* and *Z. zerumbet*[9,10] which contain saponins, flavonoids, carbohydrates provide biological activity as immunomodulators for fish. Furthermore, *Azadirachta indica*, *Ocimum sanctum* and *Curcuma longam* extracts are also able to increase the activity of phagocytosis, respiratory burst and alternative complement activity, lysozyme level in goldfish (*Carassius auratus*)[33]. Saponins are reported to play an important role as adjuvants because it can stimulate Th1 and Th2 responses[34-36]. The use of saponin from *Quillaja saponaria* Molina consisting of a mixture of more than 25 saponin

molecules in which the usage is combined with the *Edwardsiella tarda* vaccine provides a higher survival rate[37].

The use of BPE in the vaccine can increase the SR and RPS after infection with *A. hydrophila* and *P. fluorescens*, reaching 83-87% through oral administration and 83-100% through immersion method. Meanwhile, different time of challenge test also found that administration of BPE mixed with vaccines accelerated the vaccine to be work out and prolongs the vaccine protection that be accordance to the increase in white blood cell production after d7 and d21 of vaccination. This finding is in line with the statement of Ashida *et al.*[38] revealing that adjuvants increase phagocytosis activity, respiratory burst and alternative complement activity of fish and adjuvants as well as being able to modulate intrinsic antigen immunogenicity, enhancing the ability to prevent infection[39]. Guy[24] also stated that saponins from plant extracts were proven to be able to enhance fish-specific immune systems when mixed with vaccines. Furthermore, the saponin component of *Q. saponaria* extract is also able to protect the existence of rotavirus to host cells, through destruction of protein in cell membranes[40]. Saponins also trigger growth and mucosal immune responses that prevent from infections in humans[41]. In addition, previous study by Wang *et al.*[26] showed that administration of *Q. saponaria* saponin (QSS) (45 mg L^{-1}) mixed in *Vibrio anguillarum* formalin-out wiping vaccine was able to increase antibody production on the 28th day after vaccination in *Scophthalmus maximus*. Present study also found that BPE administration was able to enhance the efficacy of the *Pseudomonas* sp. vaccine, indicated by the increase in white blood cells, phagocytic activity and the level of antibody titers. Further, different methods administration of extract both through immersion and oral administration in all groups showed no significant difference but found significantly different comparing to the vaccine without extract.

Dalmo *et al.*[39] revealed that the use of *Quillaja saponaria* saponin (QSS) increased the efficacy of vaccines through immersion that is in line with the present result. The immersion and oral administration of BPE that contained saponin were able to increase the effectiveness of macrophage of cell phagocytic cells and enhance antibody production after infection with *A. hydrophila* and *P. fluorescens*[42]. Moreover, plant extracts can also boost the ability of antibodies to react with epitope antigens, so that antigens are unable to recognize host cell receptors causing failure of the process of existing antigens to the surface of the host cell (antibodies act as inhibitor). In addition, extracts can also accelerate the elimination of antigens by the process of opsonization (antibodies as opsonin). Antigens in an isolated state are more easily recognized by macrophages and are more effective to destroy[43,44].

CONCLUSION

The study showed the use of BPE combined with the *Pseudomonas* sp. vaccine improved immunity responses in tilapia in the terms of white blood cells, phagocytic activity and antibody level, which further causes the increase in protection level (RPS) and fish survival rate after *A. hydrophila* and *P. fluorescens* infections. Further, *B. pandurata* extract has the opportunity to be developed into an adjuvant in the use of vaccines in freshwater fish. For future research, deeply study about the specific components (saponin, alkaloid, steroids) in BPE that are responsible for the synergy of work with vaccines.

SIGNIFICANCE STATEMENT

The present article provides the more deeply study results on effectiveness of *B. pandurata* extract to increase the non-specific immune response and to increase protective effect of *Pseudomonas* sp. vaccine in tilapia against *A. hydrophila* and *Pseudomonas fluorescens* infection through the different methods administration (immersion and oral).

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