Immune responses and immunoglobulin gene expression of Streptococcus agalactiae infected Nile Tilapia and the effective dose of inactivated Streptococcus agalactiae vaccine for Nile tilapia (chitratalada 3 strain) in Thailand

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Abstract

*Streptococcus agalactiae* is the primary pathogen in the Nile tilapia (*Oreochromis niloticus*) culture and creates an impact through economic damage. The immune system functioned to eliminate the pathogen in infected fish. This study demonstrated the effect of different bacterial concentrations on tilapia immunity and optimal vaccine concentration to induce immunity in Nile tilapia. The experiment was performed at $10^2$, $10^4$, $10^6$, $10^8$, and $10^{10}$ CFU/fish of *S. agalactiae* compared with the control (PBS) through intraperitoneal injection for 72 h. Immunoglobulin gene expression, antibody titers, and blood smeared to measure the survival rate. The vaccine experiment investigated formalin-inactivated *S. agalactiae* vaccination and administered *S. agalactiae* injections for 14 days. The statistic revealed a significant difference ($p < 0.05$) in the $10^8$ and $10^{10}$ CFU/fish injections with high survival rates (62.22% and 53.33%, respectively). Immunoglobulin gene expression was highly represented in the $10^{10}$ CFU/fish injection; antibody titers were significantly improved from the control treatment, and antibody levels were high in the $10^{10}$ CFU/fish injection. The comparison of the blood measurement from the blood smear technique indicated gradual leucocyte enhancement, especially of lymphocytes. In addition, the erythrocyte/leucocyte ratio was reduced in the highly bacterial injection, in which the experiment disclosed that the leucocytes increased. Conversely, the erythrocytes stayed at the same number. The lymphocytes were almost two-fold in $10^{10}$ CFU/fish compared to $10^8$ CFU/fish. As depicted in the lowest concentration of $10^6$ CFU/fish, the vaccine performance had a high relative percent survival (RPS) at 86.67%. This research suggested that the tilapia infected with high *S. agalactiae* concentrations did not affect the mortality of the tilapia, and vaccine concentration was effective in $10^6$ CFU/fish.

1. Introduction

In Thailand, Chitralada 3 tilapia is a strain of tilapia developed from GIFT (Genetic Improvement of Farmed Tilapia), a Philippine ICLARM unit's fifth generation (tilapia GIFT has traditional Thai Chitralada species mixed with it). "Chitralada 3" has been consistently bred because breeding via group selection method was claimed to have achieved success most recently in 2007. With a high yield, excellent survival rate, and good growth, "Chitralada 3" is distinguished by having small, thick, dense, and highly meaty heads [1].

One of the most harmful bacterial diseases for tilapia is Streptococcosis, caused by a group B *Streptococcus agalactiae* that can lead to a mortality rate reaching up to 90% [2, 3]. The bacteria generate the streptolysin S and O for blood hydrolysis and adhesion of cell surfaces, protect lysozyme, and replicate in serum or blood into target organs [4, 5, 6]. An infected fish exhibits external signs through abnormal behavior (swirling behavior, lethargy, bent bodies, and disorientation) and eye lesions (endophthalmia or exophthalmia), abscesses, skin hemorrhages around the mouth or at the base of the fin, and ascites [7]. The internal signs include septicemia, hemorrhages, and inflammation in the liver, spleen, kidney, heart, brain, eye, intestinal tract, and peritoneum. Adhesions to the peritoneal cavity occur in severe infections, resulting in high mortality and severe economic damage. The epidemiology of this
disease is caused by stress conditions (high water temperature, suboptimal oxygen, and overcrowding), horizontal transmission from feces, bacteria via lesions, and weakfish [8]. Accordingly, several antibiotic products and probiotics have been used to control this disease, risking beneficial bacteria equivalence often administered through food and environmental conditions. More environmentally friendly methods must be developed to relieve the problem, and the S. agalactiae vaccine is an exciting option for amendments to this prevalent pandemic [9].

While S. agalactiae attacks the tilapia by leading to immune response as a pathogen exposure response, two different parts exist in the immune system: innate and acquired immunity with cell-mediated and humoral responses. The innate immune system is like a physical barrier (skin mucous) to first prevent the entry of microorganisms and chemicals (cytokines, agglutinins, precipitins, and interferons). Then it provides a generalized response (inflammation, opsonization, and phagocytosis). The macrophages and nonspecific cytotoxic cells are the effectors of the system. In contrast, acquired immunity (AC) is a system of specific antigen molecule responses, including lymphocytes and B-cells, ultimately producing immunoglobulin or antibodies. T-cells and regulator cells are the effectors of AC immunity [10].

Immunoglobulin M (IgM), a classical antibody isotype found in most vertebrates, plays a critical role in the host immune response, performing a variety of functions such as neutralizing specific antigens and activating the complement system [11], agglutination, binding of mannose binding lectin [12], and mediating cellular cytotoxicity [13]. As the main antibody generated in the immunological response to antigens and the first antibody isotype to emerge during ontogeny.

IgM is classified as the primordial immunoglobulin of the adaptive immune response and is found in monomeric and tetrameric forms in circulating blood [14]. IgM can exist in 2 forms, slgM and membrane-bound (mlgM), which are generated via alternative RNA splicing of the primary transcript of the μ gene [15]. slgM consists of the variable region and 4 constant domains in the heavy chain, whereas mlgM contains variable region, 3 constant domains and 2 additional transmembrane domains (TM1 and TM2) and acts as a B cell receptor for initial antigen binding [16]. Together with innate immunity factors, it offers the initial line of defense against microbial infection. Until date, the immunoglobulin heavy (IGH) chain gene complex has encoded three primary types of immunoglobulins (Igs) in teleost: IgM [17], IgD [18], and IgT/IgZ. The significant up-regulation of IgM expression upon bacterial challenge in Nile tilapia [13].

Although S. agalactiae vaccines are widely applied in tilapia culture. These vaccines include whole-cell inactivated, live attenuated, recombinant, or DNA vaccines; each type has different preparation and bacteria specificity [19, 20]. However, formalin-killed vaccine (FKC) is a whole-cell inactivated vaccine successfully developed for tilapia and comprises the whole cell and a subunit of dead bacterial cells [21]. The formalin-killed vaccine has been widely demonstrated in tilapia when intraperitoneally injected and has resulted in highly efficacious performance with a relative percent survival (RPS). The FKC vaccine involves adaptive immunity to release immunoglobulin M (IgM) for primary defense with immunological
memory. Therefore, the *S. agalactiae* vaccination is an extensively recognized process to prevent streptococcosis outbreaks and reduce fish mortality in the tilapia industry [22, 23, 24].

This research aimed to compare the immunoglobulin gene expression immune responses of tilapia infected with different *S. agalactiae* (serotype Ia) concentrations. We also preliminarily investigated the FKC vaccine efficacy as an alternative prevention approach in tilapia against *S. agalactiae*.

2. Materials and methods

2.1 *Streptococcus agalactiae* Isotype Ia

*S. agalactiae* (serotype Ia) obtained from Kidchakan Supamattaya Aquatic Animal Health Research Center, Department of Aquatic Science, Faculty of Natural Resources, Prince of Songkhla University, Thailand. Molecular serotyping by multiplex PCR was carried out to classify and confirm *S. agalactiae* serotype [25]. Prior to the experimental challenge, the bacterial isolation was passaged through the fish with 0.1 ml of 108 CFU/ml by intraperitoneal (IP) injection twice to enhance their virulence post storage (-80°C). The bacteria were recovered from the blood of freshly dead fish and were cultured on Trypticase soy agar (TSA) media with 5% sheep blood at 37°C for 18 h. The activated bacteria were identified using specific primers for *S. agalactiae* (Martinez et al., 2001). The bacteria were then diluted in phosphate-buffered saline, PBS (Calbiochem, USA), and the optical density (OD) at 600 nm in 1.00 (2.5×10^{12} CFU) was used to prepare the treatment.

2.2 Experimental Tilapia

Healthy Nile tilapia of chitralada 3's strain (approximately 20 grams weight) was obtained from a private fish farm in Nakhon Si Thammarat province, Thailand. They were acclimated for ten days in the experimental zone in 500-liter tanks with a density of 100 fish per tank with aeration. Tanks were supplied with flow-through dechlorinated tap water and air stones to maintain desired water temperature, dissolved oxygen (DO) and pH levels. Water quality (temperature, DO, and pH) was measured daily and maintained at 28–30°C for water temperature, >4 ppm for DO, and pH 7–8. During the acclimation period, the fish were fed with a commercial diet (THAILUXE® comprised 40.17% protein, 4.32% lipid, 18.74% ash, and 15.84% moisture) twice daily at 9.00 am and 4.00 pm at 5% of body weight and maintained and handled according to Institutional Animal Care and Use Committee–approved guidelines. The fish was randomly tested for Streptococcus infection using PCR to ensure they were free of infection before the experiments.

2.3 Investigation of severity (pathogenicity)

The acclimated Tilapia fish was used to determine mortality after *S. agalactiae* infection at different bacterial concentrations. The fish were randomly divided into six groups with four replicates per group and 15 fish for each replicate, and they were held in an 80-liter plastic tank with aeration. One group was designated as the control (PBS injection), and the others were inoculated with five different
concentrations (10^2, 10^4, 10^6, 10^8, or 10^10 CFU/fish) of the *S. agalactiae* stock. The fish in the challenge groups were intraperitoneally injected with 0.2 ml of *S. agalactiae* dilutions. In contrast, the fish in the control group were injected with 0.2 ml of PBS. During the experimental period, the fish were fed twice daily, and the water was changed every three days. Cumulative mortality, clinical signs were observed and recorded twice daily after challenge for 14 days. PCR assay F1, GAGTTTGATCATGGCTCAG and IMOD, ACCAACATGTGTTAATTACTC was performed to confirm the *S. agalactiae* infection in the dead or moribund and the surviving fish [26]. Water quality was measured daily and maintained at 28–30°C for water temperature, > 4 ppm for DO, and pH 7–8. During the acclimation period, the fish were fed with a commercial diet twice daily at 9.00 am and 4.00 pm at 5% of body weight.

For this challenge test, data from three replicates in each group were used to determine the mortality after bacterial infection. The other replicate in each group was used to study immune response.

### 2.4 Blood puncture and smear

After 72 h post-challenge, 500 to 1,000 µl of blood was collected from the caudal vein of ten infected fish from each group to measure antibody titers, gene expression, and blood smears. One drop of blood was spotted on a cleaned slide, and a glass cover equalized it. The blood slide was fixed with methanol by dipping 3–4 times and stained with commercial Dip Quick Stain (M&P IMPEX, Thailand). The fixed slide was dipped in eosin solution for ten seconds and cleaned with distilled water. Afterward, it was dropped in methylene blue for ten seconds and rewashed with water. The stained slide was air-dried for 400x microscopic observation. The erythrocyte, leucocyte, and thrombocyte counts were determined from the blood.

### 2.5 Agglutination techniques

For bacterial agglutination, 200 µl of blood was used following modified agglutination methods. Blood was collected in 200 µl samples for bacterial agglutination. The blood was centrifuged at 6,000 xg for 5 min to separate the serum, and serial dilution of the serum was started at 1:10 (10 µl serum and 90 µl 1x PBS). Two-fold serial serum dilution was prepared in 96 well-round bottom microtiter plates (Nunc™ 96-Well Polystyrene Round Bottom Microwell Plates Thermo Scientific™). The remaining well plates were dropped in 50 µl of PBS. The serum was diluted until 1:320. The 50 µl of *S. agalactiae* at OD 1.0 was added to the well. In the experiment, the serum mixed with PBS and bacteria combined with PBS were used as controls. The plates were coned with a plastic cover and incubated at room temperature for 18 h. The endpoint of cell agglutination was observed as the last serum dilution compared to the positive control. The visible agglutination was reported as log 2 of the previous serum.

### 2.6 Immunoglobulin gene expression analysis

The remaining blood was dropped into a 1,500 µl Eppendorf tube with 3.2% sodium citrate in a 9:1 proportion. The total blood was centrifuged at 6,000 xg for five min for plasma removal and RNA isolation, following the GENEzol™ reagent (Geneaid) procedure. A NanoDrop 2000c spectrophotometer measured RNA quantity and quality (Thermo Fisher Scientific, Wilmington, DE, USA) at 260 and 280 nm
wavelengths. The RNA sample was equally diluted to 500µg/µl and was combined in each treatment to synthesize the cDNA with the iScript™ cDNA Synthesis Kit protocol (BIO-RAD, USA). The cDNA quality was examined with elongation factor 1, housekeeping gene (Forward: GCACGCTCTGCTGGCCTTT, Reverse: GCGCTCAATCTTCCATCCC), and immunoglobulin gene (Forward: CCACTGGCCTGAAAGAGAAG, Reverse: GTCACGGCGGAAAATAAAAA) with PCR products of 250 and 217 bp, respectively [27]. The PCR reaction involved holding at 95°C for 15 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 30 s, and a final extension at 72°C for five min. The cDNA quality was examined via PCR products using 1% agarose gel electrophoresis. Real-time PCR examined the gene expression (ABI 7300 Real-Time PCR System (Applied Biosystems)). The amplified gene in 10 µl comprised 1.0 µl of cDNA, 2.0 µl of master mix (5× HOT FIREpol Probe qPCR Mix Plus (ROXX) (Solis Biodyne, Tartu, Estonia)), 0.25 µl of each primer (10 µM), and 6.5 µl of nuclease-free water. The cycling continued for 15 min at 95°C, followed by 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The expression level was calculated according to the $2^{-\Delta\Delta CT}$ method [28].

2.7 Formalin-inactivated *Streptococcus agalactiae* vaccine preparation

Trypticase soy broth (TSB) was used to culture active *S. agalactiae* and incubated at 37°C in a shaker for 36 h to a final concentration of 1.819 at OD 600 nm. The incubation was conducted at 37°C for 48 h and centrifuged to gather the cells before washing with sterile PBS five times. The bacteria were collected by centrifugation at 3,500 xg for ten minutes, and the cell pellets were washed with 1x PBS five times. The PBS was diluted in the bacterial solution until OD 1.000 with a total volume record. The serial dilution technique counted the bacteria cells and combined them again by adding formaldehyde at 0.5% in the solution. The vaccine was verified with TSA agar and stored at 4°C.

2.8 Vaccination performance

Tilapias were divided into seven groups for testing with *S. agalactiae* vaccination. In each group, 40 fish were injected with formalin-killed *S. agalactiae* at $10^2$, $10^4$, $10^6$, $10^8$, or $10^{10}$ CFU/fish concentrations. After 14 days of stimulation, the tilapias were injected with 200 µl of *S. agalactiae* ($2.17 \times 10^7$ CFU/fish). Conversely, the positive control was injected with active *S. agalactiae* and the negative control with PBS. Cumulative mortality was recorded for the 14-day trial to calculate the relative percent survival as follows: 

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

2.9 Statistical analysis

The SPSS Statistics for Windows (Version 20.0) software compared all the experimental data using one-way analysis of variance (ANOVA) and Duncan's multiple range test. The results were presented in triplicate, depicting mean and standard deviation values. Significant differences among treatments were determined at $P < 0.05$.

3. Results
3.1 Challenge test of *Streptococcus agalactiae* for Tilapia

The challenge test was estimated for 14 days. After infection with *S. agalactiae*, the death of tilapia occurred only around 3–12 days (Figure 1). The cumulative mortality of tilapia was 25.08 %, 21.75 %, 65.56 %, 58.41 %, and 31.75 % within 14 days post-infection with *S. agalactiae*, at doses of $10^{10}$, $10^8$, $10^6$, $10^4$ and $10^2$ CFU/fish, respectively (Figure 1). A significantly (P<0.05) higher survival rate was observed at $10^8$–$10^{10}$ CFU/fish. However, the lower (P<0.05) survival rate was revealed in $10^4$–$10^6$ CFU/fish. The accumulative mortality (Figure 1) demonstrates high mortality on Days 4–7 after infection, as the $10^4$ and $10^6$ CFU/fish injections depict survival on the sixth day of injection (Table 1).

**Table 1** The survival rate of different concentrations of *S. agalactiae* injections of Nile tilapia

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cumulative mortality (%)</th>
<th>The survival rate of different <em>S. agalactiae</em> concentrated injections (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PBS)</td>
<td>0.00 ± 0.00c</td>
<td>100.00 ± 00a</td>
</tr>
<tr>
<td>$10^2$ CFU/fish</td>
<td>31.75 ± 2.71b</td>
<td>35.56 ± 10.18c</td>
</tr>
<tr>
<td>$10^4$ CFU/fish</td>
<td>58.41 ± 6.29a</td>
<td>4.44 ± 3.858d</td>
</tr>
<tr>
<td>$10^6$ CFU/fish</td>
<td>65.56 ± 3.065a</td>
<td>6.67 ± 0.00d</td>
</tr>
<tr>
<td>$10^8$ CFU/fish</td>
<td>21.75 ± 4.11b</td>
<td>62.22 ± 7.70ab</td>
</tr>
<tr>
<td>$10^{10}$ CFU/fish</td>
<td>25.08 ± 8.39b</td>
<td>53.33 ± 13.33b</td>
</tr>
</tbody>
</table>

*Data are illustrated as mean ± standard deviation (n = 3 fish). Different superscript letters in the same row indicate statistically significant (P < 0.05) differences among the treatments after one-way ANOVA, followed by Duncan’s multiple range test.

3.2 Serum agglutination titers

We observed that the various antibody levels in the tilapia having a significant (p<0.05) difference compared to the control group. Figure 2 presents the antibody titers with the bacterial challenge. The highest titers were shown in $10^{10}$ CFU/fish of the *S. agalactiae* injection, but no significant (p>0.05) differences were found in $10^4$, $10^6$, and $10^8$ CFU/fish. The $10^2$ CFU/fish injection revealed a higher antibody level than the control group (PBS).

The total blood count results by the blood smear technique variously depicted erythrocytes and white blood cells (WBCs) of each type (Figure 3). Increased bacterial concentration caused WBCs accumulation. Therefore, statistical analysis demonstrated a significant (p < 0.05) difference between the control and injection treatments. The high bacterial inoculum concentration impacted the increase in lymphocyte, neutrophil, and monocyte counts (Figure 4). The control was revealed to have total...
lymphocytes at 0.66%, while the $10^2$ CFU/fish injection had total lymphocytes at 2.24%, with significant differences between the two groups (Table 2). Figure 5 depicts a positive correlation between the expression of the immunoglobulin gene and the total leucocyte count.

**Table 2** Percent of erythrocytes/leucocytes of different concentrations of the *S. agalactiae* injection compared with the control (PBS) under the blood smear technique at 40x microscopic performance for Nile tilapia

<table>
<thead>
<tr>
<th>Experimental variants</th>
<th>Treatments</th>
<th>Control (PBS)</th>
<th>$10^2$</th>
<th>$10^4$</th>
<th>$10^6$</th>
<th>$10^8$</th>
<th>$10^{10}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocytes (x$10^3$ cell)</td>
<td></td>
<td>0.70 ± 0.27</td>
<td>0.79 ± 0.11</td>
<td>0.79 ± 0.29</td>
<td>0.97 ± 0.43</td>
<td>0.83 ± 0.47</td>
<td>0.89 ± 0.24</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
<td></td>
<td>0.66 ± 0.56$^b$</td>
<td>0.43 ± 0.34$^b$</td>
<td>1.52 ± 1.01$^a$</td>
<td>1.87 ± 0.83$^a$</td>
<td>1.93 ± 0.73$^a$</td>
<td>2.24 ± 1.61$^a$</td>
</tr>
<tr>
<td>Neutrophils, %</td>
<td></td>
<td>0.20 ± 0.18$^c$</td>
<td>0.25 ± 0.15$^c$</td>
<td>0.28 ± 0.17$^c$</td>
<td>0.82 ± 0.81$^b$</td>
<td>0.84 ± 0.56$^b$</td>
<td>1.75 ± 1.50$^a$</td>
</tr>
<tr>
<td>Monocytes, %</td>
<td></td>
<td>0.15 ± 0.19$^b$</td>
<td>0.99 ± 0.47$^a$</td>
<td>0.99 ± 0.73$^a$</td>
<td>1.15 ± 0.94$^a$</td>
<td>1.58 ± 0.83$^a$</td>
<td>1.61 ± 2.17$^a$</td>
</tr>
<tr>
<td>Thrombocytes, %</td>
<td></td>
<td>0.33 ± 0.19$^{ab}$</td>
<td>0.51 ± 0.39$^b$</td>
<td>0.34 ± 0.16$^{ab}$</td>
<td>0.27 ± 0.17$^a$</td>
<td>0.22 ± 0.14$^a$</td>
<td>0.22 ± 0.15$^a$</td>
</tr>
</tbody>
</table>

*Data are presented as mean ± standard deviation (n =10 fish). Different superscript letters in the same row indicate statistically significant (p < 0.05) differences among the treatments after one-way ANOVA, followed by Duncan's multiple range test.

### 3.3 Immunoglobulin gene expression levels

Figure 6 depicted immunoglobulin gene expression of the tilapia blood after injection with different concentrations of bacteria. The expression gradually increased in the bacteria injection groups more than in the control group injected with PBS. However, the expression levels of the immunoglobulin gene in fish groups injected with $10^4$-10$^8$ CFU/fish did not reveal a significant difference (p>0.05) among the groups. The higher significance levels (p < 0.05) were distinguished in the $10^{10}$ CFU/fish injection. The expression of $10^2$ CFU/fish also did not significantly (p>0.05) differ from the control groups.

### 3.4 Vaccine efficacy

As depicted in Table 3, tilapia immunized with the *S. agalactiae* vaccine was demonstrated in $10^6$ CFU/fish; the survival rate was 86.67%. The vaccination with the $10^2$ and $10^4$ CFU/fish illustrated that no
fish survived after the *S. agalactiae* injection. The RPSs were highly demonstrated in 10^6 CFU/fish vaccination, but statistically significant (p>0.05) differences did not occur with 10^8 and 10^{10} CFU/fish. The corresponding RPSs were 86.67%, 83.33%, and 76.67%, respectively.

**Table3** Survival rate and relative percent survival of vaccinated performance of Nile tilapia.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival rate of vaccine experiment (%)</th>
<th>% RPS of vaccine test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (PBS)</td>
<td>100.00 ± 0.00^a</td>
<td>-</td>
</tr>
<tr>
<td>Control (<em>S. agalactiae</em>)</td>
<td>0.00 ± 0.00^c</td>
<td>-</td>
</tr>
<tr>
<td>10^2 CFU/fish</td>
<td>0.00 ± 0.00^c</td>
<td>0.00 ± 0.00^c</td>
</tr>
<tr>
<td>10^4 CFU/fish</td>
<td>0.00 ± 0.00^c</td>
<td>0.00 ± 0.00^c</td>
</tr>
<tr>
<td>10^6 CFU/fish</td>
<td>86.67 ± 15.26^a</td>
<td>86.67 ± 15.28^a</td>
</tr>
<tr>
<td>10^8 CFU/fish</td>
<td>83.33 ± 5.77^a</td>
<td>83.33 ± 5.77^a</td>
</tr>
<tr>
<td>10^{10} CFU/fish</td>
<td>86.67 ± 5.77^a</td>
<td>76.67 ± 5.77^a</td>
</tr>
</tbody>
</table>

*Data are shown as mean ± standard deviation (n = 3 fish). Different superscript letters in the same row indicate statistically significant (p < 0.05) differences among the treatments after one-way ANOVA, followed by Duncan's multiple range test.*

**4. Discussion**

*S. agalactiae* is one of the most important pathogens and causes high mortality in Nile tilapia. The immune responses of Nile tilapia infected *S. agalactiae* in this study has led to the understanding of a specific immune responses including antibody levels, hematological parameters, and immunoglobulin gene expression of Nile tilapia when infected with *S. agalactiae*. After challenge, the results indicate that the challenge with *S. agalactiae* can cause clinical signs and lesions in Nile tilapia, when the bacteria were delivered intramuscularly inoculation, mortality was initially observed within 72 h. A linear dose response was not seen. This result indicated that the high concentration of bacteria did not induce disease because tilapia survival was higher at 10^8 and 10^{10} CFU/fish than at 10^2, 10^4, and 10^6 CFU/fish. The results also showed that a high concentration of bacteria could activate the adaptive immune system concerning the expression of the IgM gene. The number of lymphocytes increased in the high bacterial concentration treatments. The decrease of mortality at higher dilution of *S. agalactiae* was possibly due to factors effecting immunological tolerance, especially the increasing of IgM level which
the expression of the IgM gene in the blood demonstrated that the high bacteria injection resulted in extremely high levels of expression. The IgM gene relates to acquired immunological response antibodies with a role in bacterial defense [29, 13]. Additionally, the IgM gene is expressed in various tissues, including the head kidney, spleen, intestine, skin, and gill. Vital tissues for producing antibodies for B and T cells in bony fish, the head kidney, and spleen had the highest IgM expression. Moreover, the IgM gene was detected in the skin and intestine's mucosal immune system. The mucosal immune system is the first line of defense against pathogen invasion and is susceptible to bacterial stimulation of phagocytosis via the mucosal surface [13]. The experiment detected bacteria from the blood throughout the target organs (brain, liver, spleen, and kidney). The IgM heavy chain gene was thoroughly expressed in all tested tissues, but higher levels were expressed in the peripheral blood leukocyte. Thus, the blood sample was the appropriate example of IgM gene expression to reduce the experimental fish mortality from tissue collection [30].

Antibody titers demonstrated that varying bacterial concentrations increased the augmentation of antibody levels; however, no statistically significant differences (p > 0.05) existed among $10^4$–$10^{10}$ CFU/fish. The lymphocyte and immunoglobulin produced antibodies with diverse functions in serum and mucus, and they participated in the B cell surface complex and antigen signaling function [31, 32]. The IgM titer should increase bacterial activation based on the quantities of bacteria found in the experiment. The dosages of bacteria also affected the immune protection response [33]. The results exhibited a strong relationship between bacterial concentrations and antibody levels.

The number of leucocytes, observed by the blood smear technique, increased directly with the quantities of bacteria, according to the study. We determined the increments of lymphocytes, neutrophils, and monocyte counts. Lymphocytes, thrombocytes, monocytes, granulocytes, and nonspecific cytotoxic cells existed among the white blood cells. Leucocytes contributed to functional retardation and pathogen elimination via the immune system during pathogen assessment, commonly suggesting a contrary relationship with the fish's condition or health [34]. Individually infected fish produced many leucocytes for antibody generation and phagocytosis. Lymphocytes appear to be made by the thymus, spleen, and kidney. The primary functions of antibody production and phagocytic activity induce the macrophage with this efficacy, where the antibodies were proportional to the number of lymphocytes [35, 36]. Therefore, excessive lymphocyte increase affects the survival of tilapia [34].

Furthermore, fish thrombocytes were accountable for the precursors of blood clotting in circulating fluids. Fish thrombocytes come in various shapes (oval, spindle, spiked, and cluster of fragmented thrombocytes), and every thrombocyte was sharp on blood smear slides [35]. A small proportion of the white blood cell population comprises monocytes or macrophages. Thought to originate in the kidney, they are competent in killing a category of pathogens and bacteria. In addition, neutrophils are one type of granulocyte elucidating up to 25% of the overall leukocyte population. Teleost produces granulocytes in the kidney and spleen [37]. Neutrophils migrate to the site of bacterial infection, where they may be phagocytic or bactericidal and frequently correlate with stress [38].
Moreover, vaccination demonstrated the effectiveness of *S. agalactiae* resistance 14 days following the induction of immunity. The intraperitoneally vaccinated injection with the whole-cell inactivated *S. agalactiae* vaccine resulted in high survival at $10^6$ CFU/fish concentration and no significant difference ($p > 0.05$) at $10^8$ and $10^{10}$ CFU/fish. In contrast, no fish survived in $10^2$ and $10^4$ CFU/fish after the *S. agalactiae* injection for 14 days of observation. Through intraperitoneal injection, formalin-killed vaccines exhibited excellent protection and a high relative percent survival (75–100%). Although no finding regarding the immune system existed, the fish experiment can be seen as a sign of success [39].

**Conclusion**

Our experiment has demonstrated the effect of highly concentrated *S. agalactiae* injections on immunological induction and survival, including an increase in the immunoglobulin gene expression and antibodies and an increase in leucocytes. The research indicated that injections of highly concentrated *S. agalactiae* reduce virulence and reverse immunological activation. Therefore, different vaccination concentrations had varying effects. We advocate vaccinating juvenile tilapia with $10^6$ CFU/fish of *S. agalactiae* vaccination to reduce *S. agalactiae* outbreaks in the tilapia farm industry and for convenience. Moreover, we suggest immersion or oral vaccination as an alternative technique for protecting a tilapia population.

**Declarations**

Ethics approval and consent to participate:

This study protocol was approved by the Animal Ethics Committee, Walailak University (Protocol No. WU-AICUC-63-042) and all of the experiments were conducted in accordance with the guidelines and regulations of the Management and Use of Laboratory Animals of the Animal Ethics Committee, Walailak University. This study did not involve endangered or protected species.

Consent for publication: NOT APPLICABLE

Availability of data and materials:

The authors declare that they do not have any shared data available.

Competing interests:

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors' contributions:

TK: conducted the experimental, data processing and manuscript preparation. CP: contributed to immunoglobulin gene expression analysis, blood counting and statistical analysis. SW: research design, implementation, and manuscript preparation. All authors have read and approved the manuscript.

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References


**Figures**

![Graph showing cumulative mortality of Nile tilapia under different concentrations of *S. agalactiae* infection. Each value is displayed as mean ± SD (n= 3)](image)

**Figure 1**

Cumulative mortality of Nile tilapia under different concentrations of *S. agalactiae* injection. Each value is displayed as mean ± SD (n= 3)
Figure 2

Serum antibody titers in tilapia following the greater bacterial agglutination techniques with varying concentrations of the *S. agalactiae* injection compared with the control (PBS injection). Different superscript letters indicate statistically significant (p < 0.05) differences among the treatments.
Peripheral blood cell morphology of Nile tilapia on blood smears was performed using light microscopy with 40x. Early immature erythrocytes (EE), Mature erythrocytes (ME), Large immature erythrocytes(LE), Clusters of fragmented thrombocytes (CT), Monocytes (M), Oval thrombocytes (OT), Neutrophils (N), Small lymphocytes (SL), and large lymphocyte (LL). Scale bars = 12.5 µm.
Figure 4

Type of blood cell of Nile tilapia under different concentrations of *S. agalactiae* injection. Each value is displayed as mean ± SD (n = 10)
Figure 5

The correlation of immunoglobulin with total leucocyte cells in tilapia is presented as mean ± SD from triplication.
Figure 6

The relative expression of the immunoglobulin gene in tilapia blood under different concentrations of the *S. agalactiae* injection compared with the control (PBS injection). Different superscript letters indicate statistically significant ($p < 0.05$) differences among the treatments.