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Efficacy of live attenuated vaccine derived from the *Streptococcus agalactiae* on the immune responses of *Oreochromis niloticus*

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ABSTRACT

Streptococcus agalactiae species have been recognized as the main pathogen causing high mortality in fish leading to significant worldwide economical losses to the aquaculture industries. Vaccine development has become a priority in combating multidrug resistance in bacteria; however, there is a lack of commercial live attenuated vaccine (LAV) against *S. agalactiae* in Malaysia. The aim of this study is to compare two methods using attenuated bacteria as live vaccine and to evaluate the efficacy of selected LAV on the immune responses and resistance of *Oreochromis niloticus* (tilapia) against *S. agalactiae*. The LAV derived from *S. agalactiae* had been weakened using the chemical agent Acriflavine dye (LAV1), whereas the second vaccine was weakened using serial passages of bacteria on broth media (LAV2). Initial immunization was carried out only on day one, given twice-in the morning and evening, for the 42 day period. Serum samples were collected to determine the systemic antibody (IgM) responses and lysozymal (LSZ) activity using ELISA. On day 43 after immunization, the fish were injected intraperitoneally (i.p) with 0.1 mL of *S. agalactiae* at $LD_{50} = 1.5 \times 10^5$ (CFU)/fish. Fish were monitored daily for 10 days. Clinical signs, mortality and the relative percent of survival (RPS) were recorded. Trial 1 results showed a significant increased ($P < 0.05$) in serum IgM titers and LSZ activity as compared to LAV2 and the control group (unvaccinated fish). The efficacy of LAV1 was proven effective as determined by the RPS values, LAV1 at 81.58% as compared to LAV2 at 65.79%. Trial 2 of LAV1 and control group were further determined by administering primary and booster doses revealed a RPS value for LAV1 of 82.05%, with the significant enhancement on the immune responses of tilapia as compared to control group. In conclusion, LAV revealed to elevate antibody IgM levels, LSZ activity and provide long-term protection when added to feed. LAV is a low-cost vaccine shown to rapidly increase the immune response of fish and increase survival rates of fish against *S. agalactiae* infection.

1. Introduction

Nile tilapia (*Oreochromis niloticus*) is the second most important fish species in aquaculture in terms of quantity. Higher production outputs through intensified farming systems involving high stock densities has rendered fish to be highly susceptible to diseases and infections. . In Malaysia, *Streptococcus agalactiae* is recognized as an important causative agent of mortality in tilapia [1,2]. Treatment of such fish infections with antibiotics often leads to bacterial drug resistance, rendering the treatment ineffective. Vaccination greatly reduces the need for drugs and chemicals and is an environmentally friendly disease control strategy to combat significant fish diseases.

As of today, the 3 widely used vaccines in controlling *S. agalactiae* infection include formalin-killed vaccine [3], live attenuated vaccine [4,5], and DNA and subunit vaccines [6]Different strategies can be used in developing modern vaccines for the prevention of *S. agalactiae* infections in fish diseases. Vaccination can be carried out before exposure to the pathogen, thus allowing adequate time for an immune response to develop. However, infectious disease of fish is most validly prevented through the use of live attenuated vaccines (LAV), which provides the most effective immunity [7]. LAV induce mucosal, cellular, and humoral immunity in the host [8]. Achieving desirable features of live vaccines requires the attenuated vaccines to be safe, efficacious and capable of stimulating a strong cellular immune response leading to

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immunosuppression [9].

LAVs hold weakened or less virulent forms of disease causing pathogens. Preparation of attenuated vaccine includes repeated laboratory passage and physical and chemical attenuation of the organism in order to cause the organism to lose their virulence without killing them. The study of Wang et al. revealed serial passages of pathogenic *S. agalactiae* strains resulted in the deletion of virulence factors, consequently causing the production of virulent strains to be highly protective when used as a live vaccine in tilapia [10]. Li et al. reported that serial passage of pathogenic strains of *S. agalactiae* lost virulence when injected as a live vaccine at 10^9 CFU/fish and orally administered at 10^{10} CFU/fish, resulting in higher protection without causing clinical disease in vaccinated fish [5]. The LAV from *S. agalactiae* provided significant protection to Nile tilapia when challenged against *S. agalactiae* [4].

The oral route is considered to be the ideal approach, with respect to handling costs and the welfare of animals, in the immunization of fish for several reasons including; ease of antigens administration, less demanding than parenteral delivery and it applies to both small and large sized fish. The oral route also provides a procedure for oral boosting during grow-out periods in cages or ponds. Mucosal and systemic responses are stimulated in the oral administration of antigens [11]. Vaccines delivered by mucosal routes have the capacity to stimulate both mucosal and systemic immunity thereby protecting the fish at the portal of entry of pathogens and preventing the spread of infection systemically [61]. Chile, Scotland and Norway are the only few countries that use oral vaccines for fish [12]. This is due to a poor understanding about the requirements for proper immune induction following oral administration [13].

There is a concern in vaccination strategy to control *S. agalactiae* infection in tilapias where some attempts have been made on feed-based vaccination to measure vaccine efficacy [14–16]. Hence, development of vaccine designs and immunization strategies are important to develop successful vaccines against *S. agalactiae* in tilapia.

This study aimed to evaluate the suitable methods of LAV introduction to fish as well as to measure the efficiency of oral vaccination regimes of LAV against *S. agalactiae* based on potential immune responses of *O. niloticus* and their survival during bacterial challenge.

2. Materials and methods

2.1. Fish maintenance

This study was carried out at the Fish Diseases Laboratory of the School of Fisheries and Aquaculture Science of University Malaysia Terengganu (UMT). Healthy *O. niloticus* (n = 900) of 70 ± 10 g were obtained from the UMT hatchery. Fish were habituated under laboratory conditions for 14 days and stocked in 400 L fiberglass tanks containing 60 fish per tank. Fish were fed twice daily with commercial feed containing 30% crude proteins. Flow through freshwater supply system with water temperature maintained at temperatures of (27 ± 2 °C), dissolved oxygen (DO) (5.57 ± 0.01 mg/L) and pH (7.2 ± 0.2) ranged on acceptable values throughout the experimental period. Water quality measurements were completed with (YS-Yellow Springs Instruments - model 85/10). The experimental fishes were confirmed to be negative from bacterial infection by bacteriological analysis of the brain and kidney samples.

2.2. Bacterial strains

Streptococcus agalactiae strain (GenBank accession number KT869025), previously isolated from the natural infections in hybrid *O. niloticus* in Temerloh, Pahang, Malaysia were used for this study [2]. Glycerol stocks of isolated strain were prepared with 20% (v/v) glycerol in Brain Heart Infusion (BHI) Broth (Merck, Germany) and stored at −80 °C. *S. agalactiae* strain was then thawed at room temperature and streaked onto blood agar (Oxoid, U.K.) at 30 °C for 24–48 h. The presence of a clear zone around the bacteria at the streaking sites was considered haemolytic; absence of a clear zone around the bacteria at the streaking site was considered non-hemolytic isolate. Haemolytic colonies were inoculated into Brain Heart Infusion (BHI) broth in a shaker incubator at 500 × *g*, 30 °C for 24 h. Bacterial cells were harvested by centrifugation (6000 × *g*, 10 min) and washed twice with phosphate-buffered saline (PBS, pH 7.2) before being re-suspended. Bacterial suspension was adjusted to McFarland turbidity standard No.5 equivalent to 1.5×10^8 CFU/mL. Ten-fold serial dilutions were completed to obtain *S. agalactiae* concentration with lethal dosage (LD_{50}) value equivalent to 1.5×10^5 CFU/mL.

2.3. Preparation of LAV

2.3.1. Acriflavine resistance assay

The acriflavine resistance of the *S. agalactiae* was evaluated by using Acriflavine solution 30 mL (1% w/v) (First Aid Supplies, China) to make various concentration of Acriflavine as follows: 0, 0.00125, 0.0025, 0.005, 0.0075, 0.015, 0.01, 0.02 0.03, 0.04 0.05, 0.06, 0.07, 0.08, 0.09, and 0.1%. *S. agalactiae* was cultivated in 10 mL Brain Heart Infusion (BHI) Broth (Merck, Germany) and incubated in a shaker incubator at 500 × *g*, 30 °C for 24 h. The overnight culture was then streaked onto BHI agar plates containing Acriflavine and incubated at 30 °C for 48 h. During the attenuation, *S. agalactiae* colony was collected at every five passages, and stored under −80 °C. The culture was thawed and streaked once more onto BHI agar plates (Merck, Germany). The attenuated procedure was done through 187 passages on (BHI) agar plates containing a gradually increasing concentration (0.00125%–0.1%) of Acriflavine dye throughout a period of three months. Acriflavine resistance was determined by the highest concentration at which a strain revealed roughly the same growth as the agar that did not contain Acriflavine [17].

2.3.2. Serial passage

The bacterial isolate was removed from the −80 °C refrigerator thawed and streaked onto Tryptic Soy Agar (TSA) with 5% Sheep Blood (Oxoid, England) before being cultured at 28 °C for 24 h. A single colony was selected, inoculated into 10 mL of Tryptic Soy Broth (TSB) medium (Merck, Germany), and cultivated at 500 × *g*, 30 °C for 24 h in the incubator shaker. After 12 h, 1 mL of bacteria was inoculated into fresh 10 mL of TSB medium and cultured continuously by shaking for another 12 h. The culture cycle (passage) was repeated every 12 h, and after 60 passages, the safety test of the vaccine was carried out according to Li et al. [10]. Attenuated bacteria using Acriflavine resistance assay was designated as live attenuated vaccine LAV1 while the second method of serial passage on media was designated as live attenuated vaccine LAV2.

2.4. Safety test of the vaccine

2.4.1. Haemolytic activity in vitro

S. agalactiae was cultivated on blood agar (Oxoid, England) at 28 °C for 24 h. The formation of clear zones around the colonies was indicative of β-hemolysis (complete lysis of the red blood cell). Green zones around the colonies signified α-hemolysis. The green appearance around a colony is an outcome of hemoglobin reduction to Meta hemoglobin in red blood cells. No change in medium, or no hemolysis, is referred to as γ-hemolysis.

2.4.2. Inducing disease in vivo

The virulence of *S. agalactiae* strain was confirmed by intraperitoneal injection (i.p) of 25 fish with 0.1 mL live attenuated vaccine of *S. agalactiae* containing 1×10^9 CFU/fish. The injected fish showed no indicative signs of the disease.

2.5. Feed - based oral vaccine

S. agalactiae was cultured as described above (section 2.2- bacterial strain). After 24 h, cells were examined for sterilization before harvesting and then washed twice with phosphate-buffered saline (PBS, pH 7.2). Harvested cells from live attenuated bacteria suspension was dehydrated using freeze dryer (Alpha 1, 4- LD Plus Martin Christ) and the parameters were set at −55 °C under vacuum condition. Prior Freeze-drying, fresh cultures were incubated for 30 min in protective media containing 2.0 M glycerol, 150 mM sucrose and 40 mM insulin in accordance to Bircher et al. (2018) [62]. The dehydrated cells were incorporated to the feed during production and mixed with food pellets by the homogenizer (ESCO-Labor AG, Germany). Food pellets were oven dried for 24 h at 70 °C in accordance to methods by Firdaus-Nawi et al. [14], with some minor modifications. The pellets were then packed and stored in a freezer at −20 °C until further use. Prior to utilization in feeding trials, the pellets, which were mixed with the vaccine at 30 °C for 48 h, had undergone bacterial counts to confirm final concentration of live cells and purity, and were further verified by streaking onto the Tryptic Soy Agar (TSA) (Merck, Germany).

2.6. Determination of median lethal dosage (LD_{50})

The LD_{50} value was determined to obtain the lowest bacterial dose of *S. agalactiae* causing 50% mortality in *O. niloticus*. Fish were divided into 6 groups, with 10 fish per group, in 20 L aquariums supplied with adequate aeration. Environmental conditions were maintained under optimum conditions. Fish were deprived of food for 24 h prior to the experiment. Fish were anesthetized using Tricaine Methanesulfonate (MS-222) (Sigma, Aldrich) before the experimental infection. Fish in groups 1, 2, 3, 4 and 5 were intraperitoneally injected (i.p) with 0.1 mL of *S. agalactiae* culture suspension containing 1.5×10^8, 15×10^7, 15×10^6, 15×10^5 and 15×10^4 CFU/mL. The control group was injected intraperitoneally (i.p) with 0.1 mL of physiological saline. Fish mortality was recorded every 24 h for 5 days. Dead fish were removed from the aquarium daily. Probit analysis was used to determine the LD_{50} after 120 h using SPSS 16.0 [18].

2.7. Collection of serum

12 tilapia fish were selected at random from each group and approximately 300 μL of blood was drawn via the caudal vein. Blood samples were stored at 4 °C for 4 h to allow time for clotting. Centrifuging was then carried out at 3000 × *g* for 10 min at 4 °C before serum collection. Sera were kept at −20 °C until further used.

2.8. Immunogenicity assay

The experiment consisted of two trials. The first trial evaluated vaccines of different methods to elicit immune response for a continuous period in tilapia. In the second trial, the efficacy of selected vaccine given orally in a primary and booster dose was done to evaluate the resistance and survival of tilapia against pathogenic *S. agalagtiae* (Fig. 1).

2.8.1. Trial 1

The first trial was conducted using a total of 450 fish that were separated into 3 groups: live attenuated vaccine (LAV1), live attenuated vaccine (LAV2) and Control group (CG). Each group experimentation was performed in replicates resulting in 50 fish per tank. Fish were starved for 24 h prior to the experiment. Vaccinated groups were orally administered with feed (oral immunization) mixed with LAV1 and LAV2 at dosages of 1.5×10^8 CFU g^{-1}, whereas the control group was treated with equal amount of commercial pellet, without bacteria. The Initial dose of both vaccines was given only once, on day 0 (twice a day; morning and evening), throughout the 6 weeks of the experimental period. Blood samples from vaccinated and control fish were taken weekly from the caudal vein using sterile plastic syringes. The challenge test was performed after 6 weeks. Fish from both the vaccinated and control groups were anesthetized using Tricaine Methanesulfonate, MS-222 (Sigma, Aldrich) and injected intraperitoneally (i.p) with 0.1 mL of *S. agalactiae* containing 1.5×10^5 CFU/fish. Fish were returned to their original tanks and observed for any clinical abnormalities. The cumulative mortality was monitored for 10 days post-challenge. The moribund fish from each group were collected, and the kidneys were inoculated aseptically onto blood agar for the isolation of *S. agalactiae*.

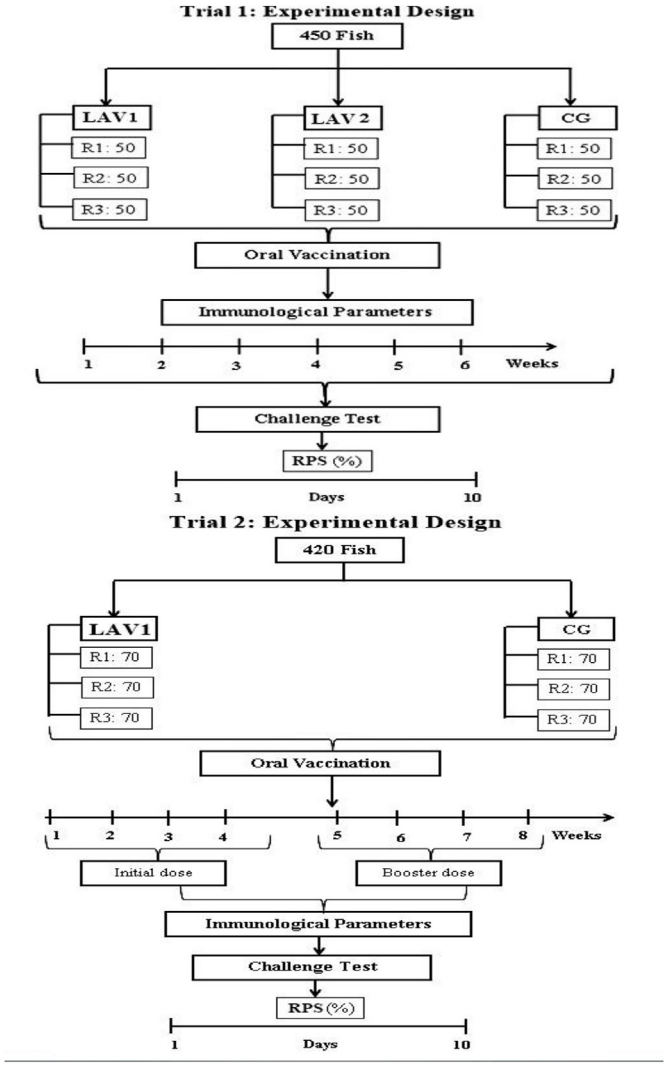

Fig. 1. Flow chart diagram of the experimental designs for Trial 1 and Trial 2 of oral vaccination.

2.8.2. Trial 2

Trial 2 consisted of a total of 420 fish divided into 2 groups; live attenuated Vaccine (LAV) and control group (CG). Each group had 210 fish for the 3 replicates; each tank containing 70 fish in 400 L fiberglass tanks. The experiment period was 4 weeks for primary dose vaccine and 4 weeks for booster dose vaccine, administered orally. The initial dose of vaccines was given once on day 0 (twice a day; morning and evening) and a booster dose vaccine was administered, once (twice a day; morning and evening) after the 4 week period. Blood samples from vaccinated and control fish were taken weekly. The challenge test with *S. agalactiae* was performed after 8 weeks and the cumulative mortality was monitored for 10 days post-challenge.

2.9. Relative percent of survival

Relative percent of survival (RPS) was determined in accordance to Amend et al. (1981) method [19]. Daily mortalities of the 50 fish per treatment were recorded for 10 days by calculating the mortality percentage of vaccinated group over the mortality percentage of the non-vaccinated group.

RPS = (1- % mortality in vaccine group/% mortality in control group) × 100

2.10. Detection of specific IgM in serum

Tilapia serum samples were tested for antibodies against live attenuated vaccine using indirect Enzyme Linked Immunosorbent Assay (ELISA) to determine the systemic antibody (IgM) titres [20]. *S. agalactiae* was cultured onto BHI broth in a shaker incubator at 500 × *g*, 30 °C for 24 h. Bacterial pellet was then re-suspended in phosphate buffer saline (PBS), sonicated at 40 Hz for 5 min (on ice), followed by centrifugation at 3000 × *g* for 60 min. Bicarbonate buffer (pH 9.6) (Sigma-Aldrich Inc.) was added to the sonicated suspension at the concentration of 5 μg antigen protein/mL and stored at −20 °C prior to use. The 96-well microtitre plate was coated with 1.0×10^8 CFU/mL of *S. agalactiae* (100 μL/well) and incubated overnight at 4 °C. Plates were washed thrice with 250 μL PBS (pH 7.4) containing 0.5% (v/v) Tween 20 (PBS-T). Then, 200 μL of 1% (w/v) bovine serum albumin (BSA) (Sigma Chemical Co., USA) was added to the plates and incubated at 37 °C for 30 min. Tilapia serum samples were diluted (1:500) in PBS-T. The resulting solution (100 μL) was combined to 3 replicate wells of microtitre plates and incubated for 2 h at room temperature on a rotary shaker and washed with PBS-T. The goat anti-tilapia hyperimmune serum (100 μL) were diluted at 1:10,000 ratios, added to the plate and placed on a shaker incubator at 500 × *g*, at room temperature for 1 h. Then, 100 μL of conjugated rabbit anti-goat IgM horseradish peroxidase (Nordic, Netherland) were diluted at 1:10,000 ratios added to the reaction and incubated at 37 °C for 1 h. After 3 washes with PBS-T, bound conjugates were detected by adding 100 μL of TMB One Solution, a substrate solution tetramethylbenzidine (TMB) (Promega, USA). Then, 0.2 mol/L sulphuric acid (H_2SO_4) was used to complete the reaction. The absorbance optical density at 540 nm (OD_{540nm}) of each well was read by BioRad 680 microplate reader (Biorad, USA). Each sample was assessed in duplicates.

2.11. Lysozyme activity

Turbidimetric assay was performed in accordance to Parry et al. with minor changes [21]. A mixture of 200 μL serum samples with 2 mL of *Micrococcus lysodeikticus* (0.2 mg/mL) suspension in 0.05 mL sodium phosphate buffer of pH 6.2 was incubated at 37 °C for 15 min. The absorbance was measured at 530 nm, between 0.5 and 4.5 min, using a spectrophotometer. The lysozyme activity causes a decline in absorbance of 0.001/min.

2.12. Statistical analysis

All data was presented as means ± standard deviation (mean ± SD) from 3 or 4 replicates, analysed by one-way ANOVA with Duncan test using SPSS Statistics 20.0 software. The significance level was defined as $P < 0.05$.

3. Results

3.1. Bacterial identification

Identification of suspected *S. agalactiae* colonies using biochemical methods observed Gram-positive chained cocci, β-hemolytic, and non-motile bacteria. Isolate confirmation of *S. agalactiae* was done by VITEK 2 (99% similarity), reconfirmed by 16S rRNA gene sequencing (99% similarity) and was deposited in GenBank with accession no. KT869025 [2].

3.2. Median lethal dose (LD_{50})

Pathogenicity test of the lethal dose (LD_{50}) of fish in groups 1 to 5 showed a 100%, 70%, 50%, 20%, and 10% mortality, respectively. Clinical characteristics of disease and mortalities in fish were observed as lethargic and loss of appetite during 24 h of post-infection (hpi). A small number of fish mortality showed an absence of clinical signs. Mortality occurred within 24 h hpi in groups 1 to 4 and reached 30% of accumulative mortality. Significant differences ($P < 0.05$) were observed amongst all groups. The LD_{50} value of the isolated bacteria was determined at 1.5×10^5 CFU/mL.

3.3. Immune responses

3.3.1. Antibody titre

The immune sera of fish were examined for their anti-*Streptococcus* antibody levels using ELISA for detecting fish response to *Streptococcus* antigen. Serum antibody levels of all groups prior to vaccination showed no significant difference ($P > 0.05$) for trial 1 and 2. Trial 1 results following vaccination showed both vaccinated groups, LAV1 and LAV2, achieved significantly higher ($P < 0.05$) IgM levels than that of the control group. Antibody level of LAV1 increased significantly ($P < 0.05$) during the second week and reached peak levels (0.23 ± 0.33) during the third week in comparison to LAV2 (0.18 ± 0.13) (Fig. 2). IgM levels began to decline but remained significantly higher ($P < 0.05$) than control group during the fourth and fifth week. All groups showed no significant difference ($P > 0.05$) during the sixth week. The results in Trial 1 where the IgM levels reached the peak in the third week suggested that the booster dose should be given on the fourth week. The live attenuated vaccine therefore resulted in a long-term protection to the end of seventh week.

In Trial 2 after primary dose of LAV, the mean of IgM levels increased significantly ($P < 0.05$) from the first, second, third and fourth week (0.18 ± 0.01, 0.20 ± 0.02, 0.21 ± 0.02 and 0.22 ± 0.02) respectively, as compared to the CG (0.15 ± 0.02, 0.15 ± 0.02, 0.14 ± 0.01 and 0.15 ± 0.01, respectively). Immediately after booster dose with LAV, the IgM levels increased rapidly and reached the peak on the fifth week (0.23 ± 0.02) with significant difference ($P < 0.05$) as compared to CG (0.15 ± 0.02). Thereafter, the IgM levels decreased slightly towards the sixth and seventh week with significant difference ($P < 0.05$) until week eight, showing no significant difference ($P > 0.05$) between both groups (Fig. 3).

3.3.2. Serum lysozyme activity

The lysozyme (LSZ) activity in Tilapia showed significant difference ($P < 0.05$) between vaccinated groups and control group for the 6 weeks period in Trial 1, with the exception that no significant difference ($P > 0.05$) was observed between LAV2 and CG during sixth week. The maximum activity of LAV2 was 15.65 ± 0.76 during the third week, whereas LAV1 (15.38 ± 0.44) showed no significant difference ($P > 0.05$). The maximum LSZ activity of LAV1 was 15.82 ± 0.51 during the fourth week and significantly different ($P < 0.05$) when compared to LAV2 (14.67 ± 0.92) in the same week (Fig. 4).

After primary dose of LAV in Trial 2, LSZ activity value increased significantly ($P < 0.05$) during the second to the fourth week (14.67 ± 0.71, 15.37 ± 0.56 and 15.61 ± 0.24, respectively) when compared to CG (13.57 ± 0.51, 13.08 ± 0.50, and 13.4 ± 0.56, respectively). The value of LSZ activity increased slightly after booster dose of LAV during fifth and sixth week with significant difference ($P < 0.05$) (15.63 ± 0.53 and 15.24 ± 0.58 respectively), as

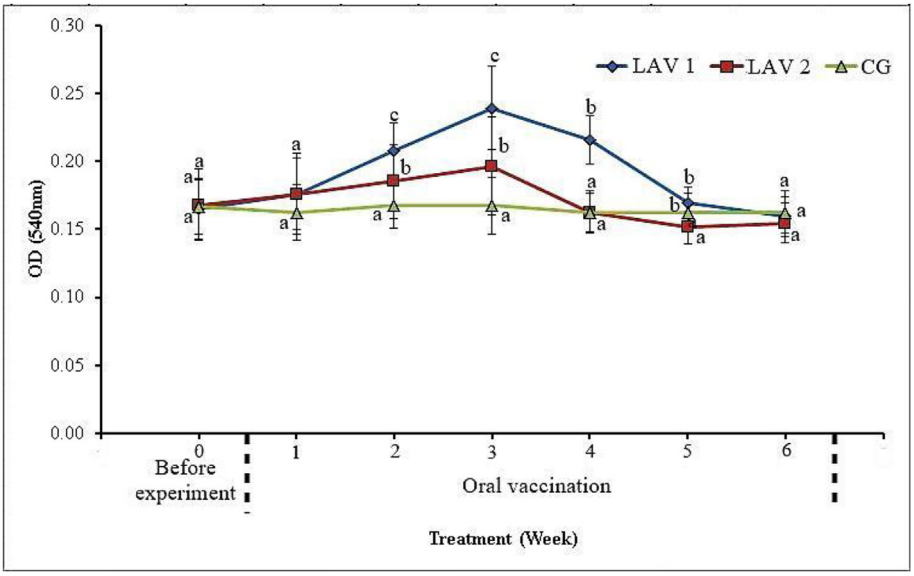

Fig. 2. Antibody titer of specific IgM in serum by Enzyme Linked Immunosorbent Assay (ELISA) for Trial 1. Different superscripts refer to significant differences between the vaccinated and un-vaccinated and recovery groups ($P < 0.05$).

compared to CG (13.1 ± 0.53 and 13.24 ± 0.58, respectively). The LSZ activity of LAV later decreased on the sixth and seventh week with significant difference ($P < 0.05$) when compared to CG. No significant difference ($P > 0.05$) was observed between both groups on the eighth week (Fig. 5).

3.4. Efficacy of the live attenuated vaccine

The occurrence of fish death within 6 h post-challenge with *S. agalactiae* was not considered in this study as the fish might be traumatized to the injection. All mortalities and clinical signs in control and vaccinated fish began within 24 h after intraperitoneal injection (i.p) of *S. agalactiae* until ten days post-challenge. Clinical symptoms were primarily apparent in control group (CG), which included loss of appetite, lethargy, erratic swimming, and inflammation at the injection site, the abdomen and fine base. Numerous fish showed ascites, severe haemorrhage inside and outside the eyes, and corneal opacity. Vaccinated groups showed loss of appetite and inflammation at the injection site, sluggish swim, and haemorrhage inside and outside the eyes. Post-mortem examination revealed haemorrhage in the liver, gastrointestinal, gills, and brain, as well as kidney congestion, gall bladder enlargement, and congestion and enlargement of the spleen. The efficacy of each vaccination type, LAV1 and LAV2, was evaluated by i.p injection of the virulent strain of *S. agalactiae* challenge model. RPS of LAV1 group was 81.58%, which was significantly higher ($P < 0.05$) than the RPS of LAV2 group at 65.79%. The survival value of CG was 5% in Trial 1 as shown in Table 1.

In Trial 2, the RPS rate of LAV was 82.05% and the survival value was 2.5% in CG (Table 2). These results suggest that the live attenuated vaccination (LAV1) provides excellent protective effects on tilapia for up to eight weeks of the experimental design.

4. Discussion

S. agalactiae is a major bacterial pathogen causing serious morbidity and mortality to *Oreochromis niloticus* (Nile Tilapia) production worldwide [22,23]. *S. agalactiae* infections cause significant economic losses to farmers in Malaysia [2]. In order to prevent and control disease in aquaculture, it is essential to use a vaccine harvested from a local strain to prevent infections of *S. agalactiae*.

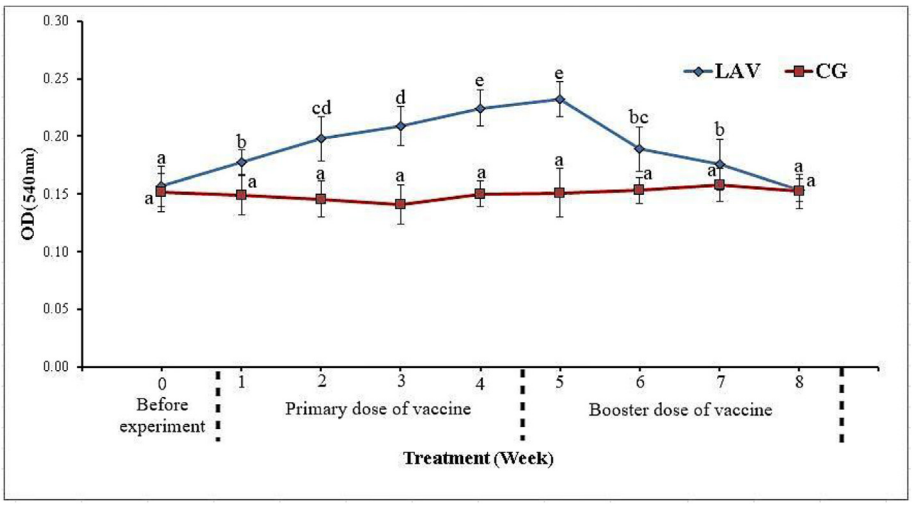

Fig. 3. Antibody titer of specific IgM in serum by Enzyme Linked Immunosorbent Assay (ELISA) for Trial 2. Different superscripts refer to significant differences between the vaccinated and un-vaccinated and recovery groups ($P < 0.05$).

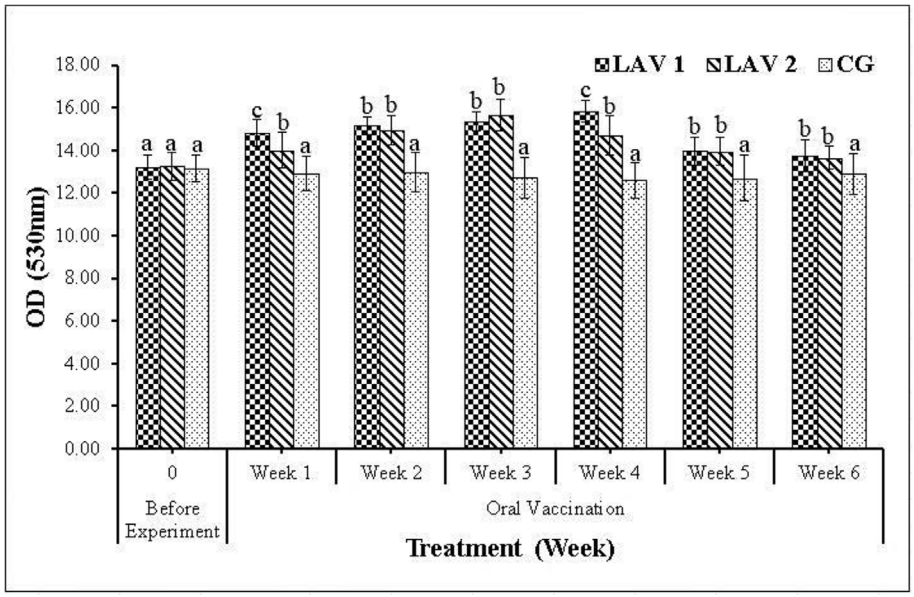

Fig. 4. Lysozyme activity (mean ± SD) in serum of *Oreochromis niloticus* before experiment and throughout oral vaccination period. Different superscripts refer to significant differences between the vaccinated and un-vaccinated and recovery groups ($P < 0.05$).

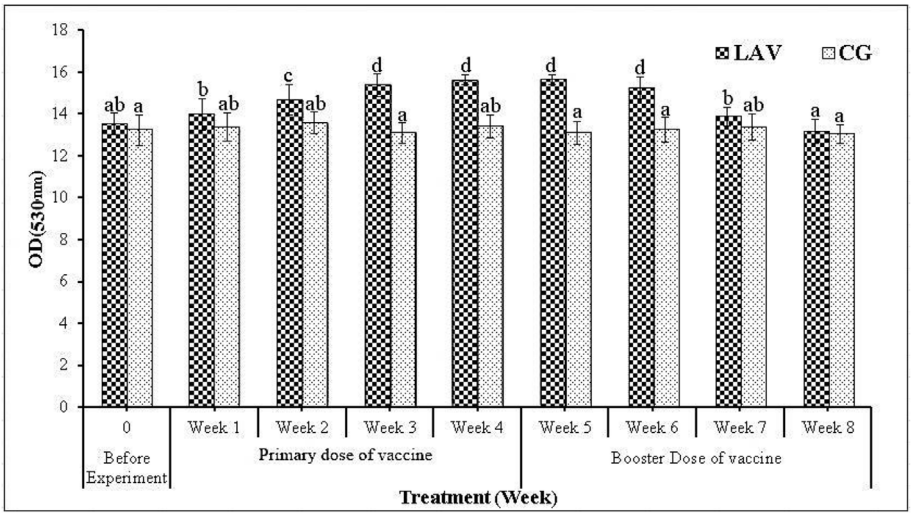

Fig. 5. Lysozyme activity (mean ± SD) in serum of *Oreochromis niloticus* before experiment and throughout oral vaccination period. Different superscripts refer to significant differences between the vaccinated and un-vaccinated and recovery groups ($P < 0.05$).

The present study tested the potential of live attenuated vaccine feed-based as a candidate vaccine against *S. agalactiae* in *O. niloticus*. Our results revealed significantly higher serum antibody titers than non-immunized Tilapia, indicating that live attenuated vaccine (LAV) could sensitize the humoral and cellular immunity, preventing *S. agalactiae* infection [4,5,24–26]. The last two decades helped construct live attenuated vaccines and these vaccines are currently being evaluated in field trials due to the vast mimicking effects of natural infections [27]. Changes in virulence have been observed in the attenuation of bacteria by continuous subculture. The use of attenuation by serial passage is the preferred approach for LAV in this study. Many important vaccines, including the *Bacillus* Calmette–Guerin (BCG) vaccine, primarily used against tuberculosis (TB), has been successfully developed from serial passage technique [28]. It is also important to consider that extended

Table 1
Cumulative mortality (%) and Relative Percent Survival (RPS) of tilapia, *Oreochromis niloticus* after being challenged with *Streptococcus agalactiae* at 37 days post vaccination from Trial 1.

Treatment	Cumulative mortality (%)										Survival (%)	RPS (%)
	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10		
[a]LAV1	5.00	10.00	12.50	15.00	17.50	17.50	17.50	17.50	17.50	17.50	82.50	**81.58**
LAV2	12.50	20.00	25.00	27.50	30.00	32.50	32.50	32.50	32.50	32.50	67.50	65.79
CG	20.00	45.00	62.50	75.00	85.00	92.50	92.50	92.50	95.00	95.00	5.00	

[a] LAV1 shows the highest RPS value compared to LAV2.

Table 2
Cumulative mortality (%) and Relative Percent Survival (RPS) of tilapia, *Oreochromis niloticus* after being challenged with *Streptococcus agalactiae* at 57 days post vaccination form Trial 2.

Treatment	Cumulative mortality (%)										Survival (%)	RPS (%)
	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10		
LAV	2.50	10.00	15.00	15.00	15.00	17.50	17.50	17.50	17.50	17.50	82.50	**82.05**
CG	35.00	50.00	57.50	75.00	87.50	92.50	97.50	97.50	97.50	97.50	2.50	

sub culturing of *M. tuberculosis* may produce mutations affecting its genetic and/or virulence characteristics [29].

The vaccine strategy used to attenuate *S. agalactiae* has been successfully resistant to the chemical agent acriflavine dye, which is in agreement with Zou et al. [17]. The strain was generated via 187 continuous passages *in vitro*. Live attenuated vaccine of *S. agalactiae* was generated via 840 continuous passage *in vitro* [10] in addition to the use of synthetic antibiotic resistance such as Novobiocin [30], Sparfloxacin [31] and Rifampicin [32]. The most commonly used attenuation system is based on replications or by simple serial passage of virulent bacteria in culture media.

Serial sub-culturing induces various types of mutations that significantly alters the virulence of the organism, or produces lesions in the host while continuing to replicate or multiply sufficiently in order to be processed by the immune system. Klesius and Shoemaker developed a modified live *Edwardsiella ictaluri* vaccine by passage of a virulent isolate on media supplemented with rifampicin [33]. Live attenuated *Renibacterium salmoninarum* strains have been used in the immunization of Atlantic salmon by Daly et al. [34]. Live attenuated *Nocardia soli*, *Nocardia fluminea*, and *Nocardia uniformis* strains have been applied to immunize yellowtail *Seriola quinqueradiata* [35]. Serial passage however, can occasionally induce fitness-increasing mutations that enhance bacterial survival [36]. The main drawbacks of a serial pathway as a strategy for the derivation of LAV strains is its inability to reveal the molecular mechanisms causing attenuation and its failure to confirm the safety and effectiveness of the vaccine [37].

In our present work, vaccines were administrated without the use of an adjuvant. This was done to allow *S. agalactiae* antigen to increase the vaccine efficacy through elicited immune response of Tilapia by stimulation of B-cells, yielding high production of IgM and resulting in higher rates of survival in immunization groups. This result is in agreement with the previous study by Jaafar et al. [38]. Feed-based bacterin without adjuvant stimulates IgM response for a period of three weeks [14]. Oral vaccination with live attenuated vaccine of *S. agalactiae* mixed with feed in production process protected the antigen. In addition, it provided a safer approach for intact delivery to the lower gut where stimulation of an immune response took place as reported previously in commercial feed pellets used as a carrier for *Vibrio anguillarum* bacterin suspension in vaccine [39]. Oral vaccination use of formalin killed bacteria of *S. agalactiae* once a week is sufficient to stimulate gut associated lymphoid tissue in *Oreochromis* sp [40]. Oral feed vaccination provides many attributes making it the preferred route of administration. These attributes include; less time, low cost, easy administration, and does not require extensive labour [15,41].

Oral feed vaccinations are limited in that the antigen may be degraded by the digestive fluids and therefore, the vaccine may not be sufficiently absorbed [3]. To overcome this problem, mixed bacterial suspension after freeze drying were used to form fish food pellets to reduce the exposure of the vaccine to the digestive fluids. One day fasting period was introduced to ensure that the fish consumed all the feed. Oral vaccination has elicited a specific and detectable immune response at local and systemic levels in Atlantic salmon *Salmo salar* [42]. This is primarily characterized by a sudden increase in the specific antibody in the blood, followed by a decline in antibody titer three weeks post-vaccination [43]. In the present study, oral route of LAV increased the antibody titer level in the vaccinated fish as a result of the mucosal surface acting as the first interface between the bacteria and the host [44]. This is an important factor in influencing the immune responses at the initial site of the pathogen invasion where protection is more effective [45]. Administration of a single dose of oral LAV resulted in a significant increase in serum IgM levels for up to three weeks [14]. In the same way, feed-based bacterin without adjuvant stimulates IgM response for a period of three weeks and elicited a systemic response [46].

Enzyme-linked immunosorbent Assay (ELISA) is an accurate and highly sensitive immunodiagnostic technique where antibody is conjugated to an enzyme and utilized to detect the presence of an antibody or an antigen in a sample. ELISA can assess the development of a humoral immune response, in addition to being more sensitive and specific in measuring antibody response against *Streptococcus iniae* [47]. Our result revealed significantly higher antibody level of vaccinated group from first week in comparison to the control group. The highest level of antibody in the vaccinated fish with primary and booster dose was shown during the fifth week of experimental period. Formalin-killed inactivated *Streptococcus* sp. whole-cell for red tilapia immunization revealed peak antibody during the first week for single and second dose of vaccine [48]. Other study has shown antibody peaking during second week after first dose and peaking levels during third week after booster dose [47]. This variability in immune response indicates the existence of antigenic heterogeneity of *streptococcal* vaccines [49].

Lysozyme activity was significantly higher in immunized groups than in non-immunized groups, suggesting that vaccines stimulate the non-specific cellular immune responses, in agreement with Marsden et al. (1996) that used live attenuated bacteria as vaccines to stimulate fish cellular immunity [24]. A primary advantage of modified live vaccines is the ability to stimulate humoral, cell-mediated, antibody and mucosal immunity responses [50,51]. The humoral and cell-mediated immune responses have been shown to be linked with systemic and mucosal vaccination in fish [52]. Suwannasang et al. used formalin-killed *S. agalactiae* vaccines in *O. niloticus* and reported a significant increase in lysozyme activity in the immunized groups when compared to the control group [53]. In our study, peak levels of lysozyme activity was observed during the third and fifth week and are in agreement with Xu et al. who reported a peak in lysozyme activity during the fourth week post vaccination [54]. The serum lysozyme levels appear to be strongly influenced by the immune status of the fish [55].

The current study chose *S. agalactiae* virulence for vaccine preparation based on the median lethal dose (LD_{50}) according to the classification by Santos et al. who reported that LD_{50} of 10^4 to 10^5 was considered virulent, 10^6 to 10^7 is weakly virulent and up to 10^8 is a virulent isolate [56]. Vaccinated and control fish groups shared similar symptoms to those caused by *S. agalactiae* infection. These clinical signs included anorexia, septicemia, exophthalmia, corneal opacity and ascites, leading to high mortalities in the infected fish [18,57]. Gram-positive bacteria produce exotoxins that can be highly toxic at very low concentration and this may be attributed to the low lethal dose concentration of *S. agalactiae.* Vaccine efficacy evaluation was performed using intraperitoneal challenge model aimed at evaluating the relative percent of survival (RPS) of vaccinated fish [58].

The results of our study showed (RPS) value was 81.58% in primary

dose after the sixth week and 82.05% in booster dose after the eighth week. Therefore, repeated vaccine dose or booster dose provides better immune responses along with lasting protection. Successful vaccines should provide more than 80% protection [59]. Similar results have been obtained by Pretto-Giordano et al. whom evaluated the efficacy of a formalin-killed vaccine of *S. agalactiae* in *O. niloticus* using primary dose and booster dose with a 21 day interval [60]. After challenge with *S. agalactiae* (i.p), the RPS of LAV1 was 81.58% in the group receiving only the primary dose of vaccine, while the other group receiving primary and booster dose was recorded as 82.05%, suggesting the vaccine strategy is efficient. Oral vaccines can be used for primary and/or as a booster vaccine to prolong the duration of protection against endemic diseases [12]. Our result further suggests that the live attenuated strains of *S. agalactiae* are capable of inducing protective immunity against *S. agalactiae* up to the fifth week. Additional antigen load from the booster immunization compromised the immune protection of the live attenuated vaccine leading to immunosuppression on the eighth week [9]. This may be due to the presence of high IgM level during the administration of antigen in booster dose, such that the vaccine is equal to the antibodies and may not be well recognized. Our data, however, proved that the primary and booster dose of LAV induced long-term protection against *S. agalactiae* and is suitable for use as a vaccine for fish. Further research is needed to assess the effects of LAV on the immunological memory in the fish.

In conclusion, live attenuated vaccines were shown to elevate both antibody IgM levels and lysozyme activity. LAV is a low cost vaccine that is able to provide long-term protection when introduced as feed and can rapidly increase the immune response of fish as well as high survival rate against *S. agalactiae* infection.

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