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Efficacy of Different Routes of Formalin-Killed Vaccine Administration on Immunity and Disease Resistance of Nile Tilapia (*Oreochromis niloticus*) Challenged with *Streptococcus agalactiae*

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Abstract: Vaccines prepared from formalin-killed *Streptococcus agalactiae* were administered to Nile tilapia (*Oreochromis niloticus)* via three different routes: immersion in a water-based vaccine, injection with an oil-based vaccine, and as a water-based oral vaccine. All vaccination treatments increased lysozyme and peroxidase activity in skin mucus of Nile tilapia by 1.2- to 1.5-fold compared to their activities in unvaccinated control fish. Likewise, alternative complement, phagocytosis, and respiratory burst activities in the blood serum of the vaccinated fish were 1.2- to 1.5-times higher than in the unvaccinated fish. In addition, the expression transcripts of interleukin-1 (*IL-1*), interleukin-8 (*IL-8*), and lipopolysaccharide-binding protein (*LBP*) were 2.3- to 2.9-fold higher in the vaccinated fish compared to those in the unvaccinated control. The unvaccinated fish challenged with *Streptococcus agalactiae* had a survival rate of 25% compared to a survival rate of 78–85% for the vaccinated fish. The differences between the unvaccinated and vaccinated fish were all statistically significant, but there was no significant difference in any of the indicators of immunity between the three vaccinated groups. Collectively, these results confirm that vaccination with formalin-killed *Streptococcus agalactiae* significantly improved the resistance of Nile tilapia to infection by the pathogen. Overall, the efficacy of oral administration of the vaccine was comparable to that of vaccine administered via injection, indicating that oral vaccination is a viable cost-effective alternative to administering vaccines by injection.

Keywords: vaccine administration; *Oreochromis niloticus*; immune response; *Streptococcus agalactiae*; lipopolysaccharide binding protein

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1. Introduction

Nile tilapia is an increasingly popular species for aquaculture due to its adaptability to a wide range of environmental conditions, and it now ranks second after the carp in terms of global fish production from aquaculture [1]. In many countries, Nile tilapia are reared in intensive cages in rivers and canals that are often polluted with drainage water [2,3]. In such conditions, fish are likely to suffer from low water quality and infectious pathogens [4], and farmed fish are frequently infected by pathogenic bacteria, such as *Streptococcus* spp., *Flavobacterium*, *Edwardsiella* spp., *Francisella* spp., and *Aeromonas* spp., causing high mortality rates and substantial economic impacts [5–12]. Antibiotics and traditional chemotherapies are usually applied to control infection and reduce mortality during disease outbreaks [4,13]. However, the use of antibiotics impairs the natural immune response of fish and promotes the development of strains of pathogenic bacteria that are resistant to antibiotics, encouraging their use in even larger quantities [14,15]. This in turn leads to the accumulation of high levels of antibiotics in the body of fish, reducing food safety and indirectly affecting human health [16–18]. The use of antibiotics to control infectious diseases also poses a serious risk to the living environment of fish [19,20]. Hence, there is a need to find alternative strategies to combat the risk of bacterial infections in fish cultures [21–23]. Vaccines can provide long-lasting protection against pathogenic bacteria and viruses [24–26] and are now widely used to control infectious diseases in many aquatic organisms [4,27]. A wide range of vaccines have been developed to protect Nile tilapia against infectious diseases; these include live attenuated vaccines against *Streptococcus agalactiae* [15,28], polyvalent vaccines against streptococcosis or lactococcosis [29], novel chimeric multiepitope for streptococcosis [30], and vaccines based on formalin-killed bacteria [31]. Vaccines can be administered in different ways, but the most common is by immersion, orally as a feed additive, and direct injection. The method of administration depends on fish size and stage of development as well as the nature of the pathogen being vaccinated against [7,32,33]. Historically, vaccines have been administered via injection, but this is time-consuming and difficult to deliver to young fish [32,34]. On the other hand, while oral and immersion vaccines are simple to administer with minimum stress to the fish, they often result in limited immune responses [7,24,33–35]. Improving immersion vaccines would go a long way to reducing the incidence and severity of infectious diseases on fish farms [36,37]. In this study, we compare the efficacy of vaccines prepared from formalin-killed *S. agalactiae* that were administered via immersion, injection, and oral routes on skin mucus and serum immune response, relative immune gene expression, and the resistance of Nile tilapia to infection by *S. agalactiae*.

2. Materials and Methods

2.1. Vaccine Preparation

Streptococcus agalactiae strain 2809 used for this research was isolated from a disease outbreak at a tilapia farm [38]. The isolate was recovered from the stock and purified by culturing on tryptic soy agar (TSA, Becton, Dickinson, ND, USA) for 48 h at 28 °C. The pure colony was then cultured in 15 mL of tryptic soy broth (TSB, Becton, Dickinson, ND, USA) for 24 h at 28 °C and 150 rpm. One percent of *S. agalactiae* was added to fresh TSB and cultured for 24 h at 28 °C and 150 rpm. The bacterial suspensions were then inactivated with 3% formalin and incubated at 4 °C overnight. To verify the inactivation of the bacteria, a volume of 0.1 mL of killed-bacterial suspension was plated on TSA and *S. agalactiae* selective agar bases (HiMedia, India) and incubated for three days at 28 °C. The inactivated bacterial suspension was washed with sterile 1× phosphate-buffered saline (PBS) three times followed by centrifuging at 4500 rpm for 5 min at 4 °C. The pellet was then resuspended in sterile 1× PBS for further use [33,39,40]. Following centrifugation, the bacterial pellets were resuspended in PBS buffer and adjusted to OD_{600nm} = 1.3 (equivalent to 10^9 CFU mL^{-1}) to provide the bacterial antigens for the vaccine formulation. This suspension of formalin-killed bacteria in PBS (bacterial antigen) was used for the water-based oral vaccine. For the oil-based injectable vaccine, the bacterial antigen was mixed with

Montanide^{TM} ISA 763A VG, a commercially available non-mineral oil adjuvant (Seppic), at a ratio of 3:7 (v/v) and homogenized at 15,600 rpm for 3 min using an IKA T25 digital ULTRA TURRAX homogenizer to form an oil-in-water vaccine with a final concentration of ~1.7 $\times$ 10^7 CFU mL^{-1}. For the immersion vaccine, the bacterial antigen was mixed with Montanide^{TM} ISA 1312 VG (Seppic) adjuvant at a ratio of 1:1 (v/v) and agitated at 250 rpm for 10 min using a low-shear mixer with a 4-blade impeller (Onilab OS20-Pro). The control was PBS without bacterial antigen. The formulated vaccines were stored at 4 °C until use.

2.2. Diet Preparation and Experimental Design

The approximate components and formulation of the experimental diets for the fish in the current study are presented in Table 1. The experimental diet consisted of the basal diet sprayed with PBS at a rate of 100 mL per kg feed; for the oral vaccine treatment, 100 mL of water-based oral vaccine was sprayed onto 1 kg of basal diet. All diets were coated with fish oil, dried for 15 min at 24 °C, and then stored at 4 °C until used.

Table 1. Approximate composition and formulation of the experimental diets (g kg^{-1}).

Constituents	Basal Diet
Soybean meal	390
Corn meal	200
Fish meal	150
Rice bran	150
Wheat flour	70
Cellulose	20
Premix	10
Vitamin C 98%	5
Soybean oil	5
Approximate component of dietary treatment (g kg^{-1} dry matter basis)	
Gross energy (Cal/g)	3892
Dry matter	991.83
Crude protein	322.28
Ash	84.90
Fiber	43.47
Crude lipid	38.56

2.3. Experimental Procedure

Healthy Nile tilapia were purchased from a commercial hatchery in Chiang Mai, Thailand. The fish were fed commercial pellets (CP, 9950) for 60 days and then fed the basal diet for 15 days. Prior to the experimental trial, 10 experimental fish were randomly selected and tested for the presence of *S. agalactiae*. After confirming that the fish were free of *S. agalactiae*, 320 fish (9.85 $\pm$ 0.35 g) were distributed into 16 glass aquaria containing 100 L of water at a stocking density of 20 fish per tank. The feeding experiment lasted for 15 days and had four replicates of each treatment arranged in a completely randomized design. There were four experimental treatments: an unvaccinated control (Treatment T1), fish given the immersion vaccine (Treatment T2), fish injected with the oil-based vaccine (Treatment T3), and fish given the oral vaccine (Treatment T4). The fish in the unvaccinated control group (T1) were injected with 0.1 mL of PBS without vaccine. The fish receiving the immersion vaccine (Treatment T2) were transferred to an identical tank with 100 L of aerated water at the same temperature (28 °C) into which 2 L of vaccine (approx. 10^9 CFU mL^{-1}) was added to give a final concentration of approximately 10^7 CFU mL^{-1}. After 30 min of exposure to the immersion vaccine, the fish were returned to their vaccine-free culture tank. Each fish in the injectable vaccine group (Treatment T3) received an intraperitoneal injection of 0.1 mL of vaccine (approx. 1.7 $\times$ 10^7 CFU mL^{-1}). For Treatment T4, the water-based oral vaccine was administered to the fish as follows: days 1–5, feeding with oral vaccine; days 6–10, feeding without vaccine; days 11–15, feeding with oral vaccine [41]. The oral vaccine treatments were given to the fish at 8:30 a.m. and 4:30 p.m. At each feeding time, a visual

examination was conducted to confirm that all feed were eaten and nothing remained in the tanks. Water temperature, pH, and dissolved oxygen were maintained at 28 ± 0.23 °C, 7.91 ± 0.31, and 5.32 ± 0.11 mg L^{-1}, respectively.

2.4. Serum, Leukocytes, and Mucus Preparation

A composite sample of fish blood from 4 fish per replicate was kept without anticoagulant in 1.5 mL microtubes for 1 h at 24 °C and then at 4 °C for 4 h. The serum was then extracted by centrifugation at 1500× *g* for 5 min and frozen at −80 °C for further analysis.

Leukocytes were isolated from the fish blood according to protocols reported previously [42] and modified according to Van Doan et al. [43]. Skin mucus was collected from the same fish following the procedures described previously. Briefly, the clove oil-anesthetized fish were gently rubbed for 2 min in a plastic bag supplemented with 10 mL of 50 mM NaCl. The mixture was immediately poured into a 15 mL sterile tube and centrifuged. The supernatant was collected and stored at −80 °C.

2.5. Immunological Assays

The lysozyme activity of the mucus and serum was assayed following the protocols previously described by [44], and the peroxidase activity was measured according to protocols previously reported by [45,46]. The protocol of Yoshida and Kitao [47] was used to assess the phagocytic activity. The blood leukocytes' respiratory burst activity was determined following the protocol of Secomebs [42], whereas ACH50 was assayed according to Yanno [48].

2.6. Nonspecific Immune-Related Gene Expression Analysis

To investigate the mRNA transcript levels of three target genes, liver tissues (3 fish/tank/treatment) were sampled for quantitative real-time PCR (RT-qPCR) after 15 days of immunization. A total of 40–50 mg of liver tissue was used for total RNA isolation using the TRIzol method. The quality and quantity of total RNA were measured using a NanoDrop One (Thermo Scientific, USA) at an OD ratio of 260:280 nm. One µg of total RNA was used to synthesize the first-strand complementary DNA (cDNA). The primers used for RT-qPCR analysis in this study are presented in Table 2. The RT-qPCR was performed in triplicate using the CFX96™ Real-Time System (Bio-Rad, USA) with 1 µL of cDNA, 10 mM of each primer, and 2 × iTaq Universal SYBR Green (Bio-Rad, USA). The mRNA transcript levels were calculated using the $2^{-\Delta\Delta Ct}$ method [49]. The primers used for qPCR were previously reported by Linh et al. [33].

Table 2. Primers used for RT-qPCR analysis.

Primers	Oligo Sequence (5′-3′)	Genes	T_m (°C)	Size (bp)
18S rRNA	F: GTGCATGGCCGTTCTTAGTT R: CTCAATCTCGTGTGGCTGAA	*18S RNA*	60	150
IL-1	F: GTCTGTCAAGGATAAGCGCTG R: ACTCTGGAGCTGGATGTTGA	*IL-1*	59	200
IL-8	F: CTGTGAAGGCATGGGTGTG R: GATCACTTTCTTCACCCAGGG	*IL-8*	59	196
LBP	F: ACCAGAAACTGCGAGAAGGA R: GATTGGTGGTCGGAGGTTTG	*LBP*	59	200

F: forward, R: reverse, bp: base pair.

2.7. Challenge Experiment

S. agalactiae was isolated and thoroughly processed as previously reported [38]. Briefly, 5 mL of the stock solution was transferred into 50 mL of TSB and incubated for 24 h at 28 °C and 150 rpm. The sub-cultures were raised in duplicate with similar conditions for the experiment. The optical density at 560 nm was used to determine growth followed by

plate counting in TSA. At 15 days post-feeding, a total of 10 fish from each replicate was intraperitoneally injected with 0.1 mL of *S. agalactiae* (approx. 10^7 CFU mL^{-1}). The survival rates were measured daily over a period of 15 days of the experimental challenge, and the relative percentage of survival (RPS) was calculated as follows: RPS = (1 − % mortality in vaccinated/% mortality in control) × 100 [50].

2.8. Statistical Analysis

The Shapiro–Wilk test was used to assess the normality of the data. Statistix v.10.1 (Analytical Software, Tallahassee, FL, USA) was used to perform the statistical analyses. The immunological responses and mRNA transcript levels of the innate immune-related genes were all evaluated using a one-way analysis of variance, and the differences between means were compared using the Least Significant Difference (LSD) test. The Kaplan–Meier estimator was used to determine the cumulative survival in the experimental challenge, and the log-rank test was used to compare statistically significant differences in survival between the different treatments. The data are presented as means ± SEM, and statistical significance was determined at the 95% confidence level ($p < 0.05$).

3. Results

3.1. Mucosal Immune Response Analysis

Overall, vaccination increased the activity of lysozyme by approximately 1.3-fold and peroxidase by approximately 1.6-fold in skin mucus compared to the unvaccinated control group, and this was statistically significant ($p < 0.05$) (Figure 1). Skin mucus lysozyme activity was marginally higher in the fish that were vaccinated by injection (1.56 μg mL^{-1}) than those vaccinated orally (1.53 μg mL^{-1}) and a little lower (1.45 μg mL^{-1}) in the fish that were vaccinated by immersion (Figure 1), but the differences between the vaccination routes were not significant ($p > 0.05$). Skin mucus peroxidase activity was similar (0.13 μg mL^{-1}) in the fish vaccinated by injection and via the oral pathway and a little lower (0.12 μg mL^{-1}) for those receiving the vaccine via immersion (Figure 1). There was no significant difference ($p > 0.05$) in skin mucus peroxidase activity between any of the vaccination treatments (Figure 1).

3.2. Immune Response Analysis in Serum

The fish in the vaccinated groups (T2, T3, and T4) had substantially greater levels ($p < 0.05$) of serum lysozyme (ca. 1.3-fold) and peroxidase activity (ca. 1.3-fold), alternative complement activity (ca. 1.4-fold), respiratory burst activity (ca. 1.5-fold), and phagocytosis activity (ca. 1.2-fold) than the unvaccinated fish in T1, the control treatment (Figure 2). Serum lysozyme (5.66 μg mL^{-1}) and peroxidase activity (0.21 μg mL^{-1}), alternative complement activity (237 μg mL^{-1}), and respiratory burst activity (0.13 μg mL^{-1}) were all marginally, but not significantly, higher in the fish vaccinated orally than those vaccinated by immersion or injection (Figure 2). There was no significant difference in any of the measured indices between the different methods of vaccine delivery ($p > 0.05$).

3.3. Immune Gene Expression Profiling

The relative transcript levels of the three target genes encoding IL-1 (interleukin-1), IL-8 (interleukin-8), and LBP (lipopolysaccharide-binding protein) were 2.5- to 3-fold higher ($p < 0.05$) in the liver tissues of the vaccinated fish compared to those in the unvaccinated control (Figure 3). The relative transcript level for IL-1 was marginally higher in the fish vaccinated orally than in the fish vaccinated via injection and, conversely, the relative transcript levels of IL-8 and LPB were slightly higher in the fish vaccinated via injection than in those vaccinated orally (Figure 3). For all three genes, the relative transcript levels were lower in the immersion treatment (T2) than in the other two vaccination treatments (T3 and T4). However, the transcript levels for all three genes were not significantly different between the three vaccination treatments (Figure 3).

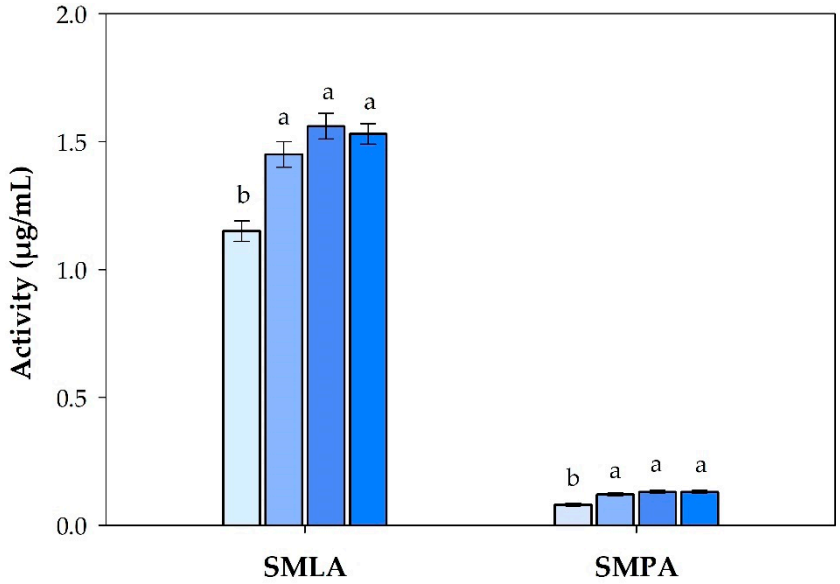

Figure 1. The activity (μg mL^{-1}) of lysozyme and peroxidase in skin mucus of Nile tilapia 15 d post-vaccination. Treatment 1 (T1)—control, Treatment 2 (T2)—formalin-killed *S. agalactiae* immersion vaccine, Treatment 3 (T3)—formalin-killed *S. agalactiae* injectable oil-based vaccine, Treatment 4 (T4)—formalin-killed *S. agalactiae* water-based oral vaccine. The presence of different letters in each group indicates a significant difference ($p < 0.05$). SMLA, skin mucus lysozyme activity; SMPA, skin mucus peroxidase activity.

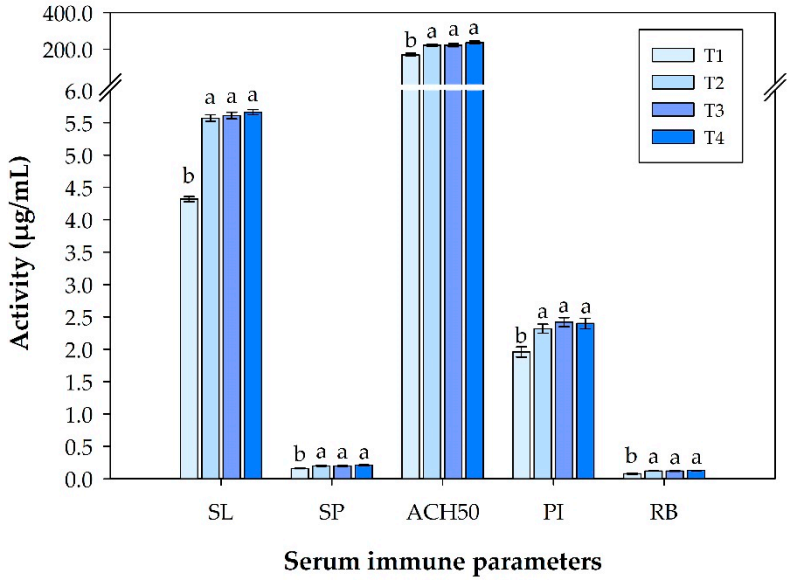

Figure 2. Serum immunity parameters of Nile tilapia post-vaccination (μg mL^{-1}). Treatment 1 (T1)—control, Treatment 2 (T2)—formalin-killed *S. agalactiae* immersion vaccine, Treatment 3 (T3)—formalin-killed *S. agalactiae* injectable oil-based vaccine, Treatment 4 (T4)—formalin-killed *S. agalactiae* water-based oral vaccine. The presence of different letters in each group indicates a significant difference ($p < 0.05$). SL, serum lysozyme activity; SP, serum peroxidase activity; ACH50, alternative complement activity; RB, respiratory burst activity; PI, phagocytosis activity.

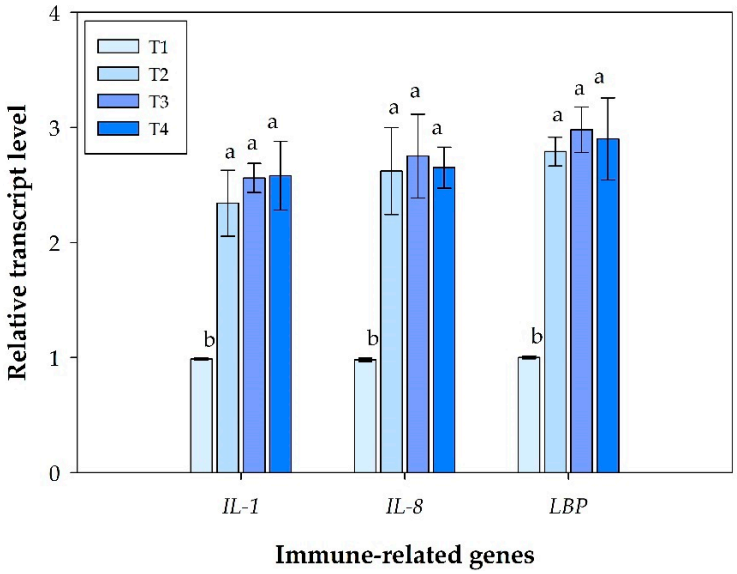

Figure 3. Comparative mRNA transcript levels of three immune-related genes (IL-1, IL-8, and LBP) in the livers of the vaccinated and control fish post-immunization. The 18S rRNA was used as an internal control gene. Significant differences between the treatment groups are denoted by different letters ($p < 0.05$).

3.4. Fish Survival Rate after S. agalactiae Challenge

For the unvaccinated fish in the T1 treatment group, mortality occurred on day 4 and continued until day 8, whereas the earliest incidence of fish mortality was recorded on day 5 for the vaccinated fish in the T2, T3, and T4 treatment groups. After 15 days of challenge with *S. agalactiae*, the relative percent survival (RPS) was 66.67% for the vaccinated fish in the T2 treatment group followed by 80% for the vaccinated fish in the T3 treatment group and 71.67% for the vaccinated fish in the T4 treatment group (Figure 4, Table 3). The fish that perished during the challenge experiment displayed clinical symptoms typical of *S. agalactiae*.

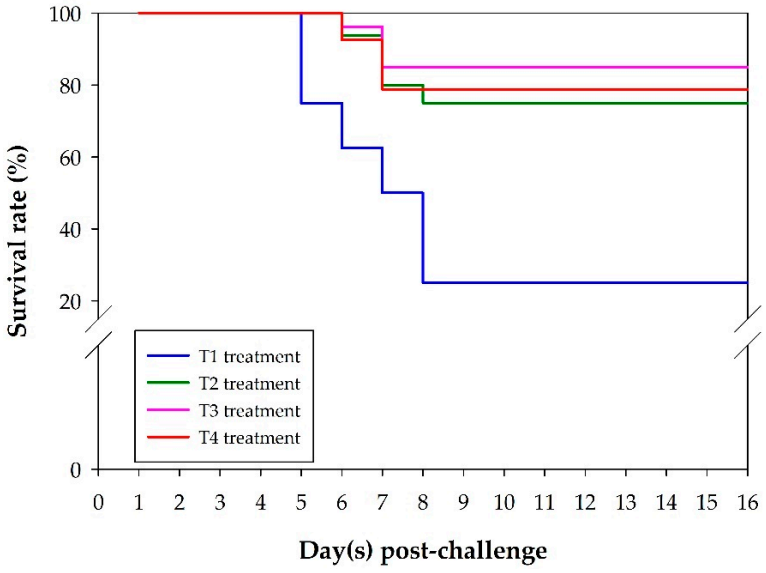

Figure 4. Kaplan–Meier analysis of *O. niloticus* ($n = 10$) challenged with *S. agalactiae* after 15 days of immunization. Treatment 1 (T1)—control, Treatment 2 (T2)—formalin-killed *S. agalactiae* immersion vaccine, Treatment 3 (T3)—formalin-killed *S. agalactiae* injectable oil-based vaccine, Treatment 4 (T4)—formalin-killed *S. agalactiae* water-based oral vaccine.

Table 3. Statistical significance among dietary treatment groups using a log-rank test.

Treatment Groups	Statistical Analysis		
	T1	**T2**	**T3**
T2	0.000 *		
T3	0.000 *	0.120 ^{ns}	
T4	0.000 *	0.624 ^{ns}	0.290 ^{ns}

"*" indicates a statistical significance ($p < 0.05$) and "^{ns}" denotes no statistically significant differences.

4. Discussion

Vaccines have been successfully applied in fish aquaculture to protect against bacterial infections [7,37,51–53]. Injection is the traditional method of administering these vaccinations, and research has shown that it is more efficient than oral administration [54]. However, injectable vaccines in aquaculture necessitate skilled vaccinator personnel, which adds to the cost of production. Moreover, injection induces handling stress, resulting in a low feed intake and loss of appetite in fish [34,37]. Following the injection of a vaccine, many fish are killed, and the remaining fish may lose some of their immunity and become more susceptible to diseases [55,56]. Therefore, for many fish, injectable vaccinations are not advised [54]. Nile tilapia is one of the species that holds the most promise for vaccine development. Given the recent extremely intensive method utilized in Nile tilapia farming, giving vaccinations orally is better than giving them via injection [7,27,34,37]. Fish oral vaccines have been thoroughly explored in recent years as an alternative to injectable immunizations, including Nile tilapia [57–59]. Even though oral vaccination seems to be the optimal way of administration, there are major limiting factors, such as larger volumes of vaccine, temperature and pressure tolerance conditions, vaccine doses, or booster vaccination for short durations of immunization [60,61]. Continuous research might greatly resolve these issues, resulting in the commercialization of effective oral vaccines for pathogenic diseases in aquaculture. Therefore, the present trial aimed at assessing injection, immersion, and oral vaccination methods in Nile tilapia. The metabolic indicators we used to assess immunological responses have all been used widely in previous studies. The immunological properties of skin mucus and blood serum provide a useful insight into the natural innate capacity of fish to resist infection [62,63]. Lysozyme is a bacteriolytic enzyme generated in the lysosome of phagocytic cells, and lysozyme activity is a crucial component of the non-specific immunological response of fish. In this investigation, vaccination through any of the three routes, immersion, injection, or oral administration, led to increased levels of the enzyme lysozyme and the antioxidant peroxidase in blood serum and skin mucus. Previously, on 7, 49 days post-vaccination and post-infection with *A. hydrophila*, lysozyme activity was considerably greater in fish inoculated with the BF vaccine (mixed *S. iniae* and *A. hydrophila*) compared to those vaccinated with the MA vaccine (monovalent *S. iniae*) [64]. Consequently, prior results indicated that, three weeks after vaccination, the lysozyme activity of vaccinated tilapia was much greater than that of unvaccinated tilapia [65]. Additionally, elevated phagocytosis, complement pathway, and respiratory burst activities implied an enhanced ability of lymphocytes and leukocytes to attack invading bacteria and increase fish survival in this study. The increase in leukocytes has a favorable effect on the generation of antibodies, resulting in a body resistance response against the foreign substance [66]. Likewise, a greater leukocyte count was closely associated with an increase in lysozyme [67]. These results suggest that the rising immunological response in vaccinated tilapia may be associated with a rise in lysozyme activity.

Interleukins 1 and 8 (*IL-1* and *IL-8*) are key pro-inflammatory cytokines associated with the immune response in fish, especially against harmful pathogens [68–70]. The results of this study display relatively high mRNA levels of *IL-1* and *IL-8* in the liver of Nile tilapia after *S. agalactiae* vaccination compared to the non-vaccinated group. *IL-1* and *IL-8* in the liver of fish vaccinated through oral, injection, and immersion routes have similar mRNA levels without significant differences. The results are in line with Jun et al. [71],

who reported upregulation of pro-inflammatory genes in Japanese eel (*Anguilla japonica*) treated with a *S. agalactiae* vaccine. The results are probably attributed to the potent immunostimulant action by the *S. agalactiae* vaccine, which enhances Nile tilapia's ability to resist infection. Besides, the lipopolysaccharide-binding protein (*LBP)* gene is another key protein involved in acute-phase immunity [68,72]. The precursor of LBP is also associated with immune resistance during infection with bacterial infections [73,74]. In this study, the more than two-fold higher levels of *IL-1* and *IL-8*, and almost three-fold rise in relative *LBP* transcript level in the vaccinated fish compared to the unvaccinated fish may have improved the survival of fish in this experiment. Taken together, the significant enhancement of pro-inflammatory gene expression in Nile tilapia is a result of vaccination, which has also been observed in Japanese eel (*Anguilla japonica*) treated with the *S. agalactiae* vaccine.

Our results showed that vaccination with formalin-killed *S. agalactiae* via immersion, injection, or orally stimulated a range of immune responses that enhanced the resistance of Nile tilapia to this pathogen and increased the survival rate in exposed populations. Whereas starch hydrogel-based oral vaccines produced a greater immune response than injection and immersion vaccination methods [75], our results for vaccination of Nile tilapia with antigen from formalin-killed *S. agalactiae* indicated that the immune response from water-based oral vaccination was very similar to that from the injectable vaccine. Similar benefits were investigated in Nile tilapia fed a live attenuated *S. agalactiae* vaccine [76]; polyvalent inactivated vaccine containing *S. agalactiae*, *S. iniae*, *L. garvieae,* and *Enterococcus faecalis* [29]; novel chimeric multiepitope vaccine for *S. agalactiae* [30]; naturally attenuated *S. agalactiae* live vaccine [28]; and inactivated *S. agalactiae* and *S. iniae* vaccines [31]. Nonetheless, the delivery of oral antigens has been reported to increase resistance in fish intestines and suppress immunity. The results of this study indicated that water-based oral vaccines can be used without reducing the pro-inflammatory immune response in Nile tilapia during infections caused by *S. agalactiae*.

In addition, the destruction of antigens by stomach acid and proteolytic enzymes in the digestive system is one of the most significant barriers to oral vaccines, which needs to be tackled to improve vaccine efficacy [59]. Several techniques are being used to protect antigens from the intestinal atmosphere for their immunogenic effects in an effort to address this challenge. Among them, encapsulation techniques by poly-biodegradable nanomaterials, such as chitosan and poly D,L-lactic-co-glycolic acid (PLGA), have demonstrated potential. The antigen is encapsulated by nanomaterials that can sustain the right epitope until it reaches the immunological site and is released in the intestine, considerably improving the immune response [77]. Vaccines, on the other hand, switch on the immune system to aid in disease resistance, and the use of these strategies to control infectious diseases is gaining relevance. Perfecting the use of adjuvants, delivery methods, and innovative technologies is needed to fulfill the demand of vaccines and ensure the safe supply of healthy fish products. It is vital for the future of the fish farming industry that vaccines can be an efficient tool for lowering the use of antibiotics in animals, therefore assisting the fight against antimicrobial resistance. In our point of view, although the formalin-killed *S. agalactiae* water-based oral vaccine shows potential in this study to improve the survival rate and activate the immune system of experimental fish, oral nano-encapsulated vaccines are promising and practical implications for aquaculture that can offer more benefits in terms of protective efficacy, time, labor, simplicity, and cost efficiency.

5. Conclusions

This study suggests that the oral vaccination of Nile tilapia with formalin-killed *S. agalactiae* stimulated the serum and skin mucus immunity and is just as effective as an injectable vaccine. By using this kind of vaccine, farmers can save time, cut down on the demand for highly skilled labor, and eliminate the stress and attendant mortality associated with direct injection. However, a larger sample size, more replicates, and an analysis of a wider range of target genes should be the focus of further studies.

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Institutional Review Board Statement: All animal experiments comply with AAALAC guidelines approved by the Chiang Mai University Committee (No. AQ006/2562[02/2562-09-16]).

Data Availability Statement: The data presented in this study are available upon request from the corresponding author.

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