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Efficacy of combined inactivated vaccines against *Vibrio alginolyticus* and *Streptococcus agalactiae* infections in Nile tilapia in Egypt

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Abstract

Vibrio alginolyticus and *Streptococcus agalactiae* are important bacterial pathogens that yielded high losses in Nile tilapia in Egypt. The present study aimed to check the protective efficacy of inactivated whole-cell bivalent vaccines against these pathogens using incomplete Freund's adjuvant and Montanide™ IMS 1312 VG as adjuvants. The antibody titers have been determined at different weeks post-vaccination (WPV). Moreover, the protection levels against the challenged bacterial pathogens have also been examined in relation to the time-dependent protection at different WPV. The results revealed that serum antibodies were generated in all immunized fish at 1st WPV, peaked at 4th WPV, continued, and gradually decreased from 6th WPV to 14th WPV in all vaccinated groups. In addition, vaccines induced significantly higher protection of the immunized tilapia, manifested by higher survival rates. We noticed that the antibody levels and survival rates of the vaccinated fish by a vaccine adjuvanted by Montanide™ IMS 1312 VG were higher than those produced by a vaccine adjuvanted by incomplete Freund's adjuvant at different time points. Moreover, no external clinical signs, visceral adhesions, or internal lesions were recorded in the vaccinated tilapia, demonstrating the safety of the formulated vaccines. According to the aforementioned findings, we could suggest that the prepared bivalent vaccines, using the two adjuvant types, are safe and highly protective and could be utilized as promising candidate vaccines to increase the resistance of Nile tilapia against *V. alginolyticus* and *S. agalactiae* infections. Moreover, Montanide™ IMS 1312 VG enhanced the immuno-protectivity and exhibited optimum immune response and earlier protection compared to the vaccine adjuvanted by incomplete Freund's adjuvant, demonstrating its added value during the preparation of tilapia vaccines.

Keywords Streptococcosis · Vibriosis · Antibody titers · Adjuvants · Challenge

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Introduction

Egyptian aquaculture is one of the largest fish producers in Africa; however, it currently faces several constraints threatening its growth and continual development (Shaalan et al. 2018), such as the wide spread of infectious diseases, particularly during the summer season (Abdel-Latif and Khafaga 2020). Bacterial pathogens contribute to heavy kills and significant economic loss in many fish farms throughout the globe (Austin and Austin 2016). In Egypt, they share the most significant cause of annual economic loss in fish farms (El-Son et al. 2021), especially when they flourish as combined infections with other pathogens (Abdel-Latif et al. 2020). Several types of bacterial pathogens have been implicated in disease outbreaks in finfish species throughout the globe (Austin 2019). From these diseases, vibriosis and streptococcosis have gained particular concerns.

Vibriosis is one of the main bacterial diseases that threaten a variety of finfish species (Ina-Salwany et al. 2019), as it causes serious pathological lesions, disease signs, and heavy kills (Manchanayake et al. 2023; Mohamad et al. 2019). This disease is caused by members of the Vibrionaceae family, including *Vibrio anguillarum*, *V. parahaemolyticus*, *V. ordalii*, *V. harveyi*, and *V. alginolyticus*. From these species, *V. alginolyticus* has been previously identified from several finfish species, such as turbot (*Scophthalmus maximus*) (Austin et al. 1993), *Mugil capito* (Khalil and Abdel-Latif 2013), cobia (Liu et al. 2004; Rajan et al. 2001), *Tilapia zillii* (El-Sayed et al. 2019), African catfish (*Clarias gariepinus*) (Abdelsalam et al. 2021), *Sparus aurata* and *Dicentrarchus labrax* (Ben Kahla-Nakbi et al. 2009), and Nile tilapia (*Oreochromis niloticus*) (Younes et al. 2016). Streptococcosis is another frequently encountered disease that affects farmed fish species and is caused by several streptococcal strains such as *Streptococcus agalactiae*, *S. iniae*, and *S. dysgalactiae* (Klesius et al. 2008; Van Doan et al. 2022). With particular concern, several researchers reported the negative impacts of *S. agalactiae* on tilapia aquaculture (Pretto-Giordano et al. 2010; Suanyuk et al. 2008; Wangkaghart et al. 2021; Zhang et al. 2020).

Vaccination could be considered a promising strategy to boost fish immunity and provide considerable protection against challenging pathogens (Sommerset et al. 2005; Austin 2012). Researchers developed and constructed many fish vaccines with proven efficacy and prominent protective roles (Adams 2019). Vaccine adjuvants have been broadly used to augment the efficacy of vaccines by improving their potency and durability of immune responses against specific antigenic materials (Brudeseth et al. 2013). Moreover, they can minimize the number of the required vaccinal doses (especially the booster doses) and help to reduce the amount of antigen needed per vaccinal dose (Wang et al. 2013; Xu et al. 2012). Since the 1990s, vaccines combined with oil-based adjuvants had been applied in aquaculture (Aucouturier et al. 2001; Ribeiro and Schijns 2010), with proven and effective roles in controlling many bacterial fish diseases. After that, a wide range of vaccine adjuvants has been used in several trials (Tafalla et al. 2013), such as chitosan oligosaccharide, aluminum hydroxide, flagellin, liposomes, astragalus polysaccharides, CpG oligonucleotides, Freund's complete adjuvant (FCA), incomplete Freund's adjuvant (IFA), and Montanide™ adjuvant (Wangkaghart et al. 2021). Reports enlightened the efficacy of such adjuvants for enhancing the immuno-protective roles of vaccines by increasing their magnitude and providing prolonged protection against several infections in many finfish species (Jiao et al. 2010; Wangkahart et al. 2019; Zheng et al. 2012).

Bivalent vaccines containing two antigenic combinations could be better than monovalent vaccines to reduce the costs and minimize the handling stress exerted on the vaccinated fish (Bastardo et al. 2012). The protective efficacy of the inactivated whole-cell

killed bivalent vaccines against two different bacterial antigens has been previously examined. For example, Shoemaker et al. (2012) reported a proven efficacy of a bivalent vaccine against *S. iniae* and *V. vulnificus* infections in hybrid tilapia (*O. niloticus* × *O. aureus*). Moreover, an effective bivalent vaccine has also been developed against *S. agalactiae* and *Aeromonas hydrophila* in Nile tilapia (Pasaribu et al. 2018). In this context, we formulated inactivated formalin-killed whole-cell bivalent vaccines against *V. alginolyticus* and *S. agalactiae* infections in Nile tilapia. We evaluated incomplete Freund's adjuvant and Montanide™ IMS 1312 VG as vaccine adjuvants. After immunization with these vaccines, the antibody titers of Nile tilapia were analyzed at different weeks post-vaccination. In addition, the protective efficacy of the constructed vaccines was also tested by monitoring the fish survival after being challenged with *V. alginolyticus* and *S. agalactiae* infections at different time points to determine the time-dependent protection of the tested vaccines.

Materials and methods

Ethical statement and approval code

Experiments were ethically approved by the Institutional Animal Care and Use Committee at Alexandria University (ALEXU-IACUC-013/2022/12/-3R/4P/187).

Experimental fish: acclimation and rearing

Healthy Nile tilapia, weighing approximately 50.0±10.0 g (as an average initial weight), were procured from a fish farm in Kafr El Sheikh, Egypt. After their arrival, fish were reared in 500-L fiberglass tanks at the Wet Laboratory belonging to the Central Laboratory for Evaluation of Veterinary Biologics, Cairo, Egypt. Before the vaccination trials, fish were left in these tanks for two successive weeks to acclimate to the laboratory conditions. During the acclimation, fish were hand-fed ad libitum daily by commercially purchased well-balanced isocaloric and isoproteinous diet (Aller Aqua Co., Egypt) to fulfill the requirements for optimum growth rates. Fish tanks were supplied with well-aerated water, and fish were maintained in static water. Water temperature, pH, dissolved oxygen, and total ammonia nitrogen were maintained at 28.50±1.0 °C, 7.8±0.1, 6.6±0.3 mg/L, and 0.05 mg/L, respectively. After acclimation, twelve individuals were randomly selected from the rearing tanks and then examined bacteriologically to confirm they tested negative for bacterial infections.

Pathogenic bacterial strains

The used bacterial strains were kindly supplied by the Department of Poultry and Fish Diseases, Faculty of Veterinary Medicine, Alexandria University. *V. alginolyticus* (Abdel-Latif 2013; Khalil and Abdel-Latif 2013) and *S. agalactiae* (Hamoury 2022) were formerly identified from naturally infected fish showing septicemic signs. The bacterial isolates were preserved in 20% glycerol at −80 °C. Before their use, the bacterial strains were cultured on tryptic soy agar (TSA, HiMedia, Maharashtra, India). The retrieved bacterial colonies were then examined and identified using microbiologically standard protocols such as Gram staining, colonial morphology, biochemical tests, and

β-hemolytic patterns for *S. agalactiae* on blood agar (BA) media. The phenotypic and molecular characterizations (PCR using specific primers) were conducted before the two bacterial pathogens were used as antigenic materials to prepare the inactivated vaccines.

Bacterial inactivation, adjuvants, and vaccine formulation

Bacterial inactivation

Pure colonies of *V. alginolyticus* and *S. agalactiae* were inoculated into brain heart infusion broth (BHIB, HiMedia, Maharashtra, India) and then incubated at 30 °C for 24–48 h. Ten-fold serial dilutions of the bacterial cultures were done. Each bacterial isolate was plated on TSA medium and then incubated at 30 °C for 24 h to estimate the CFU/mL, according to the protocol described in Abu-Elala et al. (2019). Afterward, the CFU/mL was compared to McFarland standard concentrations. For bacterial inactivation, the bacterial cultures were then suspended in a 3% formalin solution (Algomhuria, Egypt) with continuous agitation for 24 h at 25 °C. The prepared antigenic materials were centrifuged at 1800 × *g* for 30 min. Supernatants were removed, and bacterial pellets were re-suspended three times in PBS solution (phosphate-buffered saline; pH 7.4). Safety testing was conducted by plating 100 µL of the formalin-inactivated suspensions on the TSA medium and then incubating at 30 °C for 3 days to observe any bacterial growth that occurred.

Adjuvants used for vaccine formulation

Montanide™ IMS 1312 VG (SEPPIC, France) and incomplete Freund's adjuvant (Sigma-Aldrich, USA) were used to formulate bivalent vaccines. The inactivated whole-cell bivalent vaccine was formulated by mixing equal volumes of the antigenic aqueous medium containing 3×10^9 CFU/mL of each bacterial species (*V. alginolyticus* and *S. agalactiae*) with the adjuvant type at room temperature with adequate agitation. The first formulation was made using the prepared bacterial mixtures and then mixed with Montanide™ IMS 1312 VG in a ratio of 50:50 (v/v) according to the protocol described in Abu-Elala et al. (2019). In the same sense, the second formulation was made using the prepared bacterial mixtures and then mixed with incomplete Freund's adjuvant in a ratio of 50:50 (v/v). The mixing process was conducted at room temperature under gentle agitation according to the procedures provided by the manufacturer to formulate two adjuvanted whole-cell bivalent vaccines. Similarly, the control solution was prepared with PBS, which was mixed with each adjuvant type in a ratio of 50:50 (v/v). Hence, all prepared adjuvanted vaccines were stored at 4 °C until use.

Vaccine sterility test

The sterility of the formulated vaccines was tested and confirmed to be free from bacterial or fungal contamination. These procedures were conducted by spreading 100 µL from each vaccine on TSA, blood agar, and Sabouraud's dextrose agar (SDA) media. All culture plates were then incubated at 25 °C for 2 days.

Vaccine adjuvant safety test

This test examined whether the prepared vaccines caused any adverse reactions in fish before their use in the vaccination process. A double dose of the prepared vaccines was used to achieve this objective. In this regard, thirty fish (per each group) were intraperitoneally (IP) injected with 1.0 mL of each prepared adjuvanted vaccine. Fish were anesthetized with clove oil (Algomhuria, Egypt; 5 mL/L) before injection for safe handling during the injection. After injection, the inoculated fish were then monitored for 14 days to record any adverse impacts which will appear in the form of behavioral changes such as aggression, loss of appetite, isolation, color changes, lesions at the site of injection, immediate mortalities due to toxicity, and other signs related to injection at a high dose. Finally, fish were euthanized after the 14-day observation period and then aseptically necropsied to check for any postmortem (PM) lesions resulting from long-term adverse effects such as lesions or visceral adhesions. A safe vaccine could be determined not to produce the changes mentioned above.

Vaccination and blood sampling

Vaccination procedure

Fish were anesthetized with clove oil (5 mL/L) before vaccination for safe handling during the injection. Six hundred thirty healthy Nile tilapia were allocated into three groups (each one containing 210 individuals). In the 1st group (*group I*), fish were vaccinated via IP injection with 0.5 mL/fish of the formulated inactivated whole-cell bivalent vaccine adjuvanted by Montanide™ IMS 1312 VG. The IP route was selected for fish immunization according to the procedure formerly described by Abu-Elala et al. (2019) and also the approval obtained from the adjuvant supplier. In the 2nd group (*group II*), fish were vaccinated via IP injection with 0.5 mL/fish of the formulated inactivated whole-cell bivalent vaccine adjuvanted by incomplete Freund's adjuvant. In the 3rd group (*group III*), where fish were injected with 0.5 mL PBS as prepared above, this group served as the control group.

Blood sampling and serum collection

Fish were fasted for 24 h before blood sampling. Fish were then anesthetized, and blood was collected from the caudal vein using a 3-mL syringe. Blood samples were collected without anticoagulant from 5 fish per group ($n = 5$). Blood samples were then left in Eppendorf tubes on ice in a vertical position to separate the serum. Serum was collected after centrifugation at $1500 \times g$ for 10 min at 4 °C. The collected serum samples were stored at −20 °C until use. Fish sera were taken to evaluate the antibody titers at 1st, 2nd, 3rd, 4th, 6th, 8th, 10th, 12th, and 14th weeks post-vaccination (WPV).

Vaccine potency

Microtiter plate agglutination test

The agglutinating antibody titers were determined using the microtiter plate agglutination test using each antigen independently (Klesius et al. 2000; Shoemaker et al. 2011). To put it briefly, two 96-well microtiter plates (with rounded bottoms) were first plated with 25 µL of PBS.

Subsequently, 25 µL of the sampled fish serum was added in each well of the first row and then mixed well. After the mixing procedure, twofold serial dilutions were performed. Then, 50 µL of either *V. alginolyticus* or *S. agalactiae* cell suspensions in a dose of 9×10^8 CFU/mL (equal to McFarland 3) was added to and mixed with the contents in each well. Plates were covered and incubated overnight (18 h) at 28.0±2.0 °C and then for 4 h at 4 °C prior to plate reading. These steps were performed in line with the procedure described by Shoemaker et al. (2012). The endpoint of the agglutination was observed visually as the final serum dilution, whereas visible agglutination was noticed and was taken as the agglutinating antibody titer. The antibody titers were expressed as Log2 $(x + 1)$ of the mutual of the highest dilution of serum samples that exhibited noticeable agglutination in comparison with the positive control.

Vaccine efficacy

Thirty fish were selected per group (VACC and CNT groups) and were allocated in triplicates (10 fish/replicate). Fish were IP injected with 0.1 mL containing 6×10^8 CFU/mL per fish from a suspension of a single live *V. alginolyticus* or *S. agalactiae*. This dose was selected on the basis of a previously calculated lethal dose 50%. The bacterial challenge test was performed on the 2nd, 4th, 6th, 8th, 10th, 12th, and 14th WPV. All challenged fish were observed daily for 2 weeks. In addition, the challenged fish were investigated daily for the clinical signs of vibriosis and streptococcosis. Dead fish were removed daily and counted. The cumulative mortality and survival percentages were calculated for 2 weeks, and the relative percent survival (RPS) was determined (Amend 1981) in line with the following formula:

$$\mathrm{RPS} = 100 \times \left(1 - \frac{\text{Mortality \% in VACC group}}{\text{Mortality \% in CNT group}}\right)$$

Bacterial re-isolation

Bacterial re-isolation was performed from the challenged fish to verify the cause of mortalities and confirm that these mortalities originated from the pathogens used for the challenge test. To achieve this objective, bacteriological swabs were harvested from the liver samples and then subcultured on culture media, which followed the same bacteriological examination procedures described before.

Statistical analysis

Data were expressed as means ± SE. Data were examined by a one-way ANOVA. Data were analyzed using SPSS software (SPSS Inc., Chicago, IL, USA) and GraphPad prism X8 program.

Results

Vaccine sterility

The sterility of the tested bivalent vaccine was approved, whereas the examination of the inoculated culture media did not detect any bacteria other than those used as vaccinal strains. Moreover, no fungal contamination or growth was detected.

Vaccine safety

The safety studies on the prepared vaccines demonstrated that fish vaccinated with a double dose of the inactivated bivalent vaccines did not show any adverse local or systemic reactions throughout the post-vaccination period. Moreover, the fish appeared normal and healthy post-vaccination, with no clinical signs observed. PM findings also did not reveal any visceral adhesions or internal lesions.

Antibody titers

The microtiter plate agglutination test showed the serum antibody titers against *S. agalactiae* in fish groups vaccinated with bivalent vaccines using Montanide™ or incomplete Freund's adjuvants were generated at 1st WPV to 14th WPV as described in Fig. 1. The kinetics of antibody responses of vaccinated tilapia against *S. agalactiae* (Fig. 2) using Montanide™ and incomplete Freund's adjuvant were 2 and 2.1 Log2 at 1st WPV, respectively. Their values gradually increased until they peaked at the 4th WPV, whereas they reached 7.5 and 6.4 Log2 in vaccine adjuvanted by Montanide™ and incomplete Freund's adjuvant, respectively. After that, the agglutination antibody titers gradually decreased from 6th WPV to 14th WPV in fish groups vaccinated using both adjuvant types (Fig. 2). The results revealed that the agglutination antibody titers in the serum of vaccinated tilapia using a vaccine adjuvanted by Montanide™ adjuvant were significantly higher than those produced by a vaccine adjuvanted by incomplete Freund's adjuvant over different time points, as presented by Fig. 1. On the other hand, the agglutination antibody titers for Nile tilapia reared in the control group were constant at 2.3 Log2 and continued till the end of the experiment.

The microtiter plate agglutination test showed that the serum antibody titers against *V. alginolyticus* in fish groups vaccinated with a bivalent vaccine using Montanide™ or incomplete Freund's adjuvants were generated at 1st WPV to 14th WPV as described in Fig. 3. The kinetics of antibody responses of vaccinated tilapia against *V. alginolyticus* (Fig. 4) using Montanide™ and incomplete Freund's adjuvants were 3 and 2.1 Log2 at 1st WPV, respectively. Their values gradually increased until they peaked at the 4th WPV, whereas they reached 6 and 5.3 Log2 in vaccine adjuvanted by Montanide™ and incomplete Freund's adjuvant, respectively. After that, the agglutination antibody titers gradually decreased from 6th WPV to 14th WPV in fish groups vaccinated using both adjuvant types (Fig. 4). The results revealed that the agglutination antibody titers in the serum of vaccinated tilapia by a vaccine adjuvanted by Montanide™ adjuvant were significantly higher than those produced by a vaccine adjuvanted by incomplete Freund's adjuvant over different time points, as shown by Fig. 3. On the other hand, the agglutination antibody titers for Nile tilapia reared in the control group were constant at 1 Log2 and continued till the end of the experiment.

Survival (%) against bacterial challenge

As shown in Fig. 5, it was found that post-challenge survival (%) of fish experimentally infected with *S. agalactiae* was significantly increased in groups immunized with a bivalent bacterin using the two adjuvants compared to the controls. With a particular concern, it was found that the survival (%) of the vaccinated fish after being challenged with *S. agalactiae* was 60.0%, 90.0%, 80.0%, 75.0%, 70.0%, 50.0%, and 35.0% in case

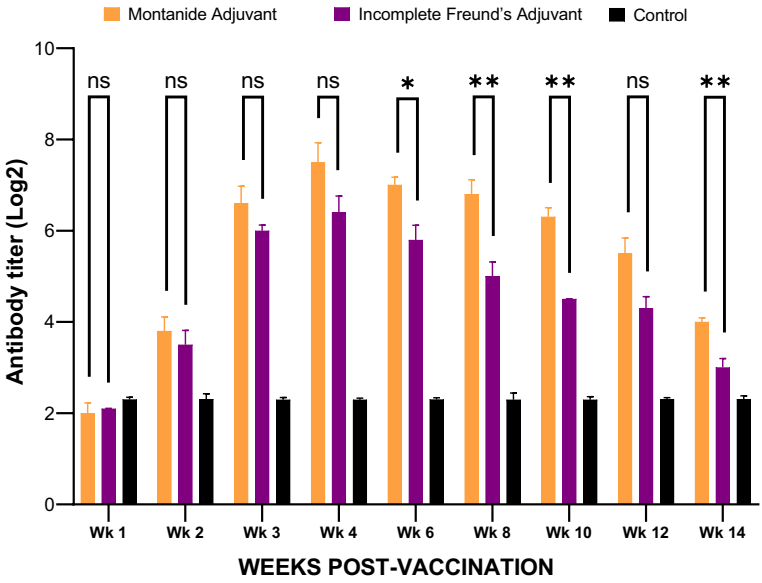

Fig. 1 Antibody titers of Nile tilapia against *S. agalactiae* as determined by the microtiter plate agglutination test. Fish were immunized with a bivalent vaccine using Montanide or incomplete Freund's adjuvant compared with the control (PBS). Sera were collected at 1, 2, 3, 4, 6, 8, 10, 12, and 14 weeks post-vaccination. Data expressed as means ± SE. Bars with asterisks (* ($P < 0.05$) and ** ($P < 0.01$) indicate significant differences between groups at each time point. Meanwhile, ns indicates non-significant differences between groups

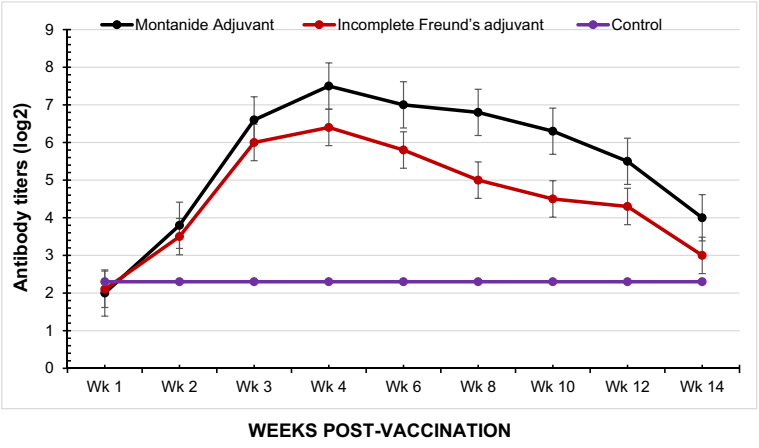

Fig. 2 The kinetics of antibody responses of Nile tilapia in the control group and the vaccinated groups with a bivalent vaccine using Montanide or incomplete Freund's adjuvant. The antibody responses against *S. agalactiae* were determined at 1, 2, 3, 4, 6, 8, 10, 12, and 14 weeks post-vaccination. Values are shown as means ± SE

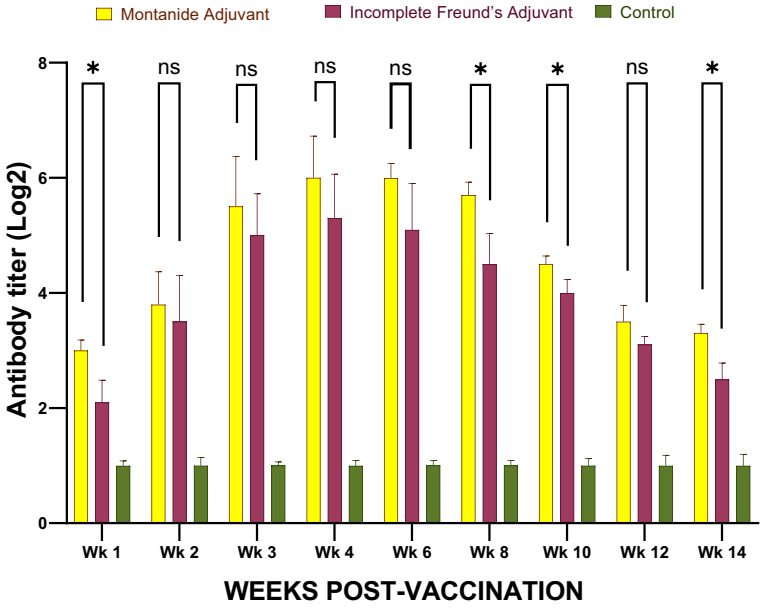

Fig. 3 Antibody titers of Nile tilapia against *V. alginolyticus* as determined by microtiter plate agglutination test. Fish were immunized with a bivalent vaccine using Montanide or incomplete Freund's adjuvant compared with the control (PBS). Sera were collected at 1, 2, 3, 4, 6, 8, 10, 12, and 14 weeks post-vaccination. Data expressed as means ± SE. Bars with asterisks (*) ($P < 0.05$) indicate significant differences between groups at each time point. Meanwhile, ns indicates non-significant differences between groups

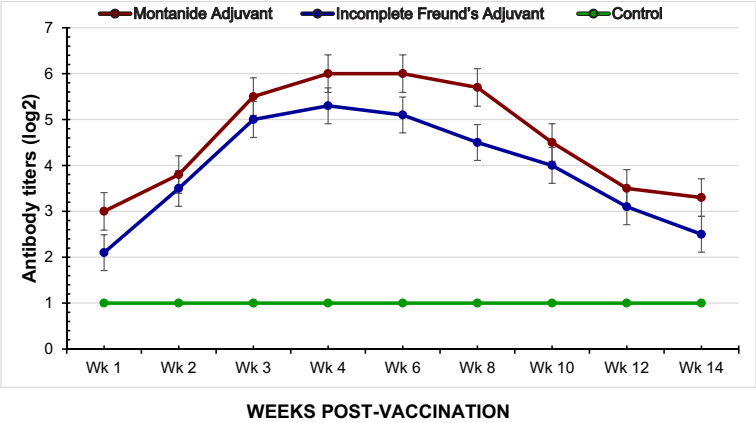

Fig. 4 The kinetics of antibody responses of Nile tilapia in the control group and the vaccinated group with a bivalent vaccine using Montanide or incomplete Freund's adjuvant. The antibody responses against *V. alginolyticus* as determined at 1, 2, 3, 4, 6, 8, 10, 12, and 14 weeks post-vaccination. Values are shown as means ± SE

of a vaccine adjuvanted with Montanide™ adjuvant at 2, 4, 6, 8, 10, 12, and 14 WPV, respectively. Moreover, the survival (%) of the vaccinated fish after being challenged with *S. agalactiae* was 45.0%, 80.0%, 75.0%, 75.0%, 55.0%, 50.0%, and 30.0% in the case of a vaccine adjuvanted with using incomplete Freund's adjuvant at 2, 4, 6, 8, 10, 12, and 14 WPV, respectively.

As shown in Fig. 6, it was found that post-challenge survival (%) of fish experimentally infected with *V. alginolyticus* was significantly increased in groups immunized with a bivalent bacterin using the two adjuvants compared to the controls. With a particular concern, it was found that the survival (%) of the vaccinated fish after being challenged with *V. alginolyticus* was 80.0%, 95.0%, 90.0%, 90.0%, 85.0%, 75.0%, and 60.0% in case of a vaccine adjuvanted with Montanide™ adjuvant at 2, 4, 6, 8, 10, 12, and 14 WPV, respectively. Moreover, the survival (%) of the vaccinated fish after being challenged with *V. alginolyticus* was 75.0%, 90.0%, 85.0%, 85.0%, 70.0%, 60.0%, and 45.0% in the case of a vaccine adjuvanted with incomplete Freund's adjuvant at 2, 4, 6, 8, 10, 12, and 14 WPV, respectively.

Bacterial re-isolation

All bacteriological swabs were harvested from the liver samples of the diseased and dead fish after being challenged by *S. agalactiae* and *V. alginolyticus*. The results confirmed that the infection and death had originated from the same pathogens used in the challenge test (Fig. S1 and Fig. S2; Supplementary Material). Freshly live fish have been protected from the infection, and no bacterial growth was recorded on the bacteriological cultures.

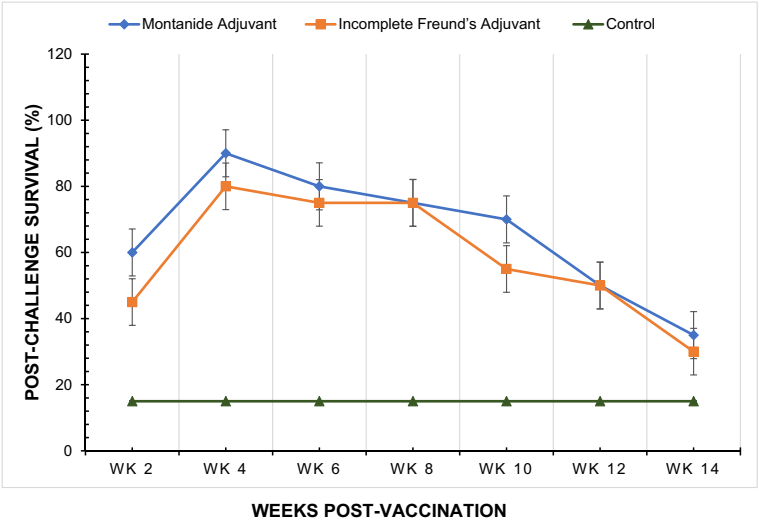

Fig. 5 Time-dependent protection of the tested inactivated bivalent vaccine. Nile tilapia were injected with PBS in the control group (control) and those immunized with inactivated bivalent vaccine using two different adjuvants: Montanide and incomplete Freund's adjuvant. Fish were monitored for survival (%) after being challenged with *S. agalactiae* at different time points (2, 4, 6, 8, 10, 12, and 14 weeks post-vaccination)

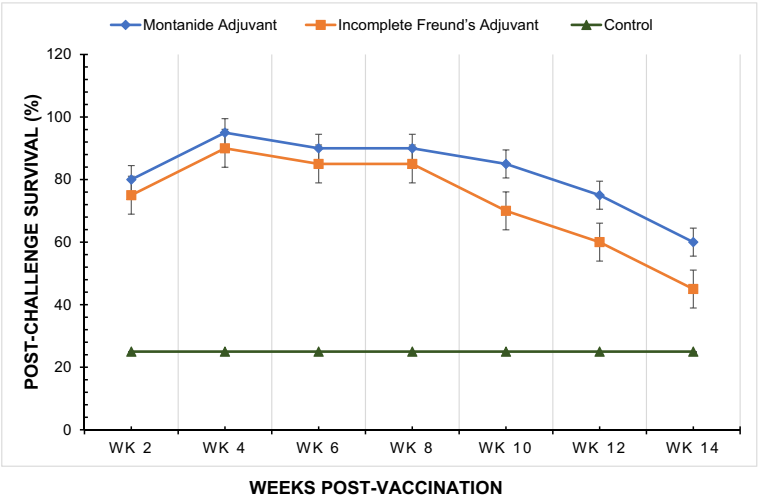

Fig. 6 Time-dependent protection of the tested inactivated bivalent vaccine. Nile tilapia were injected with PBS in the control group and those immunized with inactivated bivalent vaccine using two different adjuvants: Montanide and incomplete Freund's adjuvant. Fish were monitored for survival (%) after being challenged with *V. alginolyticus* at different time points (2, 4, 6, 8, 10, 12, and 14 weeks post-vaccination)

Discussion

Vaccination has become an effective strategy in aquaculture to prevent disease spread among farmed fish (Sommerset et al. 2005; Austin 2012). Bivalent or polyvalent vaccines are the most economical way to prevent disease caused by two or multiple infections (Brudeseth et al. 2013), as in the case of sole (*Solea senegalensis*) immunized with a bivalent vaccine (Arijo et al. 2005) and orange-spotted groupers (*Epinephelus coioides*) immunized with a polyvalent inactivated vaccine (Huang et al. 2012), besides their cost-effective benefits (Shoemaker et al. 2012). They could also minimize the handling stress on the vaccinated fish (Shoemaker et al. 2012). In the present study, we formulated formalin-inactivated whole-cell bivalent adjuvanted vaccines against *V. alginolyticus* and *S. agalactiae* infections and investigated their efficacy in Nile tilapia as a fish model. There are several examples of successful vaccination of hybrid tilapia using inactivated bivalent vaccines against two bacterial pathogens, namely, *S. iniae* and *A. hydrophila* (Monir et al. 2021; Monir et al. 2022a, b; Mohd Ali et al. 2023).

Using adjuvants in vaccine preparation has become effective for inducing prolonged protection and immunogenicity (Plant and LaPatra 2011). The incomplete Freund's adjuvant is a mixture of oil and water combined with a specific antigen to boost the immunity of the host organism against that antigen (Chang et al. 1998). Montanide™ adjuvants can be used at the industrial level in combination with a wide range of antigens (Aucouturier et al. 2000). Montanide™ IMS 1312 VG is an aqueous adjuvant that consists of water-dispersed liquid nanoparticles with an immune-stimulating compound. This adjuvant has been previously used in vaccination via the IP route with proven efficacy, as in the case of Nile tilapia against streptococcal infections (Abu-Elala et al. 2019). The IP injection for fish vaccination is a common route of vaccine delivery because of its potential ability to enhance innate and acquired immunity (Gudding et al. 2014).

Assessing the biosafety of fish vaccines is a fundamental issue that could affect the quality of the formulated vaccines. Although adjuvants are chemicals that can trigger the immune responses of fish to respond promptly to the vaccination process (Tafalla et al. 2013), these chemicals can also cause minor negligible side effects in some vaccinated fish, such as visceral adhesions or others (Romstad et al. 2013). The present study showed that the prepared adjuvanted vaccines did not cause abnormal behavior or clinical signs in the immunized fish throughout the post-vaccination period. Moreover, during this period, the vaccinated fish appeared healthy and normal. The PM findings revealed no visceral adhesions or internal lesions. The findings above propose that the tested vaccines appear nontoxic for application in Nile tilapia. It is renowned that the use of oil-based adjuvants is focused on maintaining the vaccine persistence for inducing long-term protection with a prolonged immune-boosting ability of the vaccinated fish (Tafalla et al. 2013). Certainly, reports declared that oil-based adjuvants induced side effects ranging from mild to moderate lesions in Atlantic salmon (Midtlyng et al. 1996), turbot (Noia et al. 2014), and Nile tilapia (Wangkaghart et al. 2021). Our findings were also coherent with those reported in turbot immunized by an inactivated bivalent vaccine against *V. anguillarum* and *V. harveyi* infections (Zhang et al. 2021), whereas those authors found that the immunized turbot did not show any side effects post-vaccination.

Regarding the persistence of the tested adjuvants, assessment of the adjuvant quality can depend upon its persistence in the tissues of vaccinated fish at different time points. This is an important factor in choosing the best quality adjuvant for application, with no noticeable negative impacts on the vaccinated fish (Wangkaghart et al. 2021; Wangkahart et al. 2023). In this context, our findings showed no recorded internal lesions in vaccinated fish several weeks post-vaccination. This information was consistent with that described by Wangkahart et al. (2023), who recently reported normal internal organs of Nile tilapia with no adhesions at the 5th WPV with an inactivated whole-cell vaccine against *S. agalactiae* adjuvanted using Montanide™ ISA 763 A VG or ISA 763 B VG.

Antibody titers are biomarkers for assessing the efficiency of the formulated vaccines (Zhang et al. 2021). The study showed that the bivalent vaccine increased acquired immunity and promoted protective immune responses against infection with two bacterial pathogens. These results were clarified by measuring the antibody titers against each bacterial isolate using the microtiter plate agglutination test. It was found that the immunized tilapia with this vaccine produced antibodies against both bacterial isolates, and these antibodies were expected to be implicated in the protection recorded in the challenge test. Hence, these results appeared to intensify and boost the roles of serum antibodies in the protective immunity of Nile tilapia against the challenged pathogens. Regarding these results, it was previously noticed that combining two or more different bacteria in bivalent or polyvalent vaccines can augment the antibody titers and protection levels compared to a monovalent vaccine (Hoel et al. 1997; Sun et al. 2011). In the current study, we found that the antibody titers against both bacterial pathogens started to increase earlier from 1st WPV, increased gradually, peaked at 4th WPV, and then gradually decreased from 6th WPV to 14th WPV. These results were similar in cases of the vaccine adjuvanted using Montanide™ or incomplete Freund's adjuvant when compared with the controls. However, the findings indicated that the levels of antibody titers produced and generated by tilapia vaccinated with a bivalent vaccine adjuvanted with Montanide™ were significantly higher than those produced by tilapia vaccinated with a vaccine adjuvanted with incomplete Freund's adjuvant throughout the whole observation period. A possible interpretation and explanation of these results are that the vaccine includes a combination of bacterial antigens scattered in the aqueous nanoparticle micro-emulsion of the Montanide™ adjuvant, which could increase the immunogenicity of the bivalent vaccine (Abu-Elala et al. 2019; O'Hagan and

Singh 2003). The early appearance of the antibodies may also be attributed to the ability of the adjuvant to enable the early start and initiation of the fish immune responses, boosts the adaptive immunity, and enhances the uptake of antigen by the fish mucosal surfaces with the noticeable ability to maintain a prolonged and durational immunity and antibody production (Abu-Elala et al. 2019; Soltani et al. 2014). Indeed, the degree of immune responses and antibody production varies significantly depending on the adjuvant type (Areechon et al. 1992). However, the study conducted by Bastardo et al. (2012) showed that rainbow trout generated high antibody titers after being vaccinated with aqueous and adjuvanted bivalent vaccines. Nonetheless, the mechanisms underlying the increased antibody titers in a vaccine adjuvanted with Montanide™ over those with incomplete Freund's adjuvant require further studies and warrant additional investigations.

In the present study, the antibody titers of immunized tilapia peaked at the 4th WPV and then declined gradually from the 6th WPV to the 14th WPV. The study by Bastardo et al. (2012) also declared that maximum antibody titers were detected in the sera samples of rainbow trout vaccinated by a bivalent killed bacterin at 30 days post-vaccination. It was reported that the maximum agglutination titers against *A. hydrophila* were detected in rohu (*Labeo rohita*) immunized with polyvalent and monovalent vaccines at 4 WPV (Swain et al. 2007). Our findings were also in concordance with those previously published in turbot immunized by a bivalent inactivated vaccine (Zhang et al. 2021), whereas those authors also found that specific antibody titers peaked at 4th WPV and declined at 8th WPV. Although there are aforementioned consistencies between our findings and others, we should clarify that the antibody responses may be affected by several factors and not the same among studies, such as factors related to fish (such as fish species, immunity, age, size, and physiological status), vaccine factors (adjuvant type, dose, and route of administration), and experiment conditions (rearing facilities, type of culture, period, and others). All these factors should be considered when comparing the results of different vaccines.

The post-challenge survival is another important indicator to evaluate vaccine efficacy and reflect the protective immunity of the tested vaccine. Compared to the controls, it was found that the fish survival increased significantly in vaccinated fish with a vaccine adjuvanted with Montanide™ or incomplete Freund's adjuvant at different time points after being challenged with *S. agalactiae* or *V. alginolyticus*. The protection levels and fish survivals seem closely related to the antibody titers generated in vaccinated tilapia. Antibodies can enhance the process of phagocytosis and also increase the killing activities of phagocytes and promote activation of the antigen-specific B cells (de Ståhl et al. 2003). As discussed earlier in the serum antibody titers, it was found that the fish survivals in the present study were higher in fish vaccinated with a vaccine adjuvanted with Montanide™ than those adjuvanted with incomplete Freund's adjuvant. Several other reports showed that using non-mineral oil-based adjuvants combined with injectable formalin-killed bacterin provided a prolonged and durational defense and lengthened the fish protection against the challenged bacterial pathogens (Ravelo et al. 2006). The suggested mechanism of these results may be associated with the depot effect produced by the adjuvant, which will, in turn, allow the slow and delayed release of the antigen into the fish tissue or blood, therefore augmenting prolonged humoral responses and protection against the challenged pathogens (Bastardo et al. 2012). Hence, additional mechanisms underlying the protective roles of the adjuvanted bivalent vaccine necessitate additional studies.

Several researchers previously interpreted the close relationship between the enhanced survival rates post-challenge and the antibody titers produced after vaccination in several finfish species (Bastardo et al. 2012; Wangkaghart et al. 2021). However, other researchers did not find a correlation between the antibody titers and

relative percent survival post-challenge, as previously seen in Nile tilapia (Klesius et al. 2000) and turbot (Zhang et al. 2021). These inconsistencies may be attributed to fish species differences and their response to the vaccine used. Furthermore, fish resistance following vaccination may also be affected by several factors such as vaccine (type, adjuvant type, concentration, and route of administration), fish (immune responses post-vaccination, size, physiology, age, and others), and experiment design (Wangkahart et al. 2023). All these factors necessitate additional research studies to clarify their effects on the antibody responses and fish resistance to the challenged pathogens.

Conclusions

To put it briefly, we prepared inactivated whole-cell bivalent vaccines using Montanide ™ IMS 1312 VG or incomplete Freund's adjuvant against *S. agalactiae* and *V. alginolyticus* infections in Nile tilapia. These vaccines induced higher specific antibody titers in the serum of Nile tilapia at 1st WPV, and their peak was reached at the 4th WPV. Moreover, these vaccines provoked high protective efficacy against two pathogenic bacterial pathogens, *S. agalactiae* and *V. alginolyticus*, known to cause devastating infections and high mortalities in Nile tilapia in our country. Of interest, the antibody titers and survival (%) were elevated when using a vaccine adjuvanted by Montanide™ adjuvant more than those obtained by incomplete Freund's adjuvant. Furthermore, the vaccinated Nile tilapia exhibited a normal growth pattern with no significant differences from the control non-vaccinated fish, and this vaccine was able to counteract the effects of the challenging bacterial pathogens with noticeably decreased cumulative mortalities and enhanced RPS. These findings highlighted the beneficial effects of these promising vaccine candidates for preventing both streptococcosis caused by *S. agalactiae* infection and vibriosis caused by *V. alginolyticus* infection in Nile tilapia. Accordingly, these vaccines could confer noticeable protection levels and may provide a cost-effective strategy to reduce the tilapia losses resulting from these important bacterial pathogens if they infect fish singly or in combination. Future perspectives should also be directed toward studying the immune responses of vaccinated fish at the cellular, molecular, and tissue levels to stand over solid ground when evaluating the effectiveness of a bivalent vaccine. Finally, our findings suggest the safe application and proven efficacy of this formalin-inactivated bivalent vaccine for possible use in tilapia culture.

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Declarations

Conflict of interest The authors declare no competing interests.

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