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Research article

Effects of hydrogen peroxidase-inactivated vaccine against *Streptococcus agalactiae* infection in red tilapia (*Oreochromis* sp.) via different routes of administration

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Abstract

Hydrogen peroxidase-inactivated vaccines have recently developed in controlling infectious diseases in aquaculture. The present study aimed to compare the efficacy of the hydrogen peroxidase-inactivated *Streptococcus agalactiae* vaccine via different delivery routes (injection, immersion, and oral administration) in red tilapia (*Oreochromis* sp.) without using adjuvant. Fish were randomly divided into 4 groups including G1: Control treatment (without vaccine), G2: vaccine-based diet (oral administration), G3: vaccinated by immersion and G4: vaccinated by injection. After 6 weeks of the experiment, fish were intraperitoneally injected with *S. agalactiae* and the mortality was recorded in 14 days. The results showed that lysozyme activity was differentially increased according to the delivery routes of vaccine, organs and time of sampling. However, the specific antibody levels in all vaccinated groups were only increased in week 6 post-vaccination. After the challenge test with *S. agalactiae*, the serum lysozyme levels in G3 and G4 were significantly higher than the control group (G1), while the total white blood cells and specific antibody levels were significantly increased in G2 and G4 compared to the control (G1). Similarly, the hydrogen peroxidase-inactivated vaccine statistically reduced the cumulative mortality in G2 (35.29%) and G4 (28.95%) compared to G1 (44.12%) after injected with the *S. agalactiae.* These results showed that the vaccine delivery routes by oral administration or injection may decrease the pathogen and show better protection for red tilapia than the immersion method. Further studies will be investigated to improve the efficacy of hydrogen peroxidase-inactivated vaccine via using different adjuvants.

Keywords: Hydrogen peroxidase-inactivated vaccines, immersion vaccine, injection vaccine, oral vaccine, *Oreochromis* sp., red tilapia.

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INTRODUCTION

Streptococcus agalactiae is one of the serious bacterial pathogens that cause the disease in humans, animals and aquatic species (Pang et al., 2017; Collin et al., 2020). In aquaculture, *S. agalactiae* is responsible for severe disease outbreaks in both freshwater and marine environments (Prescott et al., 2022). Particularly, *S. agalactiae* has been shown to cause significant mortality in intensive tilapia, including red tilapia (*Oreochromis* sp.) (Hernández et al., 2009; Adikesavalu et al., 2017; Wongsathein et al., 2018). The typical clinical signs in diseased fish include unilateral or bilateral exophthalmia, corneal opacity, anorexia, and body hemorrhages (Evans et al., 2015; Laith et al., 2017). Evans et al. (2006) also reported that *S. agalactiae* can cause high mortality in fish farms without any symptom detection. In Vietnam, the red tilapia infected with *S. agalactiae* was first reported by (Oanh and Phuong, 2011), which caused fast and high mortality rates up to 100% in all stages of fish. Among the 60 isolated bacterial that strains caused hemorrhagic disease in tilapia in the North of Vietnam, 52 strains (86.67%) were identified as *S. agalactiae* (Quan et al., 2013).

The great economic losses caused by *S. agalactiae* in the aquaculture industry have indicated the need to investigate more efficient prophylactic strategies to improve mortality. Vaccination is an alternative method of controlling fish disease which specifically reduced the losses as well as the use of antibiotics (Muktar et al., 2016). In aquaculture, most bacterial vaccines are inactivated vaccines that have been demonstrated to be efficacious in fish (Huang et al., 2014). Formaldehyde is the most common reagent were used to inactivate the bacterial toxin. However, (Brown, 1993) reported that formaldehyde is the cross-linking agent that could destroy the key antigenic epitopes resulting to reduce immunogenicity. For that reason, there is an unmet need for improved strategies in preparing inactivated vaccines. Recently, (Ramos-Espinoza et al., 2020b) found that the novel hydrogen peroxidase (H_2O_2)-inactivated S. agalactia vaccines possess equivalent efficacy to formaldehyde-inactivated or pH-manipulated vaccines in Nile tilapia via intraperitoneal injection. H_2O_2 is an oxidizing agent which was previously established as a potent antimicrobial and antiseptic agent (Amanna et al., 2012). Activating the pathogens by H_2O_2 resulted in lower toxicity and better preserves epitopes than formaldehyde (Fan et al., 2019). Moreover, the route of vaccine administration has also one of the major effects which contribute to the success of vaccine application. The most common way to administrate the vaccines is injection, immersion or oral administration, which depended on the fish size and stage of development as well as the kind of vaccine (Bøgwald and Dalmo, 2019; Linh et al., 2022; Dien et al., 2023). The present study aims to investigate the effects of H_2O_2-inactivated *S. agalactiae* on the innate immune response (lysozyme in head kidney, spleen and intestine mucosal), blood parameter (total red blood cells and white blood cells), adaptive immune responses (specific antibody IgM) and disease resistant to pathogens via different delivery routes (injection, immersion and feed-based administration) in red tilapia (*Oreochromis* sp.).

MATERIALS AND METHODS

Fish husbandry

Healthy red tilapia (10±2 g) were purchased from a commercial hatchery in Vinh Long province, Viet Nam. The fish were transported to the 2 m^3 composite tank and acclimatized for 2 weeks and starved for 1 day before starting the experiment. Fish were fed twice daily at 3% of body weight with standard commercial pellet feed containing 30% crude protein (Proconco) and observed carefully throughout the experiment. Prior to the experiment, 10 fish were randomly selected and tested for the presence of any pathogens.

Bacterial preparation

The *S. agalactiae* strain – SA16 isolated from the brain and head kidney of moribund cultured red tilapia in the Mekong Delta of Vietnam. The strain was cultured on brain heart infusion agar (BHI, Merck, MA, USA) plates for 48h at 28°C. Then, a single colony was collected and harvested into BHI broth (Merck, MA, USA). This suspension was shaken overnight at 180 rpm, 28°C. Bacteria were centrifuged at 5000 rpm, 4°C, 5 min and washed 3 times with 0.85% NaCl solution. The strain was stored in BHI broth containing 25% glycerol (v/v) in an ultra freezer at –80°C and then used to prepare an H_2O_2-inactivated vaccine against *S. agalactiae* for red tilapia.

Vaccine preparation

The *S. agalactiae* inactivated vaccine was prepared following (Ramos-Espinoza et al., 2020b). The bacteria were recovered and cultured on BHI agar (48h at 28°C). The single colonies were continuously cultured on BHI broth in an incubator shaker at 150 rpm, 28°C for 24h. A 30% stock solution of H_2O_2 (Fisher, Belgium) was sterilized by membrane filtration and kept in a dark sealed container. H_2O_2 was then added to inactivate the bacterial suspension with a final concentration of 5% (the optimal dose was previously determined by (Ramos-Espinoza et al., 2020b)). The mixture of H_2O_2 and bacterial suspension were incubated at 4°C for 6h. Then, the inactivated bacteria were washed three times with PBS 1X by centrifugation at 10 000g for 10 min, at 4°C. The bacteria number was adjusted by spectrophotometry at optical density (OD_{590nm} = 1.05) (SpectraMax M5, Molecular Devices, Sunnyvale) and confirmed by plate count (5.8 × 10^{10} CFU/mL) prior to activation. In order to confirm the bacterial activation, 100 µL of the inoculum was streaked onto BHI plates and incubated at 28°C, for 48 – 72h. Finally, the vaccine was stored at 4°C until use.

Experimental design

For the vaccine delivery experiment, fish were free of the bacteria pathogens were selected for the experiment. A total of 360 healthy red tilapia fingerlings were randomly assigned into four experimental groups in triplicate (30 fish per tank). The treatments were distributed as follows G1 (unvaccinated control), G2 (fish given inactivated vaccine-based diet at 1×10^9 CFU/fish), G3 (fish given immersion vaccine at 1×10^9 CFU/mL), and G4 (fish given injection vaccine at 1×10^9 CFU/mL). At day 0 (D0) after acclimation periods, the G1 group was fed with PBS-based diet, the G2 group was fed with vaccine-based diet, the G3 group was immersion with vaccine in 30 min, the G4 group was intraperitoneally injected with vaccine. In treatment G2, the fish were fed vaccine-based diet one time at 8:00 a.m. and visually observe the tank to

confirm that all feed was eaten and nothing remained in the tanks. For the treatment receiving the immersion vaccine (G3), the fish were transferred to a 100 L identical tank of aerated water including 2 L of vaccine to prepare the final concentration at approximately 1×10^9 CFU/mL. After 30 min of immersion exposure, fish were directly transferred to the free vaccine tank. For the G4 treatment, the fish were intraperitoneally injected with 0.1 mL of vaccine solution. The first and second booster doses were performed on day 14 (D14) and day 28 (D28) via feed-based diet administration at 5×10^9 CFU/mL in all vaccine treatments.

For the challenge experiment, the fish from each tank (15 fish per tank) were intraperitoneally injected (i.p.) with 0.1 mL of *S. agalactiae* suspension (LD50 – 2.1×10^5 CFU/ fish) on day 42 (D42). Cumulative mortality was recorded daily for 14 days after the challenge test. The relative percentage of survival (RPS) was calculated as follows: RPS = (1 − % mortality in vaccinated/% mortality in control) × 100 (Amend, 1981). *S. agalactiae* was re-isolated from the head kidney of the dead fish and identified by PCR confirmation to ensure that the fish mortalities were due to bacterial infection (Martinez et al., 2001).

Samples collection

Three fish per tank (9 fish/treatment) were randomly sampled on day 21 (D21), day 35 (D35), day 42 (D42) and 3 days post-challenge test. Fish were anesthetized using 0.1 ppm M222 (Sigma–Aldrich, MO, USA) before sampling. Then fish were euthanized by cervical dislocation. The protocol was also carried out in accordance with national guidelines on the protection and experimental animal welfare in Vietnam No. 79/2015/QH13 and with the approval of the Animal Ethics Committee of Can Tho University (CTU-AEC22001). Blood samples were obtained by 1 mL sterile syringes from individual fish and placed into a 1.5 mL Eppendorf tube. The blood was stored at 4°C overnight and then centrifuged (4000 rpm for 10 min at 4°C). The serum supernatant was stored at −80°C for enzyme activity analysis. Similarly, the head kidney and intestine mucus of individual fish (3 fish per tank, 9 fish per treatment) were also obtained for innate and adaptive immune parameters.

Hematological indices

Total red blood cell (RBC) counts were made with a Neubauer hemocytometer using Natt-Herrick solution as a diluent stain (Natt and Herrick, 1952). First, 10 µL of each blood sample was diluted in 1990 µL of Natt and Herrick's solution and mixed gently for at least 3 min. The cell suspension was put into the chamber and allowed to settle for 2–3 min before initiating a count under the microscope light. The RBCs were counted in 5 out of the 25 small areas. White blood cell (WBC) types were identified by smearing a small drop of whole blood on a microscope smearing slide (cover glasses 22 × 22 mm, Germany). The slide smear was quickly dried, fixed in methanol (95%, Merck, MA, USA) for 1–2 min and stained with Wright's or Giemsa (Merck, MA, USA) (Rowley, 1990).

Immunological parameters

Lysozyme activity

The lysozyme assay protocol was adapted from Ellis (1990) and Milla et al. (2010). In 96-well microplates, the lysozyme activity assay was initiated by mixing 10 µL of serum or 20 µL of sample suspension from the head kidney or intestine mucus with 130 µL of lyophilized *Micrococcus lysodeikticus* (Sigma–Aldrich, MO, USA) suspension in phosphate buffer, pH 6.2. The difference in absorbance at 450 nm was monitored between 0 and 30 min for serum (0 and 15 min for the organ samples) and used to calculate units of lysozyme activity. One unit represents the amount of lysozyme that caused a 0.001 decrease in absorbance.

The specific antibody IgM (Elisa assay)

To determine the IgM antibody levels against S. agalactiae, ELISAs using the mouse anti-tilapia (*O. niloticus*) IgM monoclonal antibody (Aquatic Diagnostics, Stirling, UK) were performed following the manufacturer's instructions. Firstly, 96-well plates were coated with inactivation *S. agalactiae* (2.5×10^8 CFU/mL) at a volume of 100 µL per well. The plate was incubated overnight at 4°C and then washed three times with low salt wash buffer (2.42 g/L Trisma base, 22.22 g/L NaCl, 0.1 g/L Merthiolate, and 0.5 ml/L Tween 20; pH 7.3). After washing, 100 µL per well of 1% (w/v) bovine serum albumin (BSA, Biobasic, Canada) diluted in PBS (pH 7.3) was added. The plate was then incubated at 28°C for 1 h. Next, 100 µl of serum diluted 1:2 in PBS was added to each well, and plate were incubated at 28 °C for 3 h. Plates were washed five times with high salt wash buffer (2.42 g/L Trisma base, 29.22 g/L NaCl, 0.1 g/L Merthiolate, and 1.0 mL/L Tween 20; pH 7.3) to wash off any unbound antibody. Then, 100 µl of mouse anti-tilapia IgM monoclonal antibody was added (1:3000) to each well, and plates were incubated for 1 h at 37 °C. Plates were then washed five times with high salt wash buffer, and 100 µl of goat anti-mouse IgG-HRP (1:8000; Proteintech Group Inc., UK) was added to each well. Plates were incubated at 28 °C for 1 h. After washing five times with high salt wash buffer, 100 µl of a tetramethylbiphenyl (TMB, Biobasic, Canada) substrate solution was added to each well. After 10 min at room temperature, the reaction was stopped by adding 100 µl of 2 M H_2SO_4 to each well. The absorbance of each well at 450 nm was measured using a microplate reader (Multiskan 354, Thermo Labsystems).

Statistical analyses

All statistical analyses were performed using SPSS version 20 (SPSS Inc, IBM, Chicago, USA). The normality of the data and the homogeneity of variance between groups were tested using Shapiro-Wilks and Levene tests. Results are presented as means ± SEM (standard error of the means). One-way ANOVA analysis of variance Duncan's multiple range test at a confidence level of 95% ($p < 0.05$) were used to determine significant differences between immunological variables in fish from the different plant extract treatments and control treatment.

RESULTS

The effects of the H_2O_2-inactivated vaccine on the red tilapia immune response via different routes of administration

Lysozyme activity

The serum lysozyme activity significantly increased in the G4 group compared to the control after one week of vaccination with the first - D21 (P = 0.036, F = 2.141) and second boosters - D35 (P = 0.038, F = 2.961) (Fig. 1A). The fish in G2 and G4 groups had a significantly higher level of serum lysozyme compared to the control (P = 0.001 and P = 0.008, respectively) and G3 group on D42 (P = 0.001 and P = 0.009, respectively) (F = 12.759). Although the inactivated vaccine did not affect the head kidney lysozyme activity on D21 and D35, all vaccine groups significantly enhanced the lysozyme levels on D42 compared to the control treatment (P = 0.007, P = 0.038, P < 0.001, respectively) (F = 13.642) (Fig. 1B). Moreover, the intestine lysozyme activity differentially increased according to the time and delivery route manners. Specifically, the lysozyme activity in all vaccinated groups was statistically higher than the control group on D42 (P = 0.005, P = 0.038, P < 0.001, respectively) (F = 15.974) (Fig. 1C).

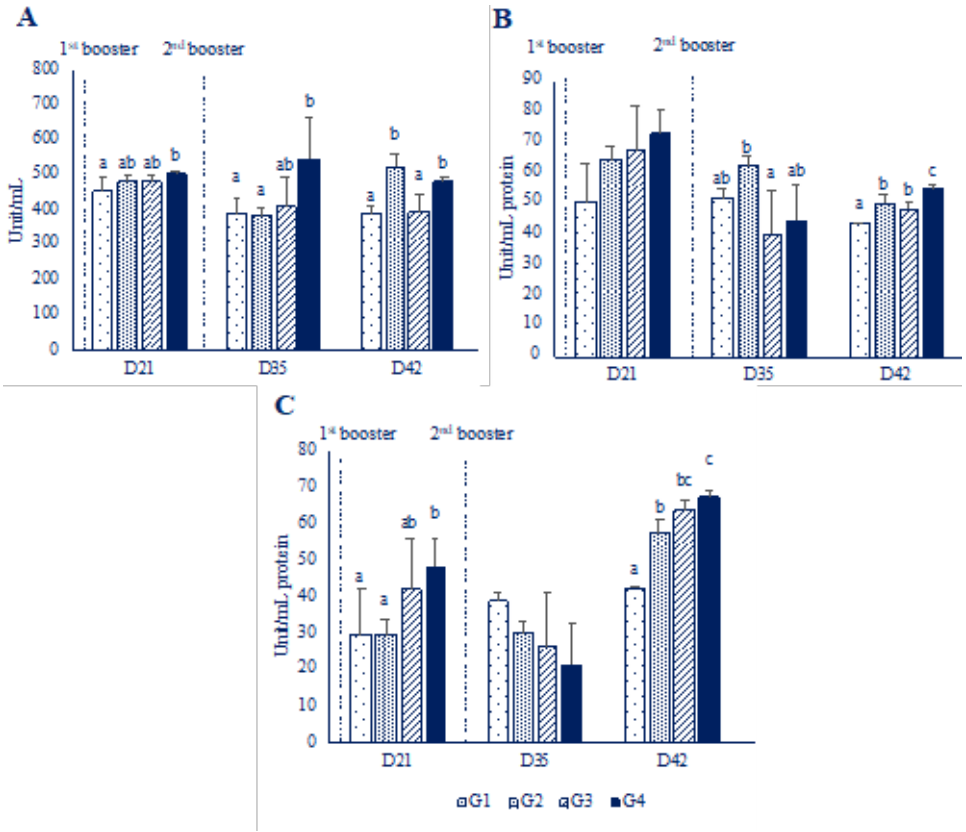

Figure 1 Effects of the H_2O_2-inactivated vaccine on the lysozyme activity in A. Serum, B. head kidney and C. intestine mucus of red tilapia before the challenge test. Data are shown as mean ± SEM, (n = 3 tanks), different letters indicate significant differences between treatments ($p < 0.05$). G1: unvaccinated (control), G2: feed-based H_2O_2-inactivated vaccine, G3: immersion H_2O_2-inactivated vaccine, and G4: injection H_2O_2-inactivated vaccine.

The specific antibody IgM

The ELISA results showed that the specific antibody IgM in the serum of red tilapia did not significantly differ between vaccinated and non-vaccinated groups at D21 and D35. However, the level of IgM was statistically enhanced in all vaccine treatments compared to the control treatment at D42 ($P = 0.012$, $P = 0.006$, $P < 0.002$, respectively) ($F = 7.927$) (Fig. 2).

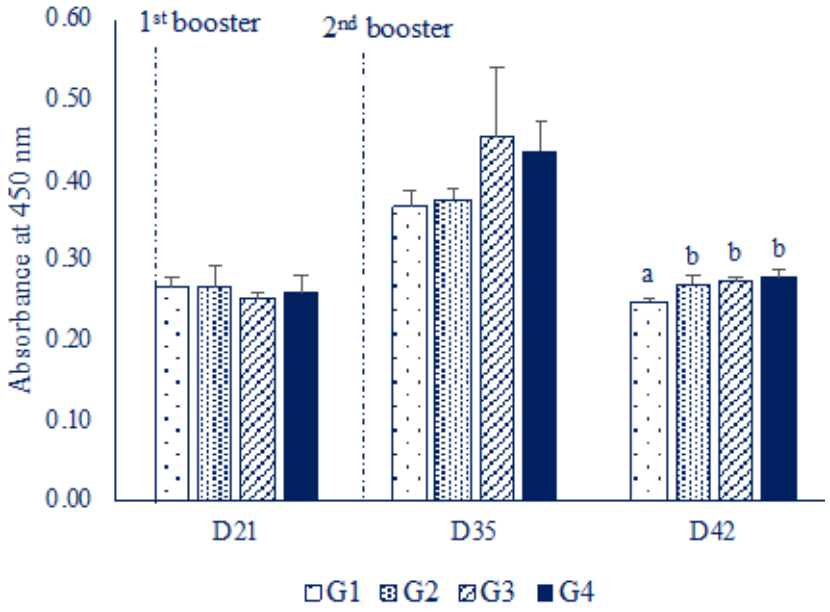

Figure 2 Effects of the H_2O_2-inactivated vaccine on the serum specific antibody IgM of red tilapia before the challenge test. Data are shown as mean ± SEM, (n = 3 tanks), different letters indicate significant differences between treatments ($p < 0.05$). G1: unvaccinated (control), G2: feed-based H_2O_2-inactivated vaccine, G3: immersion H_2O_2-inactivated vaccine, and G4: injection H_2O_2-inactivated vaccine.

The effects of the H_2O_2-inactivated vaccine on the red tilapia immune response after the challenge test

The lysozyme activity

After the challenge test with S. agalactiae, the levels of serum lysozyme activity increased in all treatments, while the lysozyme activity in the head kidney and intestine was decreased compared to those before injection. Particularly, the serum lysozyme levels in G3 and G4 treatments were significantly higher compared to the control ($P = 0.005$ and $P = 0.018$, respectively) and G2 treatments ($P = 0.009$ and $P = 0.031$, respectively) ($F = 7.113$). The lysozyme activity in the head kidney and intestine of red tilapia was not significantly different between vaccinated and non-vaccinated groups ($p>0.05$) (Fig. 3A).

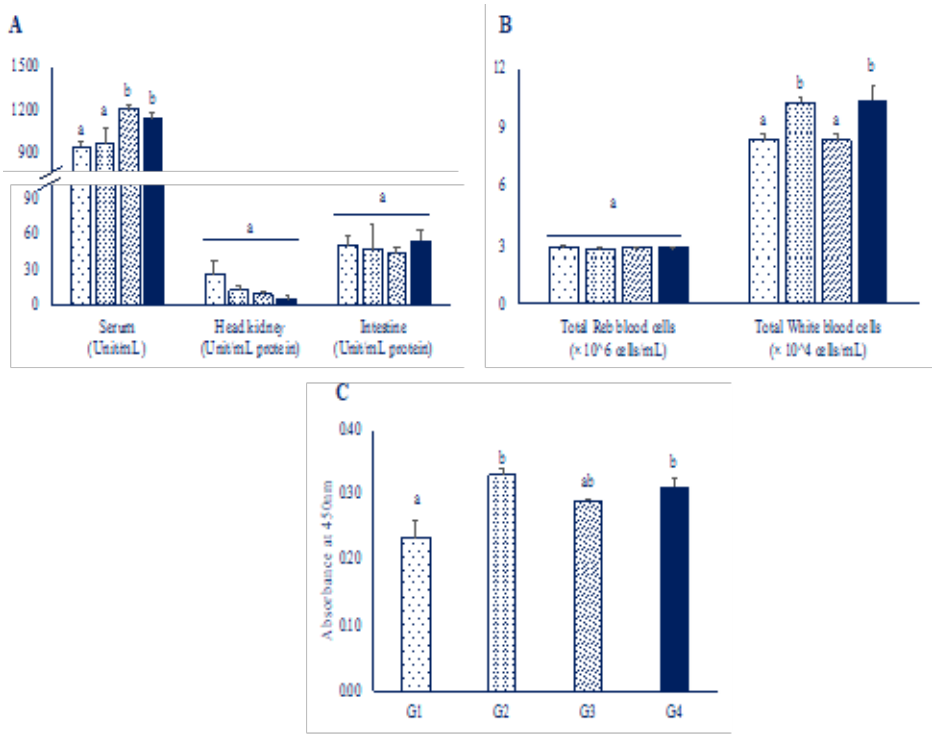

Figure 3 Effects of the H_2O_2-inactivated vaccine on A. the lysozyme activity, B. Blood parameters, and C. Serum specific antibody IgM of red tilapia after the challenge test with *S. agalactiae*. Data are shown as mean ± SEM, (n = 3 tanks), different letters indicate significant differences between treatments ($p < 0.05$). G1: unvaccinated (control), G2: feed-based H_2O_2-inactivated vaccine, G3: immersion H_2O_2-inactivated vaccine, and G4: injection H_2O_2-inactivated vaccine.

The blood parameters

The H_2O_2-inactivated vaccine did not affect the total red blood cells between treatments after intraperitoneally injected with S. agalactiae. However, the total white blood cells were statistically higher in the G2 and G4 groups compared to the G3 ($P = 0.001$ and $P < 0.001$) and control groups ($P = 0.001$, $F = 19.930$) (Fig. 3B).

The specific antibody IgM

The levels of specific antibody IgM in the G2 and G4 groups significantly enhanced compared to the control group after the challenge test with *S. agalactiae* ($P = 0.005$ and $P = 0.016$, respectively) ($F = 9.21$). Although the specific antibody IgM was slightly increased in G3, it did not differ compared to the other treatments (Fig. 3C).

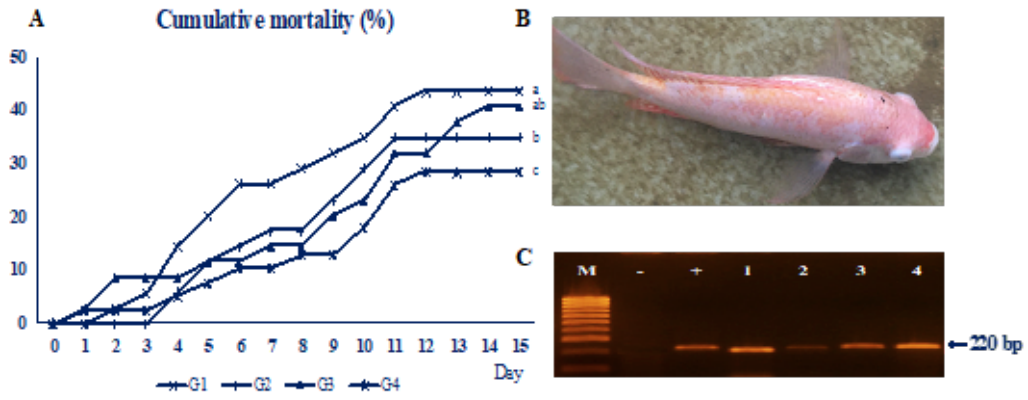

Figure 4 Effects of the H_2O_2-inactivated vaccine on fish mortality after the challenge test with *S. agalactiae*. **A.** Cumulative mortality B. Red tilapia was exophthalmia (pop-eye) after injected with *S. agalactiae*; **C.** PCR confirmation of disease fish after challenge test (-: negative control, +: positive control, 1-G1: unvaccinated (control), 2-G2: feed-based H_2O_2-inactivated vaccine, 3-G3: immersion H_2O_2-inactivated vaccine, 4-G4: injection H_2O_2-inactivated vaccine M: 100 bp marker); Data are shown as mean ± SEM, (n = 3 tanks), different letters indicate significant differences between treatments ($p < 0.05$). G1: unvaccinated (control), G2: feed-based H_2O_2-inactivated vaccine, G3: immersion H_2O_2-inactivated vaccine, and G4: injection H_2O_2-inactivated vaccine

Table 1 The relative percentage of survival (RPS) in red tilapia after challenge test with S. *agalactiae*. G1: unvaccinated (control), G2: feed-based H_2O_2-inactivated vaccine, G3: immersion H_2O_2-inactivated vaccine, and G4: injection H_2O_2-inactivated vaccine

Treatment	Mortality (%)	Relative percentage of survival (RPS-%)
G1	44.12	
G2	35.29	20.00
G3	41.18	6.67
G4	28.95	34.39

The effects of the H_2O_2-inactivated vaccine on the red tilapia mortality after the challenge test

The fish mortality did not occur during the vaccination periods (42 days of the experiment). After the challenge test with S. agalactiae, the mortality in the unvaccinated group (control treatment) started to occur on day 2 and extended until day 12, whereas the mortality in G3 and G4 groups was recorded earlier on day 1 and continued until day 12 (G4) and day 14 (G3). Moreover, the mortality in G2 was measured from day 4 to day 11 after injection (Fig. 4A). The fish clinical signs between vaccinated and non-vaccinated groups were quietly similar including exophthalmia, corneal opacity, anorexia, lethargy and erratic swimming (Fig. 4B).

The percentage of cumulative mortality in the control treatment (G1) and vaccine treatments (G2, G3 and G4) were 44.12%, 35.29%, 41.18%, and 28.95%, respectively. Specifically, the mortality in G2 and G4 groups was significantly reduced compared to the control (P = 0.009 and P = 0.001, respectively, F = 13.836). The immune protective efficacies, in terms of RPS of G2, G3 and G4 were 20.00%, 6.67% and 34.39%, respectively (Table 1.). Moreover, the PCR confirmation results showed that all treatments had a positive result with *S. agalactiae* (Fig. 4C).

DISCUSSION

A safe and effective vaccine could play an important role in maintaining aquatic animal health and reducing global infectious diseases. Most inactivated vaccines were previously performed using formaldehyde (Evans et al., 2004; Pasnik et al., 2005; Pretto-Giordano et al., 2010). However, it has many side effects on the physical and chemical characteristics of antigen epitopes resulting to reduce host immunogenicity (Fan et al., 2019). To minimize antigenic damage, there is a need to develop a new method for preparing inactivated vaccines in aquaculture. In recent years, H_2O_2 has been demonstrated to be effective in activating viruses and bacteria for production of vaccines (Amanna et al., 2012; Ramos-Espinoza et al., 2020a). (Amanna et al., 2012) also indicated that inactivating the pathogens by H_2O_2 could improve the potency and protective efficacy. Both Gram-positive and Gram-negative could be inactivated by H_2O_2 depending on its oxidizing mechanism (Fan et al., 2019). The present study aimed to investigate the effects of the H_2O_2-inactivated vaccine on red tilapia's immune responses and disease resistance before and after the challenge test via different delivery routes (oral administration, immersion and injection).

The lysozyme activity is one of the important indicators of the humoral innate immune response. It could cause cell death by breaking the N-acetylmuramic acid and N-acetylglucosamine linkages in the peptidoglycan layers of both Gram-positive and Gram-negative bacteria (Saurabh and Sahoo, 2008). The levels of lysozyme activity are varied depending on sex, age, size, season, water temperature, pH, toxicants, infections and stress (Saurabh and Sahoo, 2008). In the present study, the lysozyme activity could be stimulated by H_2O_2-inactivated vaccine via oral administration or injection compared to the control. The levels of lysozyme activity were different between the organs (serum, head kidney and intestine mucus). Specifically, fish vaccination by injection strongly enhanced the serum lysozyme activity throughout the sampling time points (D21, D35, D42 and after the challenge test). Without adding adjuvants, (Abu Nor et al., 2020) also found that the lysozyme activity significantly increased in serum, skin mucus and gut larva of red hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) injected with formalin-killed *Vibrio harveyi* compared to the unvaccinated groups. Similarly, formalin-inactivated *Edwardsiella tarda* remarkably increased the lysozyme activity in flounder (*Paralichthys olivaceus*) compared to the control after the challenge test (Wu et al., 2022). Moreover, formalin-killed *S. agalactiae* also enhanced the lysozyme activity in the serum and skin mucus of tilapia after administration with three different routes (injection, immersion and oral administration). In the present investigation, fish were immersed in H_2O_2-inactivated *S. agalactiae* did not

affect the serum lysozyme activity throughout the experiment. Without using adjuvants, our results indicated that oral feed-based and injected administration of H_2O_2-inactivated *S. agalactiae* showed a better stimulation of the innate immune response than the immersion method in red tilapia.

Vaccines induce two different stages in host-cultivated animals, the early stage of innate immune and the later stage of adaptive immune protection (Byon et al., 2006). The activation of innate immune response (lysozyme activity) in vaccinated groups could help to improve the digestive function of macrophages and then enhance the production of the specific antibody. The antibody is a crucial component in humoral adaptive immunity which could neutralize the specific antigens in fish (Burton, 2002). In the current study, H_2O_2-inactivated *S. agalactiae* did not affect the specific antibody IgM after one week of vaccination with the first and the second booster (D21 and D35). However, the levels of IgM were statistically higher in vaccinated groups (G2, G3 and G4) compared to the unvaccinated group (G1). In line with our results, (Ramos-Espinoza et al., 2020b) also demonstrated that the anti-*S. agalactiae* IgM levels did not increase at D14, but a significant increase in IgM level was detected at D28 in vaccinated groups compared to the control group. These authors also found that the stronger improvement of IgM level in the vaccinated groups was partly due to the presence of aluminum hydroxide adjuvant. Similarly, the specific antibody IgM in red hybrid tilapia also significantly increased after oral administration (feed-based vaccine) with the bivalent vaccine (combined *A. iniae* and *A. hydrophila*) and monovalent vaccine (*S. iniae*) (Monir et al., 2020). After the challenge experiment, the specific antibody IgM level in red tilapia only increased after delivering the vaccine via injection and vaccine-based diet. The results are consistent with the study of (Monir et al., 2020), the IgM levels in red hybrid tilapia were also higher in the bivalent vaccine and monovalent treatments compared to the control via feed-based vaccine after the challenge test.

Among the different blood parameters, the total white blood cell (WBC) number is a vital component for contributing to the innate and adaptive immune system of aquatic animals (Misra et al., 2006). In this study, the total WBCs significantly increased in fish that received the vaccine via injection and feed-based vaccine compared to the control group after injection with the pathogen. The same results were presented by (Monir et al., 2020) when tilapia were vaccinated with the bivalent or monovalent vaccine of *A. iniae* and/or *A. hydrophila* also enhanced the total WBCs compared to the control after the challenge test. The increase in total WBCs was strongly correlated with the enhancement of lysozyme and phagocytic activities, then activating the antibody production as well as reducing mortality in fish.

The current results showed that the cumulative mortality in vaccinated groups especially injection and oral administration groups was significantly reduced compared to the control group. Moreover, the reduction of mortality is consistent with the increase of total WBCs and specific antibody IgM in the injection and oral administration groups after the challenge test. It could be concluded that the lower percentage of cumulative mortality in vaccinated fish may be due to the improvement of the total WBCs, innate immune response (lysozyme) followed by an enhancement of the adaptive immunity (IgM). A similar lower mortality was documented in tilapia vaccinated with formalin-

killed *S. agalactiae* via three ways (injection, immersion and oral administration) (Linh et al., 2022). The Nile tilapia was injected with H_2O_2-inactivated *S. agalactiae* slightly showed lower mortality than the group injected with the pH manipulation vaccine although there was no statistical difference between these groups (Ramos-Espinoza et al., 2020b). In concordance with survival mortality in red tilapia, the RPS was recorded to reach the highest value in fish injected with the vaccine (34.39%), followed by oral administration (20.00%) and immersion (6.67%). Immersion H_2O_2-inactivated *S. agalactiae* did not improve the mortality in fish after challenge time.

CONCLUSIONS

The H_2O_2-inactivated *S. agalactiae* could provide a better improvement in the innate and adaptive immune responses (total WBCs, lysozyme and IgM) of red tilapia, and then reduce the mortality after injection with bacteria. Specifically, vaccination of H_2O_2-inactivated *S. agalactiae* via two delivery routes (injection and oral administration) had a positive effect in increasing the immune response as well as improving the capacity of red tilapia against the *S. agalactiae* pathogen. Further studies focusing on the suitable adjuvant vaccine should be developed to improve the RPS in red tilapia.

AUTHOR CONTRIBUTIONS

Tran Ngoc Bich, Bui Thi Bich Hang, Nguyen Trong Ngu, Nguyen Thanh Phuong; Conceptualization and design the experiment, investigation, supervision, editing and finalization.

Truong Quynh Nhu, Danh Thanh Duy, Nguyen Thi Thu Hang; Investigation, methodology, formal analysis, manuscript preparation

CONFLICT OF INTEREST

We have no conflict of interest.

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