Aquaculture Studies, 25(2), 93-106, AQUAST2188
http://doi.org/10.4194/AQUAST2188

RESEARCH PAPER

Formulation and Field Evaluation of Palm Oil Adjuvanted Feed-Based Streptococcosis Vaccine in Cage-Cultured Red Hybrid Tilapia

Mohd Syafiq Mohammad Ridzuan[1,2,*], Azila Abdullah[1], Nur Nazifah Mansor[2], Norazsida Ramli[3], Mohd Firdaus-Nawi[2,4,*]

[1]National Fish Health Research Centre (NaFisH), Fisheries Research Institute (FRI) Batu Maung, Department of Fisheries Malaysia, 11960 Batu Maung, Penang, Malaysia.
[2]Department of Marine Science, Kulliyyah of Science, International Islamic University Malaysia, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia.
[3]Kulliyyah of Allied Health Science, International Islamic University Malaysia, Bandar Indera Mahkota, 25200 Kuantan, Pahang, Malaysia.
[4]Faculty of Veterinary Medicine, Universitas Airlangga, Jl. Mulyorejo, Kec. Mulyorejo, 60115 East Java, Surabaya, Indonesia.
*These authors share the first authorship.

How to Cite

Ridzuan, M.S.M., Abdullah, A., Mansor, N.N., Ramli, N., Nawi, M.F. (2025). Formulation and Field Evaluation of Palm Oil Adjuvanted Feed-Based Streptococcosis Vaccine in Cage-Cultured Red Hybrid Tilapia. *Aquaculture Studies*, *25(2), AQUAST2188*. http://doi.org/10.4194/AQUAST2188

Article History
Received 04 November 2024
Accepted 21 January 2025
First Online 21 February 2025

Corresponding Author
E-mail: syafiq@dof.gov.my
firdausn@iium.edu.my

Keywords
Streptococcus agalactiae
Streptococcosis
Tilapia
Vaccine
Oral vaccine

Abstract

Streptococcosis poses a considerable threat to tilapia farming, necessitating the urgent development of an effective vaccine. Previous laboratory trials indicated the potential of palm oil as a vaccine adjuvant in a feed-based formulation. The present study further investigated the vaccine's efficacy using a similar formulation under field conditions. A total of 6.000 healthy juvenile red hybrid tilapias were divided into three groups: an unvaccinated control, a single booster group (vaccinated twice), and a double booster group (vaccinated thrice). The vaccine was administered orally at 5% of body weight. Organ samples and blood serum were collected biweekly over 16 weeks for bacterial isolation and immune response analysis, including immunoglobulin M (IgM), lysozyme, and complement C3 quantification. Serum IgM levels significantly increased (P<0.05) in all vaccinated groups, peaking at week 4. An additional booster given at week 6 in the double booster group resulted in sustained high IgM levels throughout the study. The isolation rates of *S. agalactiae* were lower in vaccinated groups, with mortality rates decreasing from 23.6% (unvaccinated) to 6.4% (double booster). These results indicate that oral administration of palm oil adjuvanted vaccine stimulates serum antibody (IgM), lysozyme, and complement C3 responses, reduces the incidence of streptococcosis, and improves the survival rate to 93.6%.

Introduction

Fish's superiority as an excellent source of protein, vitamin D, and omega-3 polyunsaturated fatty acids (PUFAs) is one of the factors furnishing to their increasing demand. The United Nations (UN) recently updated their demographic projections, predicting that the world's population would exceed 9.7 billion by 2050 (UN, 2019), implying that fish demand will consistently rise in the immediate future. As a result, aquaculture continues to expand faster than other major food-producing industries (FAO, 2018). Presently, aquaculture accounts for 49% of total fish production, with Asia contributing more than 91% of worldwide aquaculture production (FAO, 2022). With great potential and ongoing government support, Malaysia's aquaculture sector is flourishing and aiding in meeting the country's rising demand for animal protein to secure national food security.

Tilapia is one of the country's most important commercial freshwater fish species. The annual production of tilapia in Malaysia has risen from 34,822 metric tons in 2008 to 51.555 metric tons in 2012 (DOF, 2008-2012). However, recent production suffered a significant downshift, with only 33.002 metric tons in 2023, with a total estimated wholesale value of more

than RM 398 million (DOF, 2023). In general, the growth of the tilapia aquaculture industry is hindered by recurring outbreaks that cause significant mortality and morbidity to the farmers. Streptococcosis is widely recognized as a significant disease involving freshwater fish, especially tilapias, in numerous regions worldwide. In 1957, the disease was initially identified in Japanese rainbow trout Salmo gairdneri culture (Hoshina et al., 1958). Since then, there has been a significant increase in streptococcal case reports, posing a threat to freshwater aquaculture worldwide. In Malaysia, streptococcal epidemics have been continuously observed in floating net cages at Pahang River, Kenyir Lake, Pedu Lake, and Pergau Lake, resulting in high tilapia mortality (Amal & Zamri-Saad, 2011; Zamri-Saad et al., 2014). To date, various Streptococcus species have been identified as causative agents, including *S. agalactiae, S. iniae*, *S. dysagalactiae*, and *S. parauberis* (Sudpraseart et al., 2021; Mugetti et al., 2022; Maekawa et al., 2020; Han et al., 2011). However, the first two species are the most common threat to tilapia aquaculture worldwide, including Malaysia (Van et al., 2022; Amal et al., 2013; Cui et al., 2019).

Currently, streptococcal outbreaks are mitigated by a combination of effective farm management and the use of commercially accessible antibiotics. However, in recent years, antibiotics and other chemotherapeutics have lost their appeal in aquaculture due to environmental concerns, regulatory constraints, cost, and the increasing incidence of resistant microbes (Deng et al., 2020; Schar et al., 2020). For these reasons, vaccine development is increasingly viewed as a critical area of aquaculture research. The oral vaccination of fish has attracted attention due to its lack of dependence on additional manpower or specialized facilities. In Malaysia, oral vaccines are favored because 80% of local tilapia farmers are small-scale producers (Ridzuan et al., 2022). In addition, it is simple to administer (mimics natural feeding behavior), permits minimal physical contact between fish and operator, and allows flexibility in vaccine regimen formulation depending on the culture system, species, and size of fish in captivity. Despite these enticing benefits, oral immunization typically results in a shorter duration of protection than other routes and can cause inconsistent responses due to the hostile gastrointestinal environment of fish. Therefore, conjugation with an adjuvant and application of repetitive doses as boosters are essential to ensure sustained antigen release. Previously, we reported the promising results and potential use of palm oil as a fish vaccine adjuvant to replace commercially available Freund's adjuvant (Aminudin et al., 2018). Following an experimental challenge, it was determined that 10% palm oil adjuvant effectively stimulated systemic immune response and resulted in the highest survival rate of red hybrid tilapia. Therefore, in the present study, seed preparation and the vaccine's efficacy were further investigated using a similar formulation under field conditions. Evaluation of aquaculture vaccine in an actual production setting is crucial because the dynamic interaction between the host, pathogen, and environment can result in variations of immune response and vaccine efficacy.

Materials and Methods

Bacterial Strains and Growth Condition

The clinical isolates of *Streptococcus agalactiae* (SA2BKE) used as a seed vaccine in the present study were acquired from strain collection of the National Fish Health Research Centre (NaFisH), Fisheries Research Institute (FRI) Batu Maung, Malaysia. The isolates were initially isolated from a local outbreak in 2007 at Pergau Lake, Kelantan, Malaysia (Siti-Zahrah et al., 2008). The isolates were previously identified using the API®20 STREP identification system (bioMérieux, France) and confirmed using the polymerase chain reaction (PCR) technique (16s rRNA) (Nur-Nazifah et al., 2014). The strain was maintained in 1 ml aliquots at -80℃ in brain heart infusion (BHI) broth containing 10% glycerol.

Serotyping of *S. agalactiae*

The serotyping of *S. agalactiae* was carried out using ImmuLex™ Streptococcus group B antisera (SSI Diagnostica, Denmark) utilising a rapid agglutination principle as per manufacturer's instructions. Approximately one drop (10 µL) of ImmuLex™ antisera was applied on a clean glass slide. Subsequently, a loopful of freshly grown colonies from a blood agar (BA) was suspended in 100 µl saline, and a drop of this suspension was added next to the ImmuLex™ antisera. The two drops were combined with a mixing stick, equally distributed, and observed for agglutination within 5 to 10 seconds. Visible agglutination within 10 seconds suggested a positive reaction.

Development of *S. agalactiae* Growth Curve

S. agalactiae growth curve was developed according to Hall et al. (2014) with slight modification. Briefly, a single colony from an overnight culture of *S. agalactiae* was inoculated into 10 ml of BHI broth and incubated overnight at 30±2℃, 150 rpm. Then, 1 ml of bacterial suspension was inoculated onto a 1 L conical flask containing 600 ml of BHI broth. Immediately after inoculation, 3 ml of the mixture was pipetted and subjected to optical density (OD) measurement at 600 nm wavelength. Then, serial dilution was conducted using 1 ml of the suspension in 9 ml of sterile phosphate-buffered saline (PBS, pH 7.4) and plated on BA in duplicate. After overnight incubation at 30±2℃, the colonies were counted and expressed as colony-forming units (CFU ml-1). The bacterial suspension was further incubated at 30±2℃, 150 rpm and similarly repeated after 15 min, 30 min, 1 hr, 2 hr, 4 hr, 8 hr, 12 hr, 16 hr, 20 hr, and 24 hr post-incubation. The assay was

conducted at least two times independently, and the growth curve was plotted using SlideWrite™ (Advance Graphics Software Inc., USA) software.

Preparation of Formalin-Killed Bacteria

The formalin-killed bacteria used as a seed vaccine was prepared according to the method of Firdaus-Nawi et al. (2013). Briefly, the bacteria were cultured in BHI broth at 30±2℃, 150 rpm for 12 hr. Following incubation, the bacterial concentration was determined using spectrophotometer (EMCLAB Instruments, Germany) based on the previously developed growth curve and standard plate count technique. Subsequently, the cells were harvested through centrifugation at 5.000 rpm for 15 min at 4℃ and washed thrice with sterile PBS. The harvested cells were suspended in 0.5% formalin (Merck, Germany) to kill the bacteria and kept overnight at 4℃. The formalin residue was then removed through centrifugation and repetitive washing in PBS. The formalin-killed cell was re-suspended in PBS to obtain the final bacteria concentration of 109 CFU ml-1. Lastly, the suspension was streaked onto BA to ensure the entire bacterial cell was killed and stored at 4℃ until further used.

Preparation of Palm Oil Adjuvanted Feed-Based Vaccine (FBV)

The formalin-killed bacteria (109 CFU ml-1) was first diluted with an equal volume of sterile PBS and absolute ethanol (1:1 ratio). After that, palm oil was added to a final concentration of 10%. The suspension was then thoroughly mixed, sprayed onto the feed formulation, and subjected to a pressed pelleting machine. The final concentration of bacteria cells is 106 cells/kg of feed. The feed formulation is summarized in Table 1.

Proximate Analyses

Approximately 300 g of the FBV, feed formulation (FF) pellet (without vaccine), and commercial pellet (CP) were ground into a fine powder using a laboratory mill grinder for 10 minutes. The resulting powder was transferred into plastic containers and stored in a borosilicate glass desiccator until further used.

Moisture, ash, crude fat, crude fiber, and crude protein were determined following the procedures of the Association of Official Analytical Chemists (AOAC, 2012). Moisture contents were determined after oven drying at 105℃ for 3 hr. Ash compositions were calculated following the dehydration process and combustion in a muffle furnace (Fisher Scientific, USA) at 550℃ for 5 hr. The 4-step extraction protocol of boiling (135°C, 20 min), rinsing (135°C, 35 min), recovery (135°C, 10 min), and pre-drying was employed for crude fat determination and performed in a FOSS Soxtec Avanti 2055 (Foss Analytical, Denmark) using petroleum ether as solvent. Crude protein levels were quantified using the Kjeldahl method, which involves a 3-step extraction protocol of digestion, distillation using Kjeltec 2100 unit (Foss Analytical, Denmark), and titration using 876 manual titrator plus (Metrohm, Switzerland).

Experimental Design and Vaccination Protocol

The field trial was conducted on a private farm at Kenyir Lake, an artificial lake located in the province of Terengganu, Malaysia. The main fish species culture on site is red hybrid tilapia (Oreochromis spp.). Other fish species, such as catfish (Clarias spp.) and Hoven's carp (Leptobarbus hoevenii), were also cultured but in low density. The selected farm had a history of endemic streptococcal problems, which led to a cumulatively high mortality rate of cultured red hybrid tilapia during the hot and dry seasons (March to June).

The field vaccination trial was conducted according to Ismail et al. (2017) with minor modifications during the annual outbreak season. Briefly, a total of 6,000 red hybrid tilapias with an average bodyweight of 20±10 g were acquired from a local hatchery and evenly distributed into six 4 m length x 4 m width x 4 m depth floating net cages. Each cage was represented as a single booster (SB), double booster (DB), and unvaccinated control group in duplicate. Prior to the experiment, ten fish from each group were randomly screened to determine their health status.

A total of 16 weeks were devoted to the vaccination trial. The fish was fasted a day prior to each vaccine administration to ensure maximum vaccine uptake. All groups were fed with commercial pellets throughout the experimental period; however, FBV was given on day-0 and day-14 for SB group and day-0, day-14, and day-42 for DB group. The feed-containing vaccine was given at 5% of the fish's average weight. It is important to note that other husbandry practices adopted on the farm were not intervened.

Post-mortem was performed in situ from 30 fish per group at two-week intervals for 16 weeks. Brain,

Table 1. Feed formulation of the palm oil adjuvanted vaccine

Ingredients	Percentage (%)	Ingredients	Percentage (%)
Danish fishmeal	15.00	Di-calcium phosphate	1.00
Soybean meal	23.43	CMC (Binder)	0.10
Rice bran	39.77	Mineral premix	0.40
Maize / Starch	15.00	Vitamin premix	0.30
Squid meal	5.00		

eye, and kidney samples were collected and subjected to bacterial isolation and identification. Blood was also withdrawn from the caudal vein and subjected to serum antibody (IgM), lysozyme, and complement C3 determination. Fish weight, length, clinical signs, and mortality were recorded throughout the study period.

Bacterial Identification

Swab samples of the brain, eye, and kidneys were aseptically streaked on BA and incubated at 30±2℃ for 48 hours. The clinical isolates were subcultured multiple times to ensure their purity before performing the Gram staining, oxidase, and catalase test. Following manufacture instructions, the isolates were further characterized based on their biochemical profiles using the API®20 STREP identification system (bioMérieux, France).

Water Quality Measurement

During each sampling period, water quality parameters were collected at three different locations within the farm and measured on-site between 2:00 and 3:00 pm (local time in Malaysia). Using a hand-held YSI meter (YSI Inc., USA), the following parameters were measured: water temperature, pH, and dissolved oxygen. Meanwhile, nitrogen ammonia, sulfide, and nitrate concentrations were measured using salicylate, methylene blue, and cadmium reduction methods (HACH company, USA). Nitrite and iron were measured using USEPA Diazotization and the FerroVer method (HACH company, USA). On the other hand, reactive phosphorus or orthophosphate was carried out using the Molybdovanadate method (HACH company, USA).

Determination of Serum Antibody (IgM) Titer by ELISA

The blood serum was subjected to indirect ELISA to determine the antibody (IgM) levels following the method described by Firdaus-Nawi et al. (2013). Five colonies of *S. agalactiae* from the BA were briefly sub-cultured into 100 ml of the BHI broth and incubated at 30±2℃, 150 rpm for 24 hr. Following incubation, the bacterial concentration was determined using the standard plate count technique. Subsequently, the cells were harvested through centrifugation at 5000 rpm for 15 min at 4℃ and washed thrice in 40 ml of sterile PBS. The resulting pellet was then suspended in carbonate-bicarbonate buffer (Sigma-Aldrich, USA) (pH 9.6) to a final concentration of 105 CFU ml-1 and boiled in a water bath at 97℃ for 20 min. The suspension was stored at -20℃ prior to use as coating antigen.

The flat-bottomed microtitre plates were coated with 100 µl coating antigen and left overnight at 4℃. The plates were washed twice with PBS containing 0.05% Tween-20 (PBS-T) to remove unbound antigens. The coating antigens were then blocked with 200 µl of 1% bovine serum albumin (BSA) and washed twice. Then, 100 µl of each diluted serum sample (1:1.000) was added and incubated at 37℃ for 1 hr. After that, 100 µl of goat anti-tilapia immunoglobulin (GAT) serum, diluted at 1:10.000, was added to each well and incubated for 1 hr. Next, 100 µl of conjugated rabbit anti-goat (RAG) IgM-horseradish peroxidase (Nordic Immunology, Netherlands) (1:10.000) was added and incubated for another 1 hr. The bound conjugates were captured by adding 100 µl of Tetramethylbenzidine (TMB) One Substrate solution (Promega, USA) before the reaction was terminated by adding 50 µl of 2.5 M sulphuric acid. The absorbance was determined at 450 nm wavelength. Using serum from juvenile and disease-free red hybrid tilapia, the cut-off value was determined and set at mean plus three times the standard deviation value (Crowther, 2000).

Determination of Lysozyme Activity

Determination of serum lysozyme level was carried out by using a fish lysozyme (renal amyloidosis) ELISA kit (Cusabio, China), which employs the competitive inhibition enzyme immunoassay technique with a detection range between 3.12–200 ng ml-1 lysozyme. Briefly, reagents, samples, and standards were prepared per manufacturer instruction, and 50 µl of standards and sample was loaded per well. Then, 50 µl of HRP-conjugate (1x) was added to each well, excluding the designated blank well with gentle agitation for 60 sec., followed by incubation for 40 min at 37℃. The plate underwent five washes to eliminate unbound conjugate before adding 90 µl of TMB substrate to each well. The plate was incubated for 20 min at 37℃ before the reaction was terminated by adding 50 µl of stop solution. The optical density was measured by employing a microplate reader at 450 nm. The lysozyme values are expressed as µg ml-1.

Determination of Complement C3 Activity

Determination of serum complement C3 activity was carried out by using a fish Complement C3 ELISA kit (Cusabio, China), which employs a similar competitive inhibition enzyme immunoassay technique with a wider detection range of 12.5–500 µg ml-1. Briefly, reagents and samples were prepared per manufacturer instruction, and 50 µl of standards or samples were loaded per well. Then, 50 µl of HRP-conjugate mixed solution was added to each well except the designated blank well with gentle agitation and incubated for 1 hr at 37℃. Before adding 50 µl of Substrate A and Substrate B to each well, the plate was washed thrice to remove unbound conjugate. The plate was incubated for 15 min at 37℃ before the reaction was terminated by adding 50 µl of stop solution. The absorbance was measured using a microplate reader at an optical density of 450 nm. The complement C3 values are expressed as mg ml-1.

Data Analysis

The statistical analysis was performed using one-way analysis of variance (ANOVA) and Tukey's honestly significant difference (HSD) in Statistix version 10 software (Analytical Software, USA). The results were considered significant at P<0.01 or P<0.05.

Results

Serotypes of *S. agalactiae* (SA2BKE)

The serotypes of *S. agalactiae* (SA2BKE), used as a seed vaccine, were determined using a rapid latex agglutination assay. Visible agglutination within 10 sec was observed and therefore revealed that the isolates belong to serotypes III (Figure 1).

Growth Curve of *S. agalactiae*

The growth curve of *S. agalactiae* (SA2BKE) following incubation at 30±2°C in BHI broth is presented in Figure 2. Over the first two hours, the bacteria do not actively replicate, displaying a flat line; hence, this stage could be designated as the lag phase. After two hours post-incubation, the rapid growth of the bacteria was observed, marked by predictable doublings of the population, and peaked at 12 hours post-incubation. Hence, this stage of bacteria growth could be designated as the log or exponential phase. Subsequently, between 12 to 16 hours post-incubation, the growth rate of the cells appeared stagnant, resulting in a smooth horizontal linear line marking the stationary phase's duration. Prolonged incubation caused the bacteria's growth to follow a steeply declining trajectory between 16 to 24 hours post-incubation, indicating the onset of the death phase.

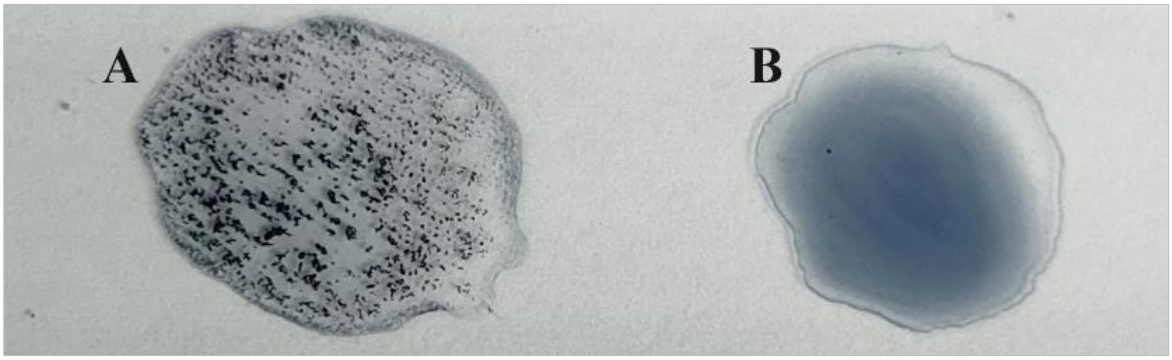

Figure 1. Serotyping of *S. agalactiae* (SA2BKE) using rapid latex agglutination test revealed serotype III identity. A positive reaction (A) has visible agglutination and clearing, whilst a negative reaction (B) is smooth and cloudy.

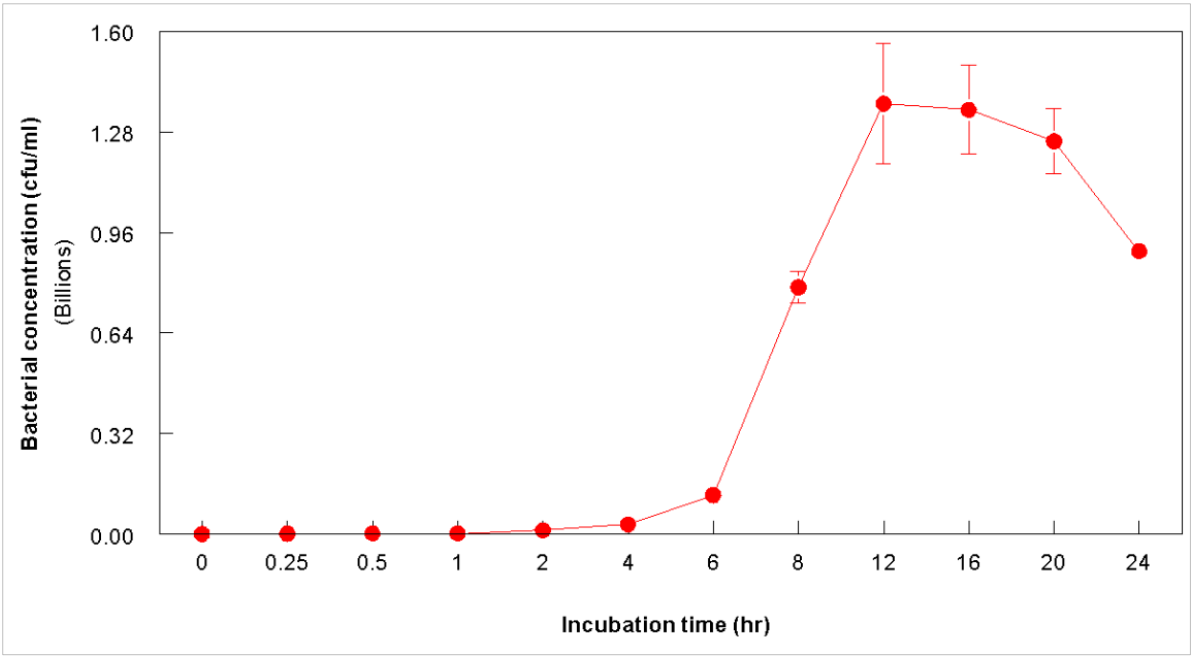

Figure 2. Growth curve of *S. agalactiae* (SA2BKE) following incubation at 30±2°C, 150 rpm in BHI Broth for 24 hours. A rapid growth was observed after 2 hours and peaked at 12 hours post-incubation.

Proximate Compositions of Feed-Based *Streptococcosis* Vaccine (FBV), Feed Formulation (FF), and Commercial Pellet (CP)

Table 2 summarizes the mean±SD proximate composition of feed-based streptococcosis vaccine (FBV), feed formulation (FF), and commercial pellet (CP). The moisture content was found to be 6.8378±0.3493, 5.2977±1.2214, and 7.8438±0.0529% in FBV, FF, and CP, respectively. A significant ($P<0.01$) difference was observed among all feed samples tested. A variation in moisture content between FBV and FF is most likely due to the seed vaccine mixture's inclusion prior to pelleting. Although the moisture was significantly higher than the control pellet (FF), it remained within an acceptable range of less than 10% (De Koning, 2002). Moisture content in animal feed should be regulated to avoid mold growth and overcome a decomposition response, allowing for safe storage and improving feed shelf life.

Ash is an indicator of the total amount of minerals present in a feed sample, following the dehydration process and combustion in a muffle furnace. This study found the highest ash content in CP with 9.6728±0.1012%, followed by FF and FBV with 8.8727±0.1493 and 8.5829±0.3474%, respectively. There was a significant ($P<0.01$) increase in the ash content of CP between FF and FBV. Meanwhile, a comparison between FF and FBV revealed insignificant ($P>0.01$) differences. The ash content of all the feed samples was found within the recommended ash content in the tilapia diet of 7-12% (Villarino et al., 2020).

The feed samples analysed contain various amounts of crude fat. The crude lipid content of FBV and FF was 7.7033±0.9735 and 6.7356±1.3329%, respectively. The addition of palm oil as a vaccine adjuvant caused a slight increase ($P>0.01$) in crude fat content in FBV compared to FF. Surprisingly, crude fat in CP was severely low (2.5625±0.0410%) and deviated from the recommended value for farmed tilapia of 5-12% (Lim et al., 2011). Dietary lipids are essential sources of highly digestible energy and provide essential fatty acids fish need for normal growth and development. Besides, lipids are primarily included in the formulated diet to maximize their protein-sparing effect by increasing their digestible energy value (Li et al., 2023).

The crude fiber was found to be 6.3110±1.8526, 5.6397±2.6170, and 6.1979±0.1216% in FBV, FF, and CP, respectively. No significant ($P>0.01$) difference was observed in the crude fiber of these feed samples, and its composition was within the suggested values for the tilapia diet of 4-8% (Ng et al., 2013).

The highest crude protein content was found in FF with 32.496±2.5750%, followed by CP and FBV with 31.561±0.3438 and 30.220±0.8907%, respectively. Although a variation was observed, there was no significant difference ($P>0.01$) in the crude protein composition of all feed samples, which fell within the recommended values for farmed tilapia of 27-37% (Khattab et al., 2000)

Isolation of *S. agalactiae*

Table 3 summarizes the isolation rate of *S. agalactiae* from each experimental group. Initially, during health screening and first sampling (week 0), the bacteria species was not isolated from any experimental groups, indicating that the fish acquired from the local hatchery was free from *S. agalactiae*. In the unvaccinated control group, *S. agalactiae* was successfully isolated from week four and throughout the

Table 2 Mean±SD proximate composition of feed-based streptococcosis vaccine (FBV), feed formulation (FF), and commercial pellet (CP)

Samples	Moisture (%)	Ash (%)	Crude fat (%)	Crude fiber (%)	Crude protein (%)
FBV	6.8378±0.3493^{a}	8.5829±0.3474^{b}	7.7033±0.9735^{a}	6.3110±1.8526^{a}	30.220±0.8907^{a}
FF	5.2977±1.2214^{b}	8.8727±0.1493^{b}	6.7356±1.3329^{a}	5.6397±2.6170^{a}	32.496±2.5750^{a}
CP	7.8438±0.0529^{c}	9.6728±0.1012^{a}	2.5625±0.0410^{b}	6.1979±0.1216^{a}	31.561±0.3438^{a}

^{a,b}Different superscript letters represent a significantly different ($P<0.01$) between the same row

Table 3 Rate of *Streptococcus agalactiae* isolated from red hybrid tilapia samples (n=30)

	Rate of isolation (%)			
Week	Unvaccinated	Single booster	Double booster	Mean±SD
0	0	0	0	0
2	0	33.3	0	11.1±19.2
4	20	26.7	16.7	21.1±5.1
6	36.7	0	0	12.2±21.2
8	20	6.7	6.7	11.1±7.7
10	60	10	20	30.0±26.5
12	0	6.7	0	2.2±3.9
14	30	10	23.3	21.1±10.2
16	10	10	6.7	8.9±1.9
Mean±SD	19.6±20.2	11.5±11.3	8.2±9.4	

sampling period, except in week 12. However, in the SB group, the bacteria species were isolated almost every sampling week starting from week 2, except in week 6. Similarly, in the DB group, *S. agalactiae* was isolated throughout the sampling period with a lower prevalence, except for week 2, week 6, and week 12. The average incidence of *S. agalactiae* during the duration of the study was 19.6%, 11.5%, and 8.2% for unvaccinated control, SB, and DB groups, respectively. As shown in Figure 3, *S. agalactiae*-infected tilapia ranged in weight from 13 to 165 g.

Clinical Signs and Symptoms of Infected Fish

Several clinical signs and gross lesions related to streptococcosis in red hybrid tilapia were observed throughout the entire study period, which included bilateral exophthalmia (pop-eye), soft and watery brain, enlarged spleen, and erratic swimming behavior (Figure 4).

Water Quality

The water quality parameters measured throughout the study period are summarized in Table 4. Most parameters were within the permissible range for farmed tilapia except the water temperature (30.73±1.08°C), which might predispose fish to infections. However, there was an insignificant correlation between monthly water quality parameters and mortality occurrence throughout the experiment (data not shown).

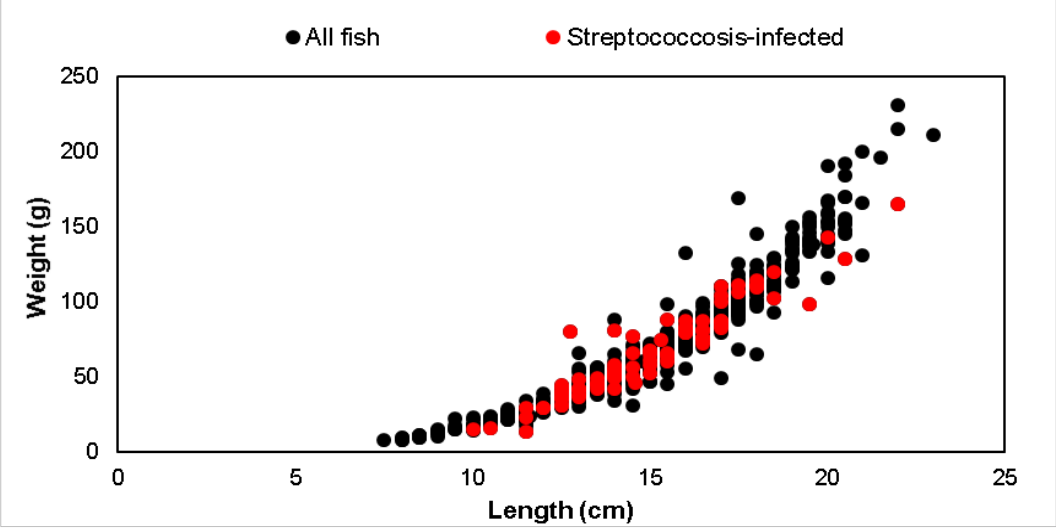

Figure 3. Range of weight and length of *S. agalactiae*-infected red hybrid tilapia.

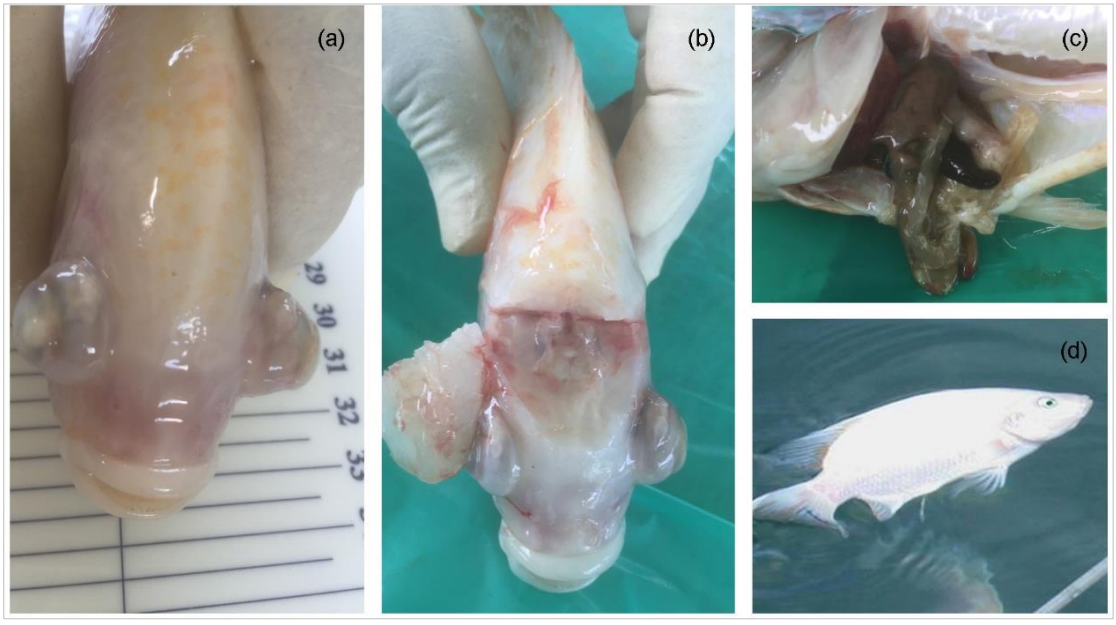

Figure 4. Several clinical signs and gross lesions were observed during routine sampling that are associated with streptococcosis: (a) bilateral exophthalmia, (b) soft and watery brain, (c) enlarged spleen, and (d) erratic swimming.

Serum Antibody (Igm) Response against *S. agalactiae* Following Vaccination Regime

Figure 5 depicts the progression of serum antibody (IgM) response and *S. agalactiae* isolation rate in each experimental group following vaccination. Prior to the vaccination, serum antibody levels against *S. agalactiae* did not exhibit significant (P>0.05) differences among groups and were marginally below the cut-off value. However, a different phenomenon was observed after vaccination, where both vaccinated groups demonstrated a significant (P<0.05) increase in the serum antibody level compared to the unvaccinated control group, reaching their peak at week 4 and subsequently declining to the non-protective cut-off value by week 12 in the SB group. Nonetheless, following a second booster at week 6, the serum antibody level in the DB group increased significantly (P<0.05) at week 8 before gradually reducing. However, it remained above the cut-off value until the end of the experimental period at week 16. Meanwhile, the serum antibody level in the unvaccinated control group rose between weeks 4 and 10 before sharply reducing, approaching the cut-off value at week 16. This event was probably due to the high infection rate of *S. agalactiae*, which started from week 4 to week 10 in the unvaccinated control group.

Lysozyme Level

The serum lysozyme concentration of red hybrid tilapia sampled during the study is presented in Figure 6. Prior to vaccine administration, the serum lysozyme level in unvaccinated control and vaccinated fish (SB and DB) showed an insignificant (P>0.05) difference. Following initial vaccine administration and first booster, the lysozyme activity in all vaccinated fish samples at weeks 2 and 4 increased significantly (P<0.05) compared to the unvaccinated fish. However, in week 6, the lysozyme level in unvaccinated fish was significantly (P<0.05) higher compared to both vaccinated groups. It remained high until week 10, likely correlating with the high infection rate of *S. agalactiae*, which was started from week 4 to week 10 in the unvaccinated control group. Moreover, following a second booster at week 6, the serum lysozyme levels of fish in the DB group were significantly (P<0.05) higher than those of fish in the SB group at week 8 before

Table 4 Water quality parameters were measured during the study period. The values are mean±SD

Parameter	Mean ± SD	Parameter	Mean ± SD
Temperature (°C)	30.73±1.08[†]	Nitrate (mg l^{-1})	1.87±1.22
pH (1-14)	7.06±0.17	Nitrite (mg l^{-1})	0.01±0.00
Dissolved oxygen (mg l^{-1})	5.56±0.84	Sulfide (µg l^{-1})	2.74±1.23
Iron (mg l^{-1})	0.03±0.01	Phosphate (mg l^{-1})	0.13±0.14
Ammonia (mg l^{-1})	0.02±0.02		

[†]Indicates not within the normal parameter

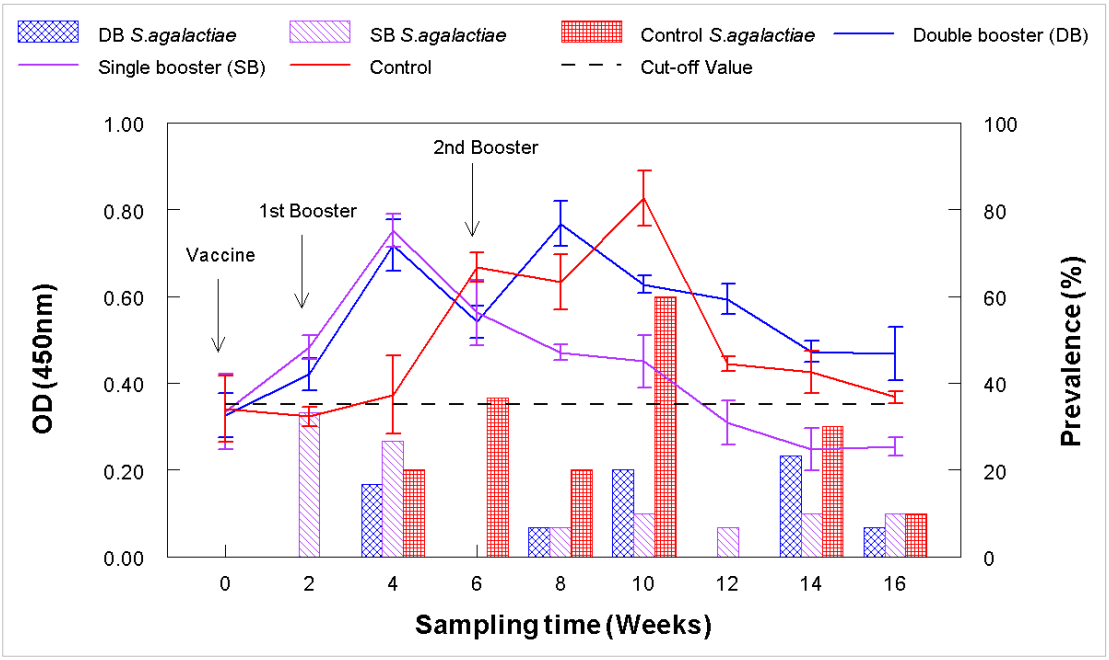

Figure 5. Serum antibody (IgM) response of red hybrid tilapia and isolation rate of *Streptococcus agalactiae* following vaccination regime (n=30). The values are mean±SD. A marked increase in IgM levels was observed in both vaccinated groups, single booster (SB) and double booster (DB) after booster dose in week-2.

gradually declining thereafter. Besides, the serum lysozyme level in unvaccinated (control) and vaccinated fish did not differ significantly (P>0.05) from week 12 through week 16 of the experiment.

Complement C3 Level

The serum complement C3 concentration of red hybrid tilapia sampled during the study is presented in Figure 7. Prior to vaccine administration, the serum complement C3 level in unvaccinated control and vaccinated fish showed an insignificant (P>0.05) difference. Following initial vaccine administration and first booster, the complement C3 activity in all samples of the vaccinated fish (SB and DB) was significantly (P<0.05) higher than the unvaccinated fish from week 2 until week 6. Besides, following another booster on week 6, the serum complement C3 level of fish in the DB group was significantly (P<0.05) more than in the SB group at week 8 before gradually declining thereafter. Nevertheless, the complement C3 level in unvaccinated control fish resulted in a significant (P<0.05) higher compared to SB groups in week 8 and remained higher (P<0.05) than both vaccinated groups (SB and DB) at week 10. This sudden increase in complement C3 activity of fish in the unvaccinated control group might be due to the high infection rate of *S. agalactiae*, which peaked at week 10. Eventually, the serum complement C3 level in unvaccinated (control) and vaccinated fish showed an insignificant (P>0.05) difference starting from week 12 until the end of the experiment in week 16.

Efficacy of The Vaccine

Table 5 summarizes fish mortality records within each of the experimental groups. The unvaccinated control group recorded the highest mortality with 23.55±1.77%, followed by SB and DB groups with 14.45±5.87% and 6.40±0.42%, respectively. The average mortality rate between the unvaccinated control group and the DB group showed significant (P<0.05) differences. It is important to note that slightly lower mortality in the unvaccinated group is expected since the incidence rate of *S. agalactiae* was relatively low.

Discussion

The streptococcal disease has posed a concern for the global tilapia aquaculture industry. Its severity is worsened by intensified cage-culture systems, especially in large waterbodies such as lakes or rivers. This disease corresponds with increasing water temperature that induced stress factors in fish under captivity. Past research has shown that an increase in temperature induces pathogenicity of *S. agalactiae* due to overexpression of β-hemolysin, pore-forming toxin (CAMP factor), and Pilus 2b backbone protein (PI-2B) (Kayansamruaj et al., 2014). The transcriptome and proteome analyses revealed the differential upregulation of genes and proteins implicated in cellular metabolic pathways, virulence factors, and bacterial adaptation (Tavares et al., 2018). Once infected, fish exhibited a significant upregulation of inflammatory-related genes, including cyclooxygenase-2, interleukin-1

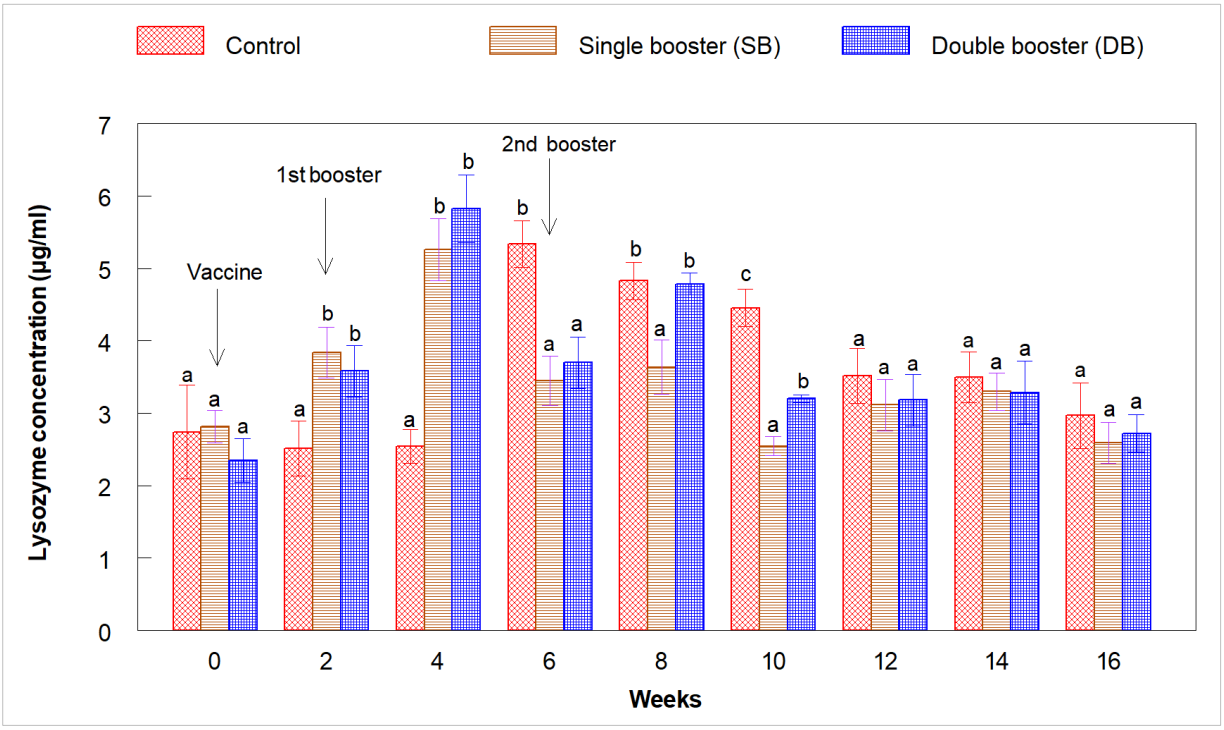

Figure 6. The serum lysozyme activity of red hybrid tilapia following the vaccination regime (n=10). The values are mean±SD. Mean values with different superscripts differ significantly at P<0.05.

beta (IL-1β), and tumor necrosis factor-alpha (TNF-α), which may result in acute mortality (Kayansamruaj et al., 2014). Due to the year-round moderate climate in Malaysia, periodic outbreaks of streptococcosis with abrupt high mortality of cultured tilapia were associated with the onset of the dry and hot season from March until June (Siti-Zahrah et al., 2008; Amal et al., 2015). Consequently, these four critical months are crucial for disease intervention, especially through vaccination.

Vaccination is one of the most significant science advancements in history. It has saved countless lives and eradicated or controlled several deadly infectious diseases in the world. Among the different types of vaccines, the whole-cell killed bacterin vaccine holds a prominent position with a long-standing history of successful disease prevention (Montero et al., 2024). One of the key advantages of whole-cell killed bacterin vaccines is that they can elicit a broad immune response. Unlike subunit vaccines that contain only specific components of the pathogen, whole-cell killed bacterin vaccines present a diverse range of bacterial antigens to the immune system. This stimulates the production of a wider array of antibodies and T-cells, contributing to a more robust and comprehensive defense against the targeted pathogen (Pollard & Bijker, 2021). Several similar types of fish vaccines against streptococcosis are available abroad, including Aquavac® Strep Sa, Aquavac® Strep Sa1, Aquavac® Strep Si (Intervet, MSD Animal Health), and Alpha Ject® micro 1 Tila (Pharmaq). All of these vaccines require an injection route for delivery, and currently, only Aquavac® Strep Si (intended against S. iniae infection) is approved for use in Malaysia. Unfortunately, most local farmers prefer not to use injection vaccines for aquaculture. This predicament might be because 80% of local tilapia farmers are small-scale operators and do not have the financial means to invest in extra labor, specialized equipment, and facilities necessary to apply injectable or immersion vaccines (Ridzuan et al., 2022). In contrast, oral fish vaccination allows for mass vaccination on the farm without unnecessary stress associated with fish handling, which can occasionally lead to mortality (Gonçalves et al., 2022; Stevens et al., 2017). In addition, oral immunization offers ease of operation and permits flexibility in formulating a vaccination regimen. Therefore, considerable effort was devoted to developing an oral vaccine.

Typically, whole-cell bacterin vaccine production entails a series of fundamental procedures culminating in the end product. The first step involves cultivating

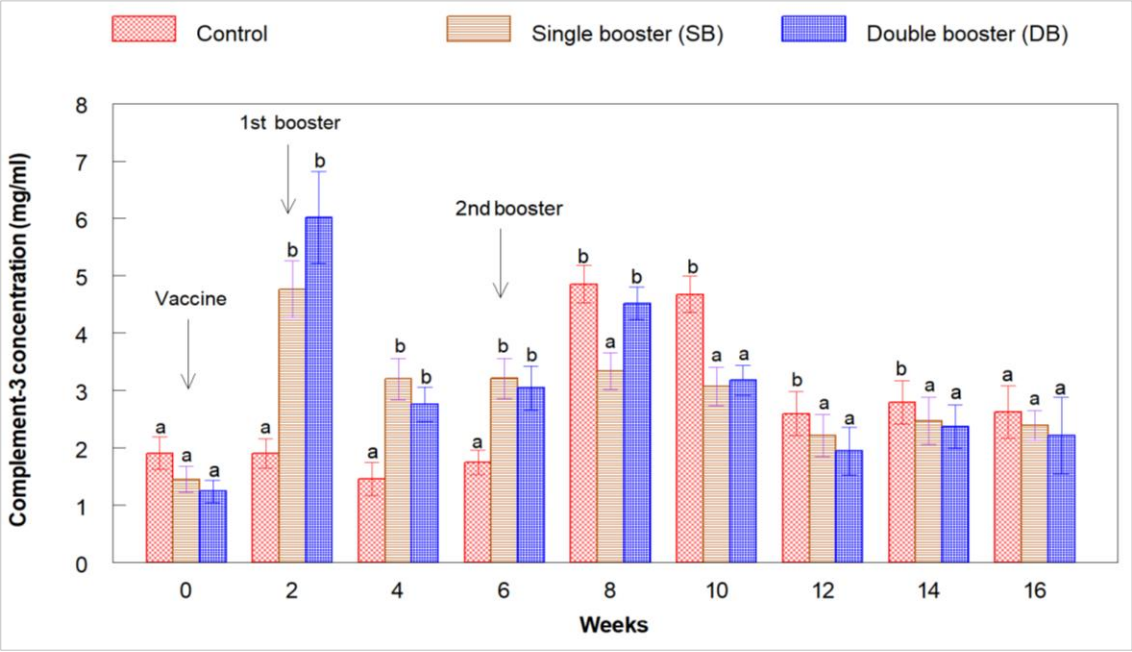

Figure 7. The serum complement C3 variations of red hybrid tilapia following the vaccination regime (n=10). The values are Mean±SD. Mean values with different superscripts differ significantly at P<0.05.

Table 5 Mortalities of red hybrid tilapia in experimental trial groups. Values are the Mean ± SD. Different superscript letters indicate a significant difference at P<0.05

Experimental groups	Cage no	Dead/Total	Mortality (%)	Average mortality (%)
Unvaccinated control	1	248/1000	24.8	23.55±1.77[a]
	2	223/1000	22.3	
Single booster (SB)	3	186/1000	18.6	14.45±5.87[ab]
	4	103/1000	10.3	
Double booster (DB)	5	61/1000	6.1	6.40±0.42[b]
	6	67/1000	6.7	

bacteria in an optimal culture medium and condition to maximize antigen yield while preserving the cell's integrity (Gomez et al., 2013). Bioreactors are commonly employed for this purpose. Huser et al. (1983) previously recommended fermenter cultivation of *S. agalactiae* in Todd-Hewitt broth containing 2% (w/v) glucose, pH 6.2, with a constant flow of CO2 for mass production. In contrast, the BHI medium was chosen in this study due to its superior sensitivity, specificity, and accuracy while reducing material and supply costs (Huu et al., 2019). The subsequent step is bacterial harvesting, which can be easily achieved by centrifugation to release the antigen from the growth substrate, followed by the inactivation procedure. Inactivating the whole-cell bacterial vaccine is crucial to destroy disease-producing capacity and is typically accomplished through exposure to heat, radiation, or chemically induced. The latter is the most widely used in human and animal vaccines, such as formaldehyde and β-propiolactone, due to its high inactivation potency without alteration of antigenic properties (Elveborg et al., 2022; Zhang et al., 2016). The toxicity mechanisms of formaldehyde and β-propiolactone toxicity have been described in detail by Delrue et al. (2012). These chemicals are alkylating agents that act through the alkylation of carboxyl and hydroxyl groups of DNAs, resulting in monohydroxymethylation of amino acids and other carboxyethyl derivatives, which blocks genome reading. In addition, formaldehyde and β-propiolactone also induce crosslinking of proteins, preventing normal transcriptional processes as a consensus mechanism and causing cell death.

In an attempt to produce a vaccine for *S. agalactiae* seed in bulk quantities, the bacterial growth curve was established following the predetermined culture conditions. After 12 hours of incubation in a nutrient-rich medium of BHI broth, the bacterial growth reached the apex of the exponential phase. At this stage, the cells are the healthiest and metabolically active and are preferred for seed vaccine preparation. In addition, a recent study disclosed higher expression levels of *S. agalactiae* virulence genes, including major nuclease, genetic pilus island (PI-2a), other virulence-associated genes involved in translation, ribosome structure, and biogenesis occurred during the exponential growth phase (Silvestre et al., 2022). Elevated virulence expression is advantageous for seed vaccine candidates as it may induce a more robust immune response from the host (Hamed et al., 2018). Therefore, incubation periods exceeding 12 hours must be avoided.

In the current investigation, the fish were not fed the day before each administration, and the vaccines were evenly distributed to ensure maximum vaccine uptake by all fish. However, it is essential to note that it is impossible to determine the exact dose of antigen each fish consumes, potentially leading to inconsistent responses. In the present study, the oral vaccine was prepared by incorporating the seed vaccine in the feed formulation before being subjected to a pressed pelleting machine. The nutritional compositions of the prepared feed-based vaccine were determined in proximate analysis, and it was revealed that the moisture, ash, crude fat, crude fiber, and crude protein of the vaccinated pellet were within the recommended nutritional value for the red hybrid tilapia diet. These dietary components in a fish's diet are essential in influencing growth, feed conversion, physiological effects, disease susceptibility, and overall health (Oliva-Tales, 2012). Besides, adding palm oil as an adjuvant did not significantly alter the vaccine's proximate composition, which is consistent with prior research by Aslah et al. (2021). The key advantages of using palm oil as a fish vaccine adjuvant is its biocompatibility and safety. Being a naturally derived product, palm oil is well-tolerated by fish and does not induce adverse reactions. It is non-toxic and does not accumulate in fish tissues, ensuring the safety of both the vaccinated fish and consumers (Ayisi et al., 2019; Ng et al., 2007). This aspect is especially important as the aquaculture industry aims to prioritize sustainability and eco-friendly practices. Other than that, palm oil is abundantly produced in Malaysia, making it readily available and cost-effective as a fish vaccine adjuvant. Compared to some synthetic adjuvants, palm oil offers a more affordable option for aquaculture producers and insignificantly increasing production costs. This affordability also makes it more accessible to small-scale farmers, who can benefit from improved disease prevention measures.

Following the vaccination trial on an endemic farm, the specific antibody (IgM), non-specific immune response activity (lysozyme and complement C3), bacterial isolation, and mortality records were thoroughly monitored over four months of the culture period. The serum IgM antibody showed a significant difference between the vaccinated and unvaccinated groups. It exhibited an ascending pattern after the initial administration and first booster, reaching a peak at week 4. The IgM level of fish that received a second booster dose displayed further elevation above the cut-off value throughout the trial period. The findings suggest that oral immunization of fish can induce a systemic response, as previously demonstrated by Firdaus-Nawi et al. (2013) and Ismail et al. (2016). Furthermore, using a subsequent dose of vaccine as a booster to the prime dose is critical for increasing the amplitude of the fish's immune response and providing extended protection (Abou-Okada et al., 2021). Both lysozyme and complement C3 were evaluated in the present study as indicators of the innate immune component. Following vaccine administration, the innate humoral system serves as the initial responder, and significant concentrations of these compounds are typically generated in response to antigen entry (Magnadóttir, 2006). The serum lysozyme and complement C3 levels revealed a significant difference between the vaccinated and unvaccinated groups following prime and booster doses. The sudden increase

in lysozyme and complement C3 in unvaccinated fish during week-6 and week-8 coincides with a high infection rate of *S. agalactiae*, reflecting the host's ongoing response against invading pathogens. An earlier study by Desvignes et al. (2002) demonstrated that an experimental infection of Atlantic salmon with salmon pancreas disease virus (SPDV) provoked increased lysozyme and complement excretion at 9- and 16-days post-infection. More recently, Charlie-Silva et al. (2019) reported that tilapia inoculated with Aeromonas hydrophila showed a significant increase in complement C3 levels 6 and 24 hours after inoculation compared to control animals.

Throughout the field trial, *S. agalactiae* was consistently recovered from various organs of the sampled red hybrid tilapia. Most notably, the presence of *S. agalactiae* was detected as early as week-2, with the highest isolation rate recorded at week-10, reaching a prevalence of 30%. A significant reduction in *S. agalactiae* isolation rate was observed between the vaccinated and unvaccinated groups, suggesting that the application of vaccine in the fish farm can lessen the incidence rate of the pathogen, as shown in the study done by Ismail et al. (2016) and Raju et al. (2023). At the end of the trial, vaccinated groups of single and double boosters recorded the highest survival rates with 85.5 and 93.6%, respectively, compared to the unvaccinated group of 76.4%. Although the study's incidence rate of *S. agalactiae* is considerably low, cumulative mortality caused a significant profit reduction and jeopardized sustainable aquaculture practices. Therefore, the application of aquaculture vaccines plays a vital role as a preventive measure and should be included as part of fish health management on fish farms.

Conclusion

This study shows that the administration of palm oil adjuvanted feed-based streptococcal vaccine as a tool to control and prevent streptococcosis in tilapia farms is a viable option as it can effectively induce both specific and non-specific immune response against *S. agalactiae*. A double booster regimen of oral immunization is recommended since it produced significantly high lysozyme and complement C3 levels and resulted in a sustained IgM level for at least 16 weeks. The field efficacy of the vaccine reduces the incidence of streptococcosis to 11.4% and improves the survival rate to 93.6%. A thorough analysis of the prospective market and sales of local fish vaccines is highly recommended. Then, with a better grasp of the industry's need, competent authority and local experts can continue their R&D of fish vaccines.

Ethical Statement

The experimental procedure was conducted in this field trial in accordance with the outlined by The Institutional Animal Care and Use Committee (IACUC), International Islamic University Malaysia, for animal utilization for scientific purposes, as indicated by permission number IACUC-2020-015.

Funding Information

This work was supported by the Department of Fisheries Malaysia (DOF) Development Fund under the 12th Malaysia Plan (P21300040170502: Research and Development Program on Fish Health in Aquaculture). MSMR was supported by Hadiah Latihan Persekutuan (HLP), a federal government scholarship for specialization programs.

Author Contribution

MSMR and MFN: Conceived and framed the main idea of this study. MSMR, AA, NNM, NR, and MFN: Project execution and data analysis. MSMR: Prepared the first draft. AA, NNM, and NR: The first draft was read, criticized, and corrected. MSMR and MFN: Proofread the second draft and finalized the manuscript.

Conflict of Interest

The authors declare that they have no known competing financial or non-financial, professional, or personal conflicts that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors wish to thank colleagues at the National Fish Health Research Centre, Fisheries Research Institute (FRI) Batu Maung, and International Islamic University Malaysia, who have helped during data collection and field sampling. The authors appreciate full cooperation from Pok Nan (farm owner) and Mr. Fazli (farm operator).

References

A.J. Pollard, A.J. & E.M. Bijker, E.M. (2021). A guide to vaccinology: From basic principles to new developments. *Nature Reviews Immunology*, *21*, 83–100. https://doi.org/10.1038/s41577-020-00479-7.

Abou-Okada, M., El-Gendy, N. M., & Elhelw, R. (2021). Effect of booster vaccination on immunoprotection in European seabass vaccinated against vibriosis. *Aquaculture Research*, *52*, 736–748. https://doi.org/10.1111/are.14930.

Amal, M.N.A. & Zamri-Saad, M. (2011). Streptococcosis in tilapia (*Oreochromis niloticus*): A review, *Pertanika Journal of Tropical Agricultural Science*, *34*, 195-206.

Amal, M.N.A., Saad, M.Z., Zahrah, A.S., & Zulkafli, A.R. (2015). Water quality influences the presence of *Streptococcus agalactiae* in cage cultured red hybrid tilapia, *Oreochromis niloticus* × *O. mossambicus*. *Aquaculture Research*, *46*, 313–323. https://doi.org/10.1111/are.12180.

Amal, M.N.A., Zamri-Saad, M., Siti-Zahrah, A., Zulkafli, A.R., & Nur-Nazifah, M. (2013). Molecular characterization of *Streptococcus agalactiae* strains isolated from fishes in

Malaysia, *Journal of Applied Microbiology*, *115*, 20-29. https://doi.org/10.1111/jam.12210

Aminudin, S.A., Kamal, F.M., Zamri-Saad, M., Siti-Zahrah, A., Ridzuan, M.S., Yusoff, H.M., Hashim, S., Sudirwan, F., Salihin, I.M., & Sulaiman, S.A. (2018). Effect of incorporating different concentrations of palm oil as adjuvant in fish vaccine. *International Journal of Biosciences*, *12*, 35–41. https://doi.org/10.12692/ijb/12.1.35-41.

Aslah, M., Zamri-Saad, M., Amal, M. N. A., Al-Saari, N., Monir, M. S., Chin, Y. K., & Ina-Salwany, M. Y. (2021). Vaccine efficacy of a newly developed feed-based whole-cell polyvalent vaccine against vibriosis, streptococcosis and motile aeromonad septicemia in Asian seabass, *Lates calcarifer*. *Vaccines, 9*, 368. https://doi.org/10.3390/vaccines9040368.

Ayisi, C. L., Zhao, J., Yame, C., Apraku, A., & Debra, G. (2019). Effects of replacing fish oil with palm oil in diets of Nile tilapia (*Oreochromis niloticus*) on muscle biochemical composition, enzyme activities, and mRNA expression of growth-related genes. *Fisheries and Aquatic Sciences*, *22*, 25. https://doi.org/10.1186/s41240-019-0139-y.

Charlie-Silva, I., Klein, A., Gomes, J. M. N, Prado, E. J. R, Moraes, A. C., Eto, S. F., Fernandes, D. C., Fagliari, J. J., Junior, J. D. C., Lima, C., Lopes-Ferreira, M., Conceição, K., Manrique, W. G., & Belo, M. A. A. (2019). Acute-phase proteins during inflammatory reaction by bacterial infection: Fish-model. *Scientific Reports*, *9*, 4776. https://doi.org/10.1038/s41598-019-41312-z.

Cui, M., Zhang, H., Li, J., Liu, R., Wu, M., Xu, D., & Zhang, Q. (2019). Differential PCR detection of *Streptococcus agalactiae* and *Streptococcus iniae* in tilapia (*Oreochromis niloticus*) by a single primer pair. *Journal of Fish Diseases*, *42*, 1211–1216. https://doi.org/10.1111/jfd.13014

De Koning, A.J. (2002). Quantitative quality tests for fish meal. II. An investigation of the quality of South African fish meals and the validity of a number of chemical quality indices. *International Journal of Food Properties*, *5*, 495–507. https://doi.org/10.1081/JFP-120015487.

Delrue, I., Verzele, D., Madder, A., & Nauwynck, H.J. (2012). Inactivated virus vaccines from chemistry to prophylaxis: Merits, risks and challenges. *Expert Review of Vaccines*, *11*, 695–719. https://doi.org/10.1586/erv.12.38.

Deng, Y., Xu, L., Liu, S., Wang, Q., Guo, Z., Chen, C., & Feng, J. (2020). What drives changes in the virulence and antibiotic resistance of *Vibrio harveyi* in the South China Sea?. *Journal of Fish Diseases*, *43*, 853–862. https://doi.org/10.1111/jfd.13197

Desvignes, L., C. Quentel, C., F. Lamour, F., & le, V. A. (2002). Pathogenesis and immune response in Atlantic salmon (*Salmo salar* L.) parr experimentally infected with salmon pancreas disease virus (SPDV). *Fish & Shellfish Immunology*, *12*, 77–95. https://doi.org/10.1006/fsim.2001.0356.

DOF (Department of Fisheries Malaysia). (2008-2012). Annual fisheries statistic. https://www.dof.gov.my/en/resources/fisheries-statistics-i/. (Accessed 21 January 2024).

DOF (Department of Fisheries Malaysia). (2023). Annual fisheries statistic. https://www.dof.gov.my/en/resources/fisheries-statistics-i/. (Accessed 16 January 2025).

Elveborg, S., Monteil, V. M., & Mirazimi, A. (2022). Methods of inactivation of highly pathogenic viruses for molecular, serology or vaccine development purposes. *Pathogens*, *11*, 271. https://doi.org/10.3390/pathogens11020271.

FAO (Food and Agriculture Organization of the United Nations). (2018). The state of world fisheries and aquaculture 2018–Meeting the sustainable development goals. https://www.fao.org/3/i9540en/i9540en.pdf. (Accessed 21 January 2024).

FAO (Food and Agriculture Organization of the United Nations). (2022). The state of world fisheries and aquaculture 2022. Towards blue transformation. https:// doi.org/10.4060/cc0461en.

Firdaus-Nawi, M., Yusoff, S.M., Yusof, H., Siti-Zahrah, A., & Zamri-Saad, M. (2013). Efficacy of feed-based adjuvant vaccine against *Streptococcus agalactiae* in *Oreochromis* spp. in Malaysia. *Aquaculture Research*, *45*, 87–96. https://doi.org/10.1111/j.1365-2109.2012.03207.x.

Gomez, P.L., Robinson, J.M., & Rogalewicz, J.A. (2013). Vaccine manufacturing. In *Vaccines* (6th ed.) (pp. 44-57). W.B. Saunders https://doi.org/10.1016/B978-1-4557-0090-5.00019-7.

Gonçalves, G., Santos, R.A., Coutinho, F., Pedrosa, N., Curado, M., Machado, M., Costas, B., Bonneville, L., Serrano, M., Carvalho, A.P., Díaz-Rosales, P., Oliva-Teles, A., Couto, A., & Serra, C.R. (2022). Oral vaccination of fish against vibriosis using spore-display technology. Frontiers in immunology, 13, 1012301. https://doi.org/10.3389/fimmu.2022.1012301.

Hall, B.G., Acar, H., Nandipati, A., & Barlow, M. (2014). Growth rates made easy. *Molecular Biology and Evolution*, *31*, 232-238. https://doi.org/10.1093/molbev/mst187.

Hamed, S. B., Ranzani-Paiva, M. J. T., Tachibana, L., Dias, D. C., Ishikawa, C. M., & Esteban, M. A. (2018). Fish pathogen bacteria: Adhesion, parameters influencing virulence and interaction with host cells. *Fish Shellfish Immunology*, *80*, 550-562. https://doi.org/10.1016/j.fsi.2018.06.053.

Han, S.Y., Kang, B.K., Kang, B.J., Shin, S.P., Soen, B.H., Kim, J.M., Kim, J.H., Choresca, C.H., Jr, Han, J.E., Jun, J.W., & Park, S.C. (2011). Prevalence and different characteristics of two serotypes of *Streptococcus parauberis* isolated from the farmed olive flounder, *Paralichthys olivaceus* (Temminck and Schlegel), in Korea. *Journal of Fish Diseases*, *34*, 731-739. https://doi.org/10.1111/j.1365-2761.2011.01289.x

Hoshina, T., Sano, T., & Moritomo, J. (1958). A *Streptococcus* pathogenic to fish. *Journal of the Tokyo University of Fisheries*, *44*, 57-68.

Huser, H., Goeke, L., Karst, G., & Fehrenbach, F.J. (1983). Fermenter growth of *Streptococcus agalactiae* and large-scale production of CAMP factor. *Microbiology*, *129*, 1295–1300. https://doi.org/10.1099/00221287-129-5-1295.

Huu, D.T., Le, D.T.H., Ha, N.T., & Minh, H.L.N. (2019). A comparison of two enrichment broth medium for the isolation and identification of *Streptococcus agalactiae* from vaginal swabs. *Journal of Medical Microbiology & Diagnosis*, *8*, 300. https://doi.org/10.4172/2161-0703.1000300.

Ismail, M. S., Siti-Zahrah, A., Syafiq, M. R. M., Amal, M. N. A., Firdaus-Nawi, & M., Zamri-Saad, M. (2016). Feed-based vaccination regime against streptococcosis in red tilapia, *Oreochromis niloticus* × *Oreochromis mossambicus*. *BMC Veterinary Research*, *12*, 194. https://doi.org/10.1186/s12917-016-0834-1

Ismail, M.S., Syafiq, M.R., Siti-Zahrah, A., Fahmi, S., Shahidan, H., Hanan, Y., Amal, M.N.A., & Zamri Saad, M. (2017). The effect of feed-based vaccination on tilapia farm endemic for streptococcosis. *Fish & Shellfish Immunology*, *60*, 21–24. https://doi.org/10.1016/j.fsi.2016.11.040.

Kayansamruaj, P., Pirarat, N., Hirono, I., & Rodkhum, C. (2014). Increasing of temperature induces pathogenicity of *Streptococcus agalactiae* and the up-regulation of inflammatory related genes in infected Nile tilapia (*Oreochromis niloticus*). *Veterinary Microbiology*, *172*, 265–271. https://doi.org/10.1016/j.vetmic.2014.04.013.

Khattab, Y.A.E., Ahmad, M.H., Shalaby, A.M.E., & Abdel-Tawwab, M. (2000). Response of Nile tilapia (*Oreochromis niloticus*) from different locations to different dietary protein levels. *Egyptian Journal of Aquatic Biology and Fisheries*, *4*, 295–311.

Li, P., Song, Z., Huang, L., Sun, Y., Sun, Y., Wang, X., & Li, L. (2023). Effect of dietary protein and lipid levels in practical formulation on growth, feed utilization, body composition, and serum biochemical parameters of growing rockfish *Sebastes schlegeli*. *Aquaculture nutrition*, 2023, 9970252. https://doi.org/10.1155/2023/9970252.

Lim, C., Yildirim-Aksoy, M., & Klesius, P. (2011). Lipid and fatty acid requirements of tilapias. *North American Journal of Aquaculture*, *73*, 188–193. https://doi.org/10.1080/15222055.2011.579032.

Maekawa, S., Wang, T.Y., Yoshida, T., Wang, P.C., & Chen, S.C. (2020). Group C *Streptococcus dysgalactiae* infection in fish. *Journal of Fish Diseases*, *43*, 963-970. https://doi.org/10.1111/jfd.13211.

Magnadóttir, B. (2006). Innate immunity of fish (overview), Fish & Shellfish Immunology, *20*, 137–151. https://doi.org/10.1016/j.fsi.2004.09.006.

Montero, D.A., Vidal, R.M., Velasco, J., Carreño, L.J., Torres, J.P., Benachi, O.M.A., Tovar-Rosero, Y.Y., Oñate, A.A., & O'Ryan, M. (2024). Two centuries of vaccination: historical and conceptual approach and future perspectives. *Frontier in Public Health*, *11*, 1326154. https://doi.org/10.3389/fpubh.2023.1326154.

Mugetti, D., Colussi, S., Pastorino, P., Varello, K., Tomasoni, M., Menconi, V., Pedron, C., Bozzetta, E., Acutis, P. L., & Prearo, M. (2022). Episode of mortality associated with isolation of *Streptococcus iniae* in Adriatic sturgeon (*Acipenser naccarii* Bonaparte, 1836) reared in Northern Italy. *Journal of Fish Diseases*, *45*, 939–942. https://doi.org/10.1111/jfd.13608

Ng, W. K., Tocher, D. R., & Bell, J. G. (2007). The use of palm oil in aquaculture feeds for salmonid species. *European Journal of Lipid Science and Technology*, *109*, 394–399. https://doi.org/10.1002/ejlt.200600209.

Ng, W.K. & Romano, N. (2013). A review of the nutrition and feeding management of farmed tilapia throughout the culture cycle. *Reviews in Aquaculture*, *5*, 220–254. https://doi.org/10.1111/raq.12014.

Nur-Nazifah, M., Sabri, M.Y., & Siti-Zahrah, A. (2014). Development and efficacy of feed-based recombinant vaccine encoding the cell wall surface anchor family protein of *Streptococcus agalactiae* against streptococcosis in *Oreochromis* sp. *Fish & Shellfish Immunology*, *37*, 193–200. https://doi.org/10.1016/j.fsi.2014.01.011.

Oliva-Tales, A. (2012). Nutrition and health of aquaculture fish. *Journal of Fish Diseases*, *35*, 83–108. https://doi.org/10.1111/j.1365-2761.2011.01333.x

Raju, T., Manchanayake, T., Danial, A., Zamri-Saad, M., Azmai, M. N. A., Yasin, I. S. M., Mohd Nor, N., & Salleh, A. (2023). Evaluating the intestinal immunity of Asian seabass (*Lates calcarifer*, Bloch 1790) following field vaccination using a feed-based oral vaccine. *Vaccines*, *11*, 602. https://doi.org/10.3390/vaccines11030602.

Ridzuan, M.S.M., Abdullah, A., Ramly, R., Mansor, N.N., Ramli, N., Firdaus-Nawi, M. (2022). Current status and advances of fish vaccines in Malaysia. *Veterinary World*, *15*, 465–482. https://doi.org/10.14202/vetworld.2022.465-482.

Schar, D., Klein, E.Y., Laxminarayan, R., Gilbert, M., & Boeckel, T.P.V. (2020). Global trends in antimicrobial use in aquaculture. *Scientific Reports*, *10*, 21878. https://doi.org/10.1038/s41598-020-78849-3.

Silvestre, I., Borges, V., Duarte, S., Nunes, A., Sobral, R., Vieira, L., Gomes, J. P., & Borrego, M. J. (2022). Global gene expression analysis of *Streptococcus agalactiae* at exponential growth phase. *Cellular and Molecular Biology*, *68*, 1–8. https://doi.org/10.14715/cmb/2022.68.7.1.

Siti-Zahrah, A., Padilah, B., Azila, A., Rimatulhana, R., & Shahidan, H. (2008). Multiple streptococcal species infection in cage-cultured red tilapia but showing similar clinical signs. In R. P. Bondad-Reantaso, M.G., Mohan, C.V., Crumlish, M., and Subasinghe (Eds.), *Diseases in Asian Aquaculture VI. Fish Health Section of Asian Fisheries Society*, (pp. 313–320). Manila

Stevens, C.H., Croft, D.P., Paull, G.C., & Tyler, C.R. (2017). Stress and welfare in ornamental fishes: what can be learned from aquaculture?. *Journal of Fish Biology*, *91*, 409–428. https://doi.org/10.1111/jfb.13377.

Sudpraseart, C., Pei-Chi, W., & Shih-Chu, C. (2021). Phenotype, genotype and pathogenicity of *Streptococcus agalactiae* isolated from cultured tilapia (*Oreochromis* spp.) in Taiwan. *Journal of Fish Diseases*, *44*, 747–756. https://doi.org/10.1111/jfd.13296

Tavares, G.C., Carvalho, A.F., Pereira, F.L., Rezende, C.P., Azevedo, V.A.C., Leal, C.A.G., & Figueueiredo, H.C.P. (2018). Transcriptome and proteome of fish-pathogenic *Streptococcus agalactiae* are modulated by temperature. *Frontier in Microbiology*, *9*, 2639. https://doi.org/10.3389/fmicb.2018.02639.

UN (United Nations). (2019). World population prospects 2019: Highlights (ST/ESA/SER.A/423). https://doi.org/10.18356/13bf5476-en. (Accessed 21 January 2024).

Van, D.H., Soltani, M., Leitao, A., Shafiei, S., Asadi, S., Lymbery, A.J., & Ringo, E. (2022). Streptococcosis a re-emerging disease in aquaculture: Significant and phytotherapy. *Animals*, *12*, 2443. https://doi.org/10.3390/ani12182443.

Villarino, R.T. (2020). Formulated feeds for genetically improved farmed tilapia (GIFT). *Fisheries and Aquaculture Journal*, *11*, 277. https://doi.org/10.35248/2150-3508.20.11.277.

Zamri-Saad, M., Amal, M.N.A, Siti-Zahrah, A., & Zulkafli, A.R. (2014). Control and prevention of streptococcosis in cultured tilapia in Malaysia: A review, *Pertanika Journal of Tropical Agricultural Science*, *37*, 389-410.

Zhang, L., Ma, J., Fan, Y., Zhou, Y., Xu, J., Liu, W., Gu, Z., & Zeng, L. (2016). Immune response and protection in gibel carp, *Carassius gibelio*, after vaccination with β-propiolactone inactivated cyprinid herpesvirus 2. Fish & Shellfish Immunology, *49*, 344-350. https://doi.org/10.1016/j.fsi.2016.01.003.