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Research Article

Oral vaccination of Grass carp (*Ctenopharyngodon idella*) against *Aeromonas veronii* as a sustainable strategy for disease control and biodiversity conservation

Nimra Mubeen[1], Farzana Abbas[1*], Margaret Crumlish[2], Muhammad Hafeez-ur-Rehman[1], Arshad Javid[3], Imran Altaf[4]

[1]Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Lahore, Punjab 54000, Pakistan

[2]Institute of Aquaculture, Faculty of Natural Sciences, University of Stirling, Stirling FK9 4LA, United Kingdom

[3]Department of Wildlife and Ecology, University of Veterinary and Animal Sciences, Lahore, Punjab 54000, Pakistan

[4]Quality Operations Laboratory, University of Veterinary and Animal Sciences, Lahore, Punjab 54000, Pakistan

[*]Email: farzana.abbas@uvas.edu.pk

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Asbract

Ctenopharyngodon idella (*C. idella*), a freshwater species of significant ecological and economic value, plays a vital role in aquaculture and aquatic ecosystem management. However, bacterial pathogens such as *Aeromonas veronii* (*A. veronii*) represent a major threat to fish survival, biodiversity, and sustainable aquaculture production. The current research assessed the protective potential of a monovalent oral vaccine formulated against *A. veronii* in grass carp (*C. idella*). Experimental feeds were formulated by two delivery methods: spraying and incorporation with either fish oil or mineral oil, included as adjuvants (10%). Healthy fingerlings (20 ± 5 g) were randomly assigned to five dietary groups: spray vaccine + mineral oil (SV-MO), incorporated vaccine + mineral oil (IV-MO), spray vaccine + fish oil (SV-FO), incorporated vaccine + fish oil (IV-FO), and unvaccinated control (C). The feeding trial lasted 60 days. After 56 days of dietary vaccination, vaccinated fish demonstrated markedly ($p < 0.05$) improved growth, antioxidant capacity, serum biochemical profiles (including total protein, albumin, and globulin) and immunological responses (lysozyme activity and antibody agglutination) relative to the control group. The dietary group IV-FO achieved the highest growth performance (12.7 ± 0.15^{a} g) and exhibited superior immune responses relative to other treatments. Challenge with live *A. veronii* demonstrated that IV-FO provided the greatest protection (87%), with only 13% mortality and a

relative percent survival (RPS) of 85%. Protection rates were comparatively lower in SV-FO (67%, RPS 62%), SV-MO (67%, RPS 62%), and IV-MO (54%, RPS 70%), while the control group displayed minimal protection (14%) with 86% mortality. The findings indicate that oral delivery of vaccines, particularly via incorporation with fish oil, provides an effective strategy to enhance fish resilience against bacterial infection. Fish oil acted as an immunomodulatory adjuvant that strengthened antibody production, balanced oxidative stress, and improved mucosal defense, whereas mineral oil mainly stabilized the antigen and prolonged its immune-stimulating effect. These differences explain the superior vaccine performance observed in the incorporated fish-oil formulation (IV-FO). By improving fish health and reducing disease-induced mortality, this approach supports sustainable aquaculture practices while contributing to biodiversity conservation in freshwater ecosystems.

Keywords: Grass carp; oral vaccine; *Aeromonas veronii*; fish oil adjuvant; immunity

Introduction

Grass carp (*C. idella*) is the most extensively produced freshwater fish species globally, representing nearly 16% of total freshwater aquaculture output. It serves as a key economic species, particularly in China and several other Asian countries, where it is intensively farmed. However, with the rapid expansion of the grass carp industry, outbreaks of motile *Aeromonas* septicemia (MAS) caused by *Aeromonas* spp. have become increasingly problematic (50-90 percent mortalities in uncontrolled conditions) in recent years (Rasmussen-Ivey et al., 2016).

Fish diseases caused by bacterial infections are among the most critical challenges in aquaculture, leading to considerable economic losses through high rates of mortality and morbidity. Intensive fish farming practices, characterized by high stocking densities, facilitate the transmission and proliferation of pathogenic bacteria, thereby triggering large-scale outbreaks (Rohani et al., 2022). Among these pathogens, motile species of the genus *Aeromonas* are the most commonly encountered, producing acute and chronic infections associated with severe mortalities and economic impacts in numerous cold- and warm-water fish species (Paul et al., 2015; Ullah et al., 2023). The Gram-negative, rod-shaped bacterium *A. veronii* is often isolated from aquaculture species. (Singh et al., 2012). It is responsible for bacterial hemorrhagic septicemia (BHS), motile *Aeromonas* septicemia (MAS), and epizootic ulcerative syndrome (EUS) in freshwater species. Typical clinical manifestations include ulcerative skin lesions, abdominal distension, exophthalmia, hemorrhagic septicemia, and fin rot (Rahman et al., 2004; Cui et al., 2007).

Traditionally, antibiotics have been widely applied to control bacterial diseases in aquaculture (Assane et al., 2019). However, the rapid emergence of antimicrobial resistance is undermining

their effectiveness (Amal & Zamri-Saad, 2011; Monir et al., 2020). The accumulation of antibiotic residues and the spread of resistant bacteria through trophic pathways threaten public health and ecological safety (Watts et al., 2017). Consequently, vaccination has emerged as an eco-friendly and effective strategy for mitigating bacterial diseases in aquaculture (Assefa & Abunna, 2018).

Based on preparation strategies, aquaculture vaccines can be grouped into live attenuated, inactivated, subunit, and nucleic acid vaccines (Wang et al., 2020). Among these, inactivated vaccines are particularly valued due to their safety, strong immunogenicity, and ability to induce protective responses (Farias et al., 2020). Despite these benefits, effective inactivated vaccines against many bacterial fish pathogens remain scarce, and the development of stable vaccines for non-culturable pathogens continues to present significant challenges. Only a few studies in recent years have investigated inactivated vaccines against *A. veronii*, underscoring the need for further exploration and evaluation of their protective efficacy (Matsuura et al., 2019).

Vaccines in aquaculture are commonly delivered through injection, immersion, or oral routes. While injection and immersion methods can be effective, they are impractical for large-scale farming due to high costs and labor demands (Ismail et al., 2016). Oral vaccination, particularly through feed-based formulations, offers a more feasible alternative as it is cost-effective, less labor-intensive, and capable of eliciting both mucosal and systemic immunity (Firdaus-Nawi et al., 2014; Han et al., 2018). The inclusion of adjuvants is critical for enhancing the immunogenicity of oral vaccines. Adjuvants act by potentiating immune responses and improving antigen recognition (Aucouturier et al., 2001). Fish oil, in particular, has been shown to modulate immune function by improving survival under inflammatory conditions and reducing acute and chronic inflammatory responses. Its application in clinical and experimental contexts suggests beneficial roles in managing inflammation and enhancing immune outcomes (Calder, 1998).

The present study aimed to develop a cost-effective oral vaccine against *A. veronii* for carp species, as this pathogen is a major cause of mortality and financial losses in aquaculture. In many developing countries, injectable vaccines face practical barriers such as limited technical expertise and high labor costs. Therefore, this research focused on designing an efficient feed-based vaccine, incorporating suitable adjuvants, to provide a practical and sustainable solution for disease prevention in carp aquaculture.

Material and methods

Ethical approval

This study was approved by the Institutional Ethics Committee of the University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan (Approval No. dr/486; dated 15 November 2023).

Bacterial recovery

The bacterial strain used for vaccine preparation, *A. veronii* (AV-201022), was initially isolated from diseased carps. was derived from a previously characterized *A. veronii* strain (AV-201022), originally isolated from naturally infected carps. The strain is deposited in the NCBI GenBank (accession no. NR-118947.1) and preserved at UVAS, Lahore (Mubeen et al., 2025a). The isolate's identity was reconfirmed through conventional morphological, physiological, and biochemical profiling (Bowman et al., 2005; Mahmood et al., 2024). For molecular confirmation, the 16S rRNA gene was amplified using the universal primer pair 27F (5′-AGAGTTTGATCATGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Manzoor et al., 2023). Sequence analysis indicated 98.43–100% homology with *A. veronii* reference sequences (Queipo-Ortuño et al., 2008; Sughra et al., 2021).

Vaccine Formulation

For vaccine preparation, frozen bacterial stocks were streaked with 100 µL on tryptic soy agar (TSA; Sigma-Aldrich®, Switzerland) and incubated at 30 °C for 24 h. A single colony was then inoculated into 5 mL of tryptic soy broth (TSB; Sigma-Aldrich®, Switzerland) and cultured for an additional 24 h at 30 °C (Legario et al., 2020). This primary inoculum was transferred to two 500 mL TSB flasks and incubated for 24 h at 30 °C with orbital shaking at 150 rpm. Bacterial cells were harvested at 5000 rpm, washed with sterile 0.9% (w/v) saline and adjusted to 3.3×10^9 CFU/mL corresponding to $OD_{600} = 0.67$ (Argayosa et al., 2024). Inactivation was performed with 1% (v/v) formalin, followed by repeated washing to ensure removal of residual formalin. Sterility was confirmed by in vitro culture tests (Sughra et al., 2021). Fish oil (Nutrifactor Laboratories Pvt. Ltd., Pakistan) and mineral oil (Montanide ISA 201 VG, Thermo Fisher Scientific, Waltham, MA, USA) were used as adjuvants, each added at a concentration of 10% (Mubeen et al., 2025b).

Vaccine-formulated feed

A commercial powdered feed containing 30% crude protein (Hi-Tech Feeds Mill Pvt. Ltd., Lahore, Pakistan) served as the basal diet. The inactivated bacterial suspension was mixed into the feed at a ratio of 1 L/kg, providing an estimated concentration of 10^9 cells/g (Monir et al., 2020). The prepared mixture was then pelletized using a mini-pelleting machine (GEMCO Model ZLSP150).

Experimental fish and design

Two hundred clinically healthy grass carp (*C. idella*; 20 ± 5 g) were obtained from the UVAS Fish Farm, Ravi Campus, Pattoki. Fish were acclimatized for 15 days on a commercial diet before the experiment. After acclimation, fish were randomly distributed into five groups with two replicates each: (i) spray vaccine + mineral oil (SV-MO), (ii) incorporated vaccine + mineral oil (IV-MO), iii) spray vaccine + fish oil (SV-FO), iv) incorporated vaccine + fish oil (IV-FO) and v) unvaccinated control (C). Fish were fed their assigned diets at 3% of body weight, twice daily, for a period of 2 months (Table 1 & Fig. 1).

Table 1. Experimental treatment groups of spray- and incorporation-based vaccine feeds

Treatment 1	Treatment 2	Treatment 3	Treatment 4	Treatment 5
Control (commercial feed)	Spray vaccine + mineral oil (SV-MO)	Incorporated vaccine + mineral oil (IV-MO)	Spray vaccine + fish oil (SV-FO)	Incorporated vaccine + fish oil (IV-FO)

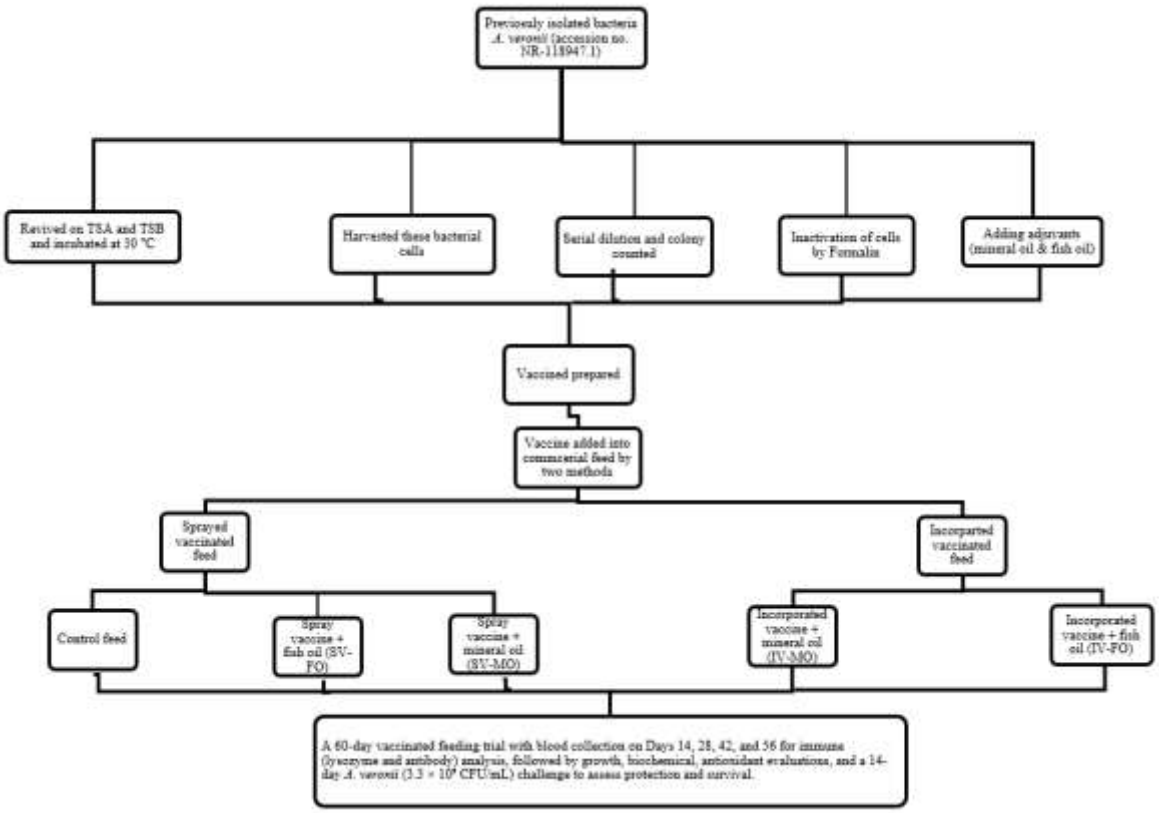

Figure 1. Schematic representation of experimental design for oral immunization of grass carp against *A. veronii.*

Feed quality tests

Before starting the trial, the experimental diets were subjected to proximate composition analysis for both feed and fish according to AOAC (2016). Palatability was examined following Dong et al. (2016), while diet safety was assessed as outlined by Abu-Nor et al. (2020). Additionally, the water stability of the prepared pellets was evaluated by Obaldo et al. (2002).

Growth performance

Growth indices including net weight gain (NWG), specific growth rate (SGR), and feed conversion ratio (FCR) were recorded at the end of the trial as described by Salas-Leiton et al. (2010).

Serum sampling

Blood was collected from ten randomly selected fish per treatment group, with two fish processed simultaneously. Blood samples were centrifuged at 6000 rpm for 20 min and the collected serum were preserved at –20 °C until subsequent analyses (Kaur et al., 2020).

Biochemical analysis

Serum biochemical indicators including total protein, albumin, and globulin were measured. Total protein levels were determined using the Biuret method (Reinhold, 1953), while albumin was quantified through the bromocresol green dye-binding method (Doumas et al., 1971), with absorbance recorded at 630 nm using a spectrophotometer. Globulin concentration was obtained by subtracting albumin values from total protein.

Determination of antioxidant activity

The activities of key antioxidant enzymes glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD) were determined following standard methods (Wendel, 1981; Aebi, 1984; Sun et al., 1988).

Immunological parameters

Immunological parameters were analyzed such as agglutination antibody titer test following the protocol of Swain et al. (2007) and serum lysozyme activity test followed by Ruckenstein & Zeng (1997).

Challenge test

To assess protective efficacy, fifteen fish from each group were intraperitoneally injected with 0.1 mL of *A. veronii* suspension (3.3×10^9 CFU/mL), following Argayosa et al. (2024). Mortality was monitored for 14 days post-challenge, and relative percent survival (RPS) was calculated using the formula proposed by Amend (1981):

$$RPS = 1 - [(vaccinated\ fish\ mortality\ \%age) \div (control\ fish\ mortality\%age)] \times 100$$

Statistical analysis

Values are presented as mean ± SD. Normal distribution of data was assessed using the Shapiro–Wilk test, and variance equality was checked via Levene's test. Non-parametric statistics were applied using the Kruskal–Wallis test. A one-way ANOVA based on a completely randomized design (CRD) was applied to identify treatment effects, followed by Duncan's Multiple Range Test (DMRT) for multiple comparisons where applicable. All statistical procedures were carried out in SPSS version 20.0, considering $p < 0.05$ as statistically significant (Duncan, 1955).

Results

Vaccinated feed quality

Feed quality assessment revealed that stability values were significantly higher ($p < 0.05$) in all vaccinated groups compared with the control. Palatability, measured through ingestion ratio, was also markedly improved in vaccinated groups, with the incorporated vaccine containing fish oil (IV-FO) showing the highest acceptance among fish. Following one week of feeding, microbiological examination of fish organs (gills, intestine, and mouth) was performed by swabbing and culturing on TSA plates. No bacterial growth was observed in any sample after 24 h incubation at 30 °C (Table 2).

Table 2. Water stability and feed palatability of control and vaccinated diets

Parameters	Treatment diets				
	C	SV-MO	IV-MO	SV-FO	IV-FO
Stability (%)	72.0±1.41^{e}	73.1±0.07^{d}	75.2±0.64^{c}	76.0±0.71^{b}	82.0±2.12^{a}
Palatability	0.45±0.02^{e}	0.48±0.01^{d}	0.54±0.02^{c}	0.63±0.02^{b}	0.84±0.01^{a}

Control (C); spray vaccine + mineral oil (SV-MO), incorporated vaccine + mineral oil (IV-MO), spray vaccine + fish oil (SV-FO), incorporated vaccine + fish oil (IV-FO). Results are shown as mean values ± SD, with n = 2 fish per group. One-way ANOVA was applied to determine statistical differences, and significance was accepted at $p < 0.05$. Groups with different superscript letters differ significantly.

Proximate analysis of feed

Feed proximate results revealed that crude protein was significantly evaluated ($p < 0.05$) in group IV-FO in comparison to the sprayed vaccinated and the control diet. Additionally, the crude fat content exhibited an increased level in the vaccinated diets than the control diet (Table 3).

Table 3. Proximate compositions of treatment diets

Parameters	Treatment diets				
	C	SV-MO	IV-MO	SV-FO	IV-FO
Crude protein (%)	30.2±0.21^{c}	32.2±0.21^{b}	32.8±0.15^{a}	31.60±0.84^{b}	33.30±0.49^{a}
Crude lipid (%)	4.20±0.28^{c}	5.7±0.01^{bc}	5.6±0.01^{c}	6.00±0.28^{a}	5.25±0.77^{b}
Moisture (%)	8.1±0.02^{a}	8.3±0.02^{a}	8.2±0.02^{a}	8.4±0.01^{a}	8.5±0.01^{a}
Ash (%)	10.1±0.14^{d}	12.2±0.02^{c}	12.1±0.01^{c}	12.8±0.13^{b}	13.4±0.01^{a}

Control (C); spray vaccine + mineral oil (SV-MO), incorporated vaccine + mineral oil (IV-MO), spray vaccine + fish oil (SV-FO), incorporated vaccine + fish oil (IV-FO). Results are shown as mean values ± SD, with n = 2 fish per group. One-way ANOVA was applied to determine statistical differences, and significance was accepted at $p < 0.05$. Groups with different superscript letters differ significantly.

Proximate compositions of experimental fish

Proximate compositions of the experimental fish indicated significant variation in crude protein content between vaccinated and control groups. All vaccinated groups showed elevated crude protein levels compared with the control, with the IV-FO group exhibiting the highest value (25.5 ± 2.80%, $p < 0.05$). The dietary treatments ranked in descending order for crude protein content as follows: IV-FO (25.5 ± 2.80) > SV-FO (24.1 ± 1.05) > IV-MO (22.0 ± 0.62) > SV-MO (20.5 ± 2.40) > Control (16.8 ± 0.45) (Table 4).

Table 4. Proximate compositions of experimental fish

Parameters	Experimental fish groups				
	C	SV-MO	IV-MO	SV-FO	IV-FO
Crude protein (%)	16.8 ± 0.45^{c}	20.5 ± 2.40^{bc}	22.0 ± 0.62^{ab}	24.1±1.05^{ab}	25.5 ± 2.80^{a}
Crude lipid (%)	2.1 ± 0.70^{ab}	2.5 ± 0.35^{ab}	1.5 ± 0.78^{b}	2.6 ± 0.82^{ab}	2.8 ± 0.16^{a}
Moisture (%)	1.2 ± 0.04^{a}	1.3 ± 0.06^{a}	1.1 ± 0.10^{a}	1.2 ± 0.09^{a}	1.2 ± 0.06^{a}
Ash (%)	76.8 ± 0.70^{b}	76.2 ± 0.72^{c}	78.9 ± 0.50^{ab}	77.4±0.52^{bc}	79.8 ± 0.45^{a}

Control (C); spray vaccine + mineral oil (SV-MO), incorporated vaccine + mineral oil (IV-MO), spray vaccine + fish oil (SV-FO), incorporated vaccine + fish oil (IV-FO). Results are shown as mean values ± SD, with n = 2 fish per group. One-way ANOVA was applied to determine statistical differences, and significance was accepted at $p < 0.05$. Groups with different superscript letters differ significantly.

Growth performance

The growth performance of fish fed with vaccinated feed was significantly better growth performance. The SGR significantly ($p < 0.05$) elevated during the 2-month trial in the vaccinated group IV-FO, which also showed the lowest FCR (1.16) (Table 5).

Table 5. Growth performance of vaccinated and control diets.

Parameters	Treatment diets				
	C	SV-MO	IV-MO	SV-FO	IV-FO
NWG (g)	4.6±0.14^{e}	7.5±0.07^{d}	9.7±0.05^{c}	10.4±0.21^{b}	12.7±0.15^{a}
SGR (%)	0.42±0.01^{e}	0.64±0.01^{d}	0.79±0.01^{c}	0.83±0.02^{b}	1.45±0.06^{a}
FCR	1.04±0.01^{a}	1.17±0.02^{b}	1.16±0.02^{b}	1.16±0.04^{b}	1.12±0.01^{b}

Control (C); spray vaccine + mineral oil (SV-MO), incorporated vaccine + mineral oil (IV-MO), spray vaccine + fish oil (SV-FO), incorporated vaccine + fish oil (IV-FO). Net weight gain (NWG); Specific growth rate (SGR); Feed conversion ratio (FCR). Results are shown as mean values ± SD, with n = 2 fish per group. One-way ANOVA was applied to determine statistical differences, and significance was accepted at $p < 0.05$. Groups with different superscript letters differ significantly.

Biochemical analysis

A serum total protein, albumin, and globulin test was performed on day 14th, 28th, 42^{th,} and 56th. All treatment groups showed higher levels of total protein than the control (Fig. 2). The IV-FO group had a significantly ($P < 0.05$) greater total protein value than the SV-FO group at day 56. The control group recorded the lowest value. Similarly, the treatment groups' albumin and globulin contents were considerably ($P < 0.05$) greater than the control.

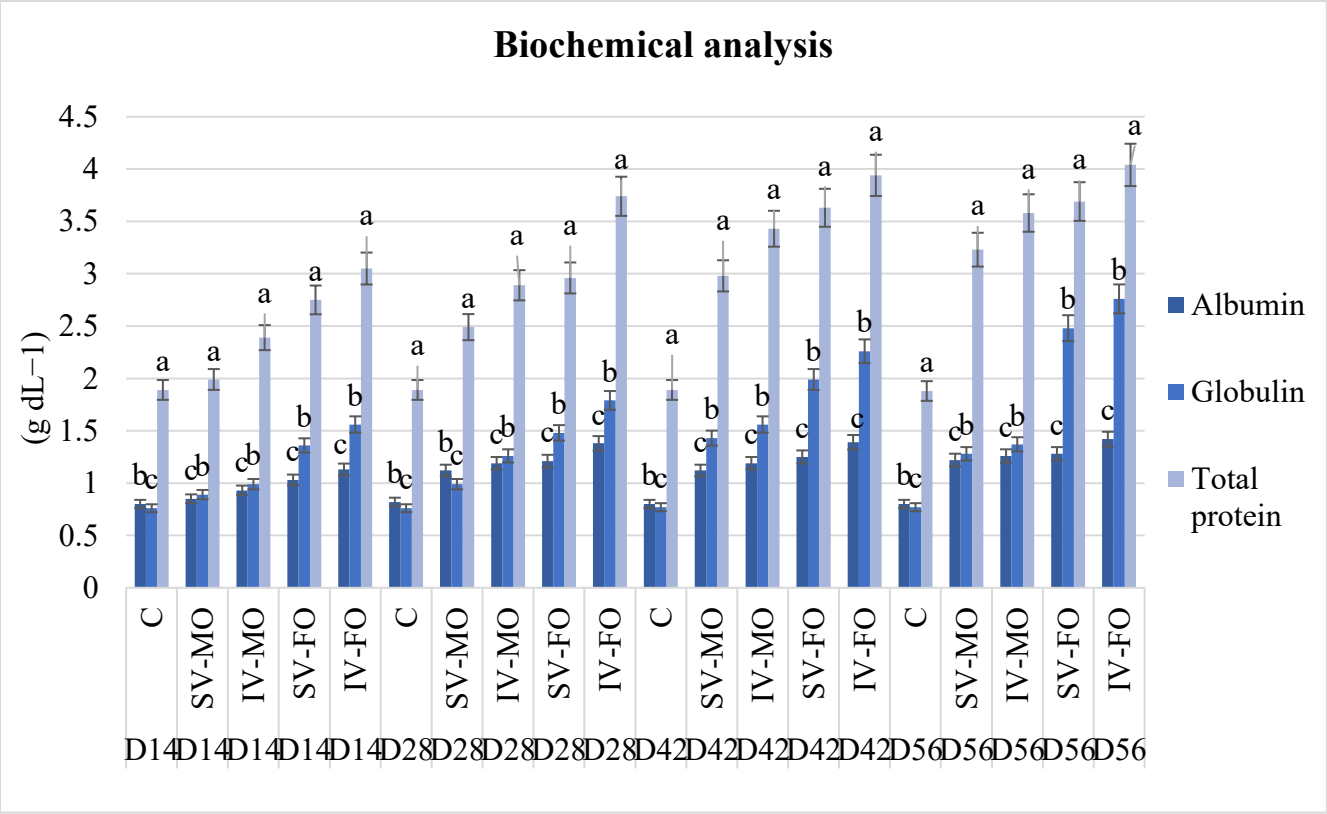

Figure 2. Graphical representation of biochemical analysis of *C. idella*. Results are shown as mean values ± SD, with n = 2 fish per group. Statistical differences were assessed by one-way ANOVA and differences between groups were considered significant when denoted by distinct superscript letters ($p < 0.05$).

Antioxidant enzyme activity

Antioxidant activity (SOD, GR, GPx, GST, CAT) was performed on days 14th, 28th, 42th and 56th. All treatment groups showed higher levels of SOD, GR, GPx, GST, and CAT compared to the control (Fig. 3). The group IV-FO had a significantly ($P < 0.05$) greater SOD, GR, GPx, GST, and CAT level than the SV-FO group at day 56. The control group recorded the lowest value of SOD, GR, GPx, GST, and CAT.

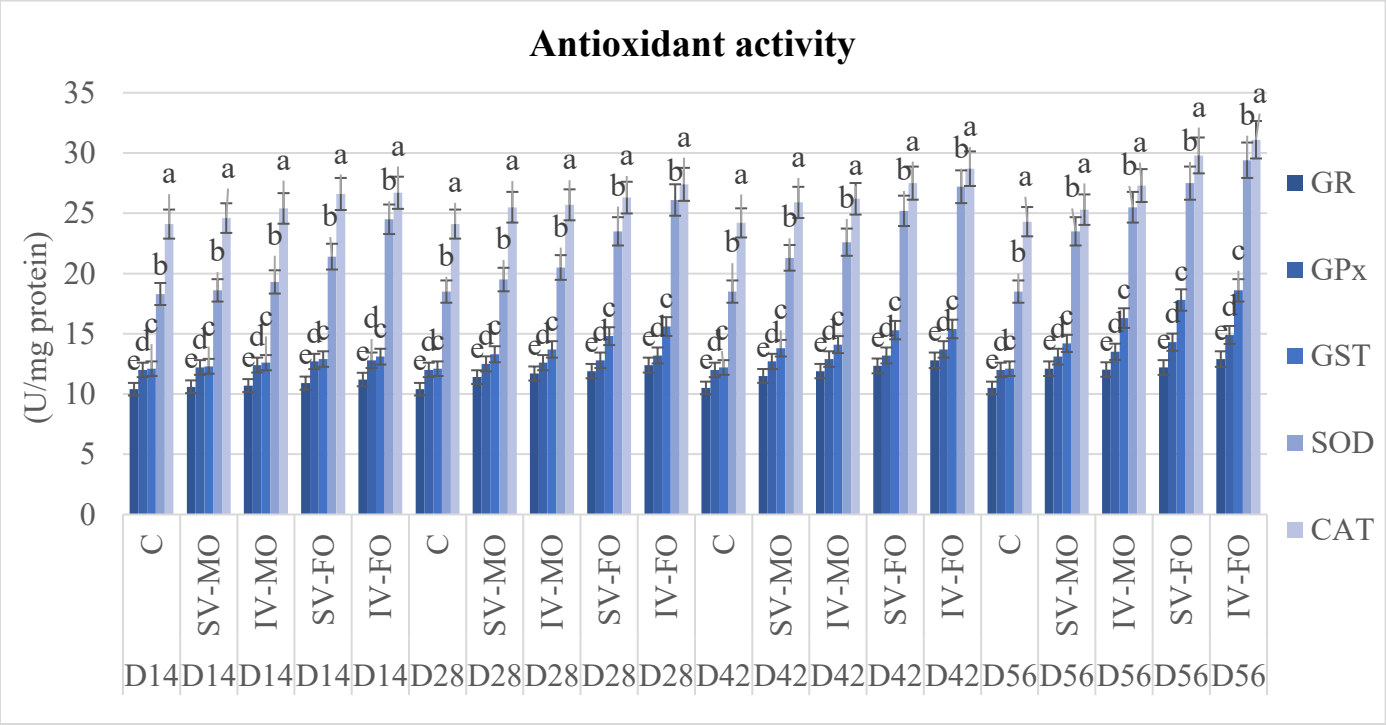

Figure 3. Graphical representation of antioxidant activity of *C. idella*. Results are shown as mean values ± SD, with n = 2 fish per group. Statistical differences were assessed using one-way ANOVA, and differences between groups were considered significant when denoted by distinct superscript letters ($p < 0.05$).

Agglutination antibody titer test

An antibody titer test conducted on day 14th, 28th, 42th and 56th revealed a progressive increase in mean antibody titer values over time. Vaccination diets exhibited significantly higher ($p < 0.05$) than to the control diet. Notably, the IV-FO group demonstrated the highest mean agglutination antibody titer value of 0.71 in *C. idella*, surpassing other vaccinated diets (Fig. 4).

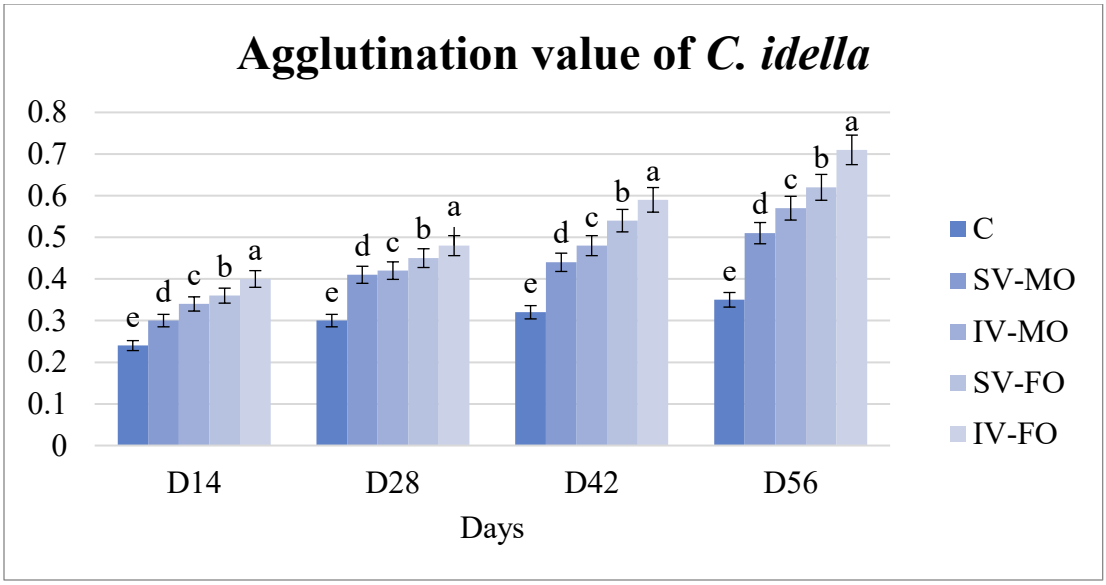

Figure 4. Graphical representation of the agglutination mean antibody titer of *C. idella*. Values are presented as mean ± SD, with n = 2 fish per group. Statistical differences were assessed using one-way ANOVA, and differences between groups were considered significant when denoted by distinct superscript letters ($p < 0.05$).

Serum lysozyme activity

A lysozyme activity test was performed on days 14th, 28th, 42^{th,} and 56th. The mean lysozyme activity values exhibited a gradual increase from 14th to 56th days. Lysozyme activity was notably higher ($p < 0.05$) in fish receiving vaccinated diets than in the control group. The incorporated vaccination diet differed significantly from the spray vaccination diet. When comparing diet IV-FO to the control, *C. idella* exhibited the highest mean lysozyme activity values (5760) within this group (Fig. 5).

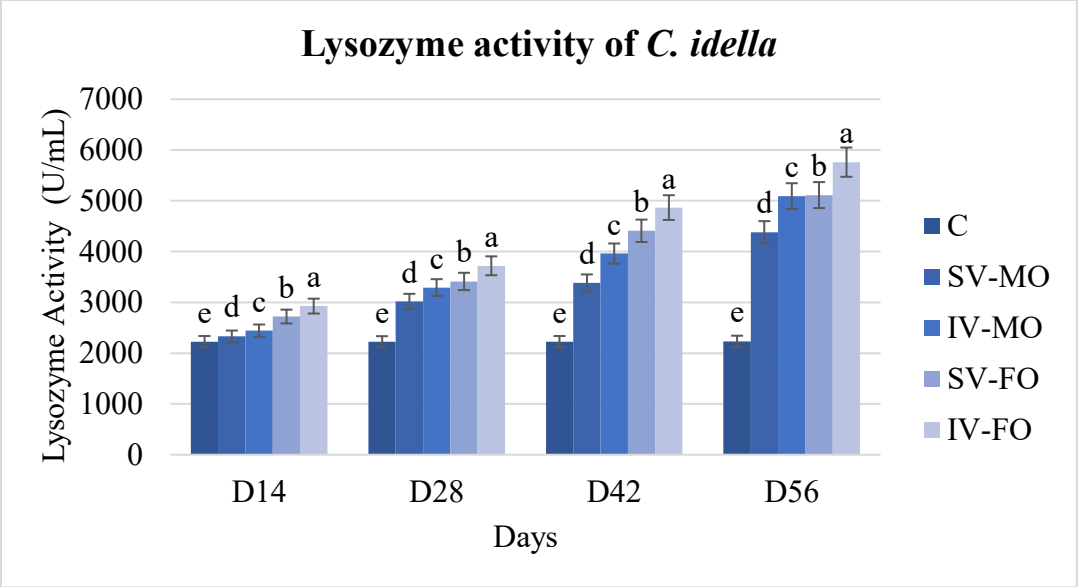

Figure 5. Graphical representation of the lysozyme activity of *C. idella*. Values are presented as mean ± SD, with n = 2 fish per group. Statistical differences were evaluated using one-way ANOVA, and differences between groups were considered significant when denoted by distinct superscript letters ($p < 0.05$).

Relative percentage survival rate

Fish were challenged with live *A. veronii* at 56 days post-vaccination and monitored for two weeks to record clinical signs such as dropsy, mortality, and other disease manifestations. On the 14th day post-challenge, the incorporated fish-oil vaccine group (IV-FO) exhibited the lowest mortality (13%), followed by SV-MO and SV-FO (33% each), and IV-MO (46%), and the control group recorded the highest mortality rate (86%) (Table 6).

Table 6. Relative percentage survival rate of *C. idella*

Treatments	Total fish	No. of dead	Protection (%)	Mortality (%)	RPS (%)
C	15	13	14	86	---
SV-MO	15	5	67	33	62
IV-MO	15	9	54	46	70
SV-FO	15	5	67	33	62
IV-FO	15	2	87	13	85

Control (C); spray vaccine + mineral oil (SV-MO), incorporated vaccine + mineral oil (IV-MO), spray vaccine + fish oil (SV-FO), incorporated vaccine + fish oil (IV-FO); Relative percentage survival rate (RPS).

Table 7. Comparative summary of growth, biochemical, immune, and survival responses of grass carp fed oral vaccines prepared with different adjuvants.

Parameter	Control	SV-MO	IV-MO	SV-FO	IV-FO
Feed Stability (%)	72.0 ± 1.41^{e}	73.1 ± 0.07^{d}	75.2 ± 0.64^{c}	76.0 ± 0.71^{b}	82.0 ± 2.12^{a}
Feed Palatability (g feed/fish)	0.45 ± 0.02^{e}	0.48 ± 0.01^{d}	0.54 ± 0.02^{c}	0.63 ± 0.02^{b}	0.84 ± 0.01^{a}
Crude Protein in Feed (%)	30.2 ± 0.21^{c}	32.2 ± 0.21^{b}	32.8 ± 0.15^{a}	31.6 ± 0.84^{b}	33.3 ± 0.49^{a}
Crude Protein in Fish (%)	16.8 ± 0.45^{c}	20.5 ± 2.40^{bc}	22.0 ± 0.62^{ab}	24.1 ± 1.05^{ab}	25.5 ± 2.80^{a}
Net weight gain (g)	4.6 ± 0.14^{e}	7.5 ± 0.07^{d}	9.7 ± 0.05^{c}	10.4 ± 0.21^{b}	12.7 ± 0.15^{a}
SGR (%)	0.42 ± 0.01^{e}	0.64 ± 0.01^{d}	0.79 ± 0.01^{c}	0.83 ± 0.02^{b}	1.45 ± 0.06^{a}
FCR	1.04 ± 0.01^{a}	1.17 ± 0.02^{b}	1.16 ± 0.02^{b}	1.16 ± 0.04^{b}	1.12 ± 0.01^{b}
Serum total protein (g dL^{-1})	3.8 ± 0.2^{d}	4.2 ± 0.3^{c}	4.5 ± 0.2^{b}	4.9 ± 0.1^{b}	5.4 ± 0.3^{a}
Agglutination antibody titer	0.35 ± 0.2^{e}	0.51 ± 0.3^{d}	0.57 ± 0.1^{c}	0.62 ± 0.2^{b}	0.71 ± 0.2^{a}
Lysozyme activity (U mL^{-1})	3180 ± 145^{e}	4210 ± 210^{d}	4930 ± 190^{c}	5110 ± 165^{b}	5760 ± 220^{a}
RPS (%)	—	62	70	62	85

The table summarizes the principal results across all treatment groups: growth, serum biochemistry, immune activity, and post-challenge survival to allow direct comparison of vaccine efficacy among adjuvant types and delivery methods.

Discussion

The increasing prevalence of antibiotic resistance highlights the urgency of identifying effective alternatives for the prevention of *Aeromonas* infections, particularly those caused by hypervirulent strains. Alternative strategies under investigation include probiotics, phytochemicals, bacteriophages, and vaccination. A wide array of vaccines has been designed for protection against motile *Aeromonas* septicemia (MAS), encompassing attenuated (Pridgeon et al., 2013), recombinant protein (Peepim et al., 2016), formalin-inactivated (Sukenda et al., 2017) and DNA vaccines (Thirumalaikumar et al., 2021). These immunization strategies are fundamental to enhancing host immune responses and reducing the impact of bacterial diseases (Abu-Elala et al., 2019). In the present research work, an oral inactivated vaccine targeting *A. veronii* in

Ctenopharyngodon idella was developed and administered at ~10^9 CFU/mL with fish oil incorporated as an adjuvant. The immunoprotective efficacy of the vaccine was subsequently assessed through challenge with live *A. veronii*.

Feed-based vaccine formulations must possess both high stability and palatability to ensure efficacy. Adequate feed intake is essential for maintaining growth performance and efficient antigen delivery, whereas reduced palatability may compromise both nutrition and vaccine uptake (Gencer, 2025). The bivalent vaccine pellets tested in this study demonstrated significantly greater palatability ($P<0.05$) compared to commercial tilapia feed, with no negative impact from palm oil inclusion, which has also been shown to enhance immune responses (Dong et al., 2016). In the current investigation, the monovalent vaccine exhibited higher palatability relative to the control, indicating its potential to improve ingestion and vaccine delivery.

Water stability of feed pellets is another critical parameter for aquaculture feed quality. Rapid disintegration may result in nutrient leaching, water quality deterioration, and reduced antigen delivery (Obaldo et al., 2002). The vaccine pellets in this study showed significantly greater stability ($P<0.05$) compared with the control feed, highlighting their suitability for maintaining pellet integrity and reducing environmental impacts (Table 2).

Proximate analysis revealed no significant differences in protein, lipid, carbohydrate, or ash contents between vaccinated and control feeds, although moisture content was slightly higher in vaccine pellets due to additional water used during the incorporation process (Table 3). Nevertheless, moisture levels remained within acceptable limits (Mohamad et al., 2021). Crude protein and lipid contents differences were observed, but ash and moisture content did not differ significantly.

The proximate composition analysis revealed significant differences in nutrient content between vaccinated and control groups, with the most notable improvement observed in crude protein levels. Fish receiving the incorporated fish-oil vaccine diet (IV-FO) exhibited the highest protein content, indicating that dietary vaccination not only enhanced immune performance but also promoted better nutrient assimilation (Mubeen et al., 2025b). Similar findings have been reported in carp and tilapia, where feed-based vaccines and functional diets improved protein deposition and nutrient utilization (Reyes et al., 2017; Monir et al., 2020;). Lipid levels also varied among the treatment groups, with higher lipid content in the IV-FO diet suggesting that fish oil acted as both an adjuvant and an additional energy source, supporting growth and immunity (Calder, 2017). Ash

content remained statistically comparable across groups, aligning with earlier studies that reported minimal vaccine-related influence on mineral deposition (Kumar et al., 2007). Moisture levels were slightly elevated in vaccinated feeds, likely due to the incorporation process, but remained within acceptable ranges for aquafeeds, consistent with observations by Mohamad et al. (2021). Overall, the proximate composition results confirm that oral vaccination, particularly with fish oil as an adjuvant, did not compromise the feed nutrient profile and, in fact, enhanced protein and lipid retention, thereby supporting both growth and immune responses in *C. idella* (Table 4).

Growth performance did not differ significantly ($P > 0.05$) between fish receiving vaccinated diets and those in the control group, indicating that vaccination did not compromise growth (Table 5). Similar results have been reported in Nile tilapia (Kahiesh-Esfandiari et al., 2019; Mamun et al., 2020). Some other researches have suggested possible growth reduction due to the metabolic costs of immune activation (Fraser et al., 2014), other reports support neutral or even positive growth effects following oral vaccination (Reyes et al., 2017). The incorporation of fish oil as an adjuvant may have supported the enhanced growth observed in vaccinated fish relative to controls.

Serum biochemistry further supported enhanced immune status. Vaccinated fish exhibited significantly higher levels of TP, globulin and albumin compared with controls (Fig. 2). These proteins play essential roles in non-specific defense, osmotic regulation, and immune protection (Kumar et al., 2007; Asadi et al., 2012). The observed increases suggest improved non-specific immune responses and enhanced serum bactericidal activity (Citarasu et al., 2006; Maqsood et al., 2009; Poline et al., 2023).

Antioxidant enzyme activities (SOD, CAT, and GPx) were also significantly elevated in vaccinated groups, reflecting improved oxidative stress regulation (Fig. 3). Similar trends have been documented in other fish species following vaccination against bacterial pathogens (Tkachenko et al., 2014; Jomova et al., 2023; Nasr-Eldahan et al., 2024). The highest antioxidant activities were recorded in the IV-FO group, underscoring the immunomodulatory role of fish oil.

Humoral immune parameters confirmed the immunogenicity of the oral vaccine. Antibody titers were significantly elevated ($P<0.05$) in the group (IV-FO) (Fig. 4), demonstrating effective antigen-specific antibody production against *A. veronii* (Monir et al., 2020). Lysozyme activity, a key component of the innate immune response, was also enhanced significantly ($P<0.05$) in all vaccinated fish against both *Streptococcus iniae* and *A. veronii* when compared with unvaccinated groups, consistent with earlier findings (Magnadóttir, 2006; Nayak et al., 2004). In *C. idella*,

lysozyme activity remained elevated from day 14 to day 56 post-vaccination, particularly in groups receiving fish oil adjuvants (Fig. 5).

Challenge experiments confirmed the protective efficacy of the vaccine. Survival rates following *A. veronii* challenge were 0% in the control, 62% in SV-MO, 70% in IV-MO, 62% in SV-FO, and 85% in IV-FO (Table 6). These results demonstrate superior protection conferred by oral feed-based vaccination, particularly when fish oil was used as an adjuvant. Protection rates exceeding 70% are generally considered effective (Chettri et al., 2015), aligning with prior reports on *Aeromonas* vaccination (Kalita et al., 2018; Anantasuk et al., 2024).

Environmental factors such as temperature, dissolved oxygen levels, and feed stability influenced the overall vaccine response observed in this study. Fluctuations in temperature can alter fish metabolism and immune efficiency, while variations in oxygen concentration can impose physiological stress that may interfere with antigen uptake or immune stimulation. Likewise, feed stability directly affects the persistence and bioavailability of the vaccine antigen, potentially impacting the degree of protection achieved. Therefore, future research should include continuous monitoring of environmental parameters during vaccination trials to better understand their relationship with immune and growth responses, thereby improving the reliability and reproducibility of results.

For on-farm use, the preparation and storage of vaccine-coated feed require careful attention to maintain antigen quality. Farmers are advised to prepare small quantities of vaccine-coated feed daily and keep it refrigerated or stored in shaded, cool places to minimize antigen degradation. Consistent feeding over several weeks before the onset of the disease season can help build stronger immunity in fish populations. Previous research has demonstrated that feed-based oral vaccines can maintain their stability and protective efficacy under varied storage conditions in *Oreochromis* sp. (Mohd Ali et al., 2024), and that encapsulated antigens remain active despite temperature and pH fluctuations in salmonids infected with *Piscirickettsia salmonis* (Sotomayor-Gerding et al., 2022). These findings support the practical use of oral vaccines as a reliable disease prevention strategy in aquaculture.

Conclusion

The results of this study confirm that oral feed-based vaccination is a practical and sustainable method for protecting fish against *A. veronii* infections. The vaccine improved growth, biochemical balance, antioxidant activity, immune response, and survival without negative effects

on fish health. The use of fish oil as an adjuvant further enhanced the immune response, demonstrating its potential as an affordable and easily applicable approach for farm-level disease control. These findings indicate that oral vaccination can be effectively adapted and scaled for freshwater aquaculture systems, reducing reliance on antibiotics and supporting environmentally sound production practices. Future research should aim to refine vaccine dosage, delivery methods, and feeding frequency to improve consistency and immune protection across different aquaculture environments and fish species. Long-term studies assessing immune-memory duration, feed stability, and cost-effectiveness is recommended to ensure practical application in commercial systems. The study's findings can also guide the development of evidence-based policies that encourage the use of oral vaccines within national aquaculture health frameworks. Such policies would promote sustainable production, minimize antibiotic dependence, and support biodiversity conservation through responsible aquaculture expansion.

References

Abu-Elala, N. M., Samir, A., Wasfy, M., & Elsayed, M. (2019). Efficacy of injectable and immersion polyvalent vaccine against streptococcal infections in broodstock and offspring of Nile tilapia (*Oreochromis niloticus*). Fish & Shellfish Immunology, 88, 293–300. * https://doi.10.1016/j.fsi.2019.02.042

Abu-Nor, N., Zamri Saad, M., Md Yasin, I. S., Salleh, A., Mustaffa Kamal, F., Matori, M. F., & Azmai, M. N. (2020). Efficacy of whole cell inactivated *Vibrio harveyi* vaccine against vibriosis in a marine red hybrid tilapia (*Oreochromis niloticus* × *O. mossambicus*) model. Vaccines, 8(4), 734. * https://doi.org/10.3390/vaccines8040734

Aebi, H. (1984). Catalase in vitro. In L. Packer (Ed.), Methods in enzymology (Vol. 105, pp. 121–126). Academic Press.

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

Amend, D. F. (1981). Potency testing of fish vaccines. Developments in Biological Standardization, 49, 447–454.

Anantasuk, N., Phurahong, T., Pumchan, A., Hirono, I., & Unajak, S. (2024). Molecular characterization and bivalent vaccine development of *Aeromonas hydrophila* and *Aeromonas veronii* in Nile tilapia (*Oreochromis niloticus*). Aquaculture, 590*,* 741042. * https://doi.org/10.1016/j.aquaculture.2024.741042Get rights and content

AOAC International. (2016). Official methods of analysis (20th ed., G. W. Latimer Jr., Ed.). AOAC International.

Argayosa, A. M., Santos, M. N., Argayosa, V. B., Pakingking, R. V., Jr., Buhian, W., Salvador, M. L., & Teh, R. E. (2024). Pathogenicity of *Aeromonas veronii* from Nile tilapia (*Oreochromis niloticus*) and efficacy of fish oral vaccine against motile aeromonad

septicemia in tank trials. Aquaculture, 4(3), 163–179. * https://doi.org/10.3390/aquacj4030012

Asadi, M. S., Mirvaghefei, A. R., Nematollahi, M. A., Banaee, M., & Ahmadi, K. (2012). Effects of watercress (*Nasturtium nasturtium*) extract on selected immunological parameters of rainbow trout (*Oncorhynchus mykiss*). Open Veterinary Journal, 2, 32–39.

Assane, I. M., Gozi, K. S., Valladão, G. M., & Pilarski, F. (2019). Combination of antimicrobials to reduce their use in aquaculture: Thiamphenicol/florfenicol against *Aeromonas hydrophila*. Aquaculture, 507, 238–245.

* https://doi.org/10.1016/j.aquaculture.2019.04.021

Assefa, A., & Abunna, F. (2018). Maintenance of fish health in aquaculture: Review of epidemiological approaches for prevention and control. Veterinary Medicine International, 2018, 5432497. * https://doi.org/10.1155/2018/5432497

Aucouturier, J., Dupuis, L., & Ganne, V. (2001). Adjuvants designed for veterinary and human vaccines. Vaccine, 19(17), 2666–2672. * https://doi.org/10.1016/S0264-410X(00)00498-9

Bowman, J. P. (2005). Genus I. *Aeromonas*. In D. J. Brenner, N. R. Krieg, J. T. Staley, & G. M. Garrity (Eds.), Bergey's manual of systematic bacteriology (2nd ed.). Springer.

Calder, P. C. (1998). Immunoregulatory and anti-inflammatory effects of n-3 polyunsaturated fatty acids. Brazilian Journal of Medical and Biological Research, 31, 467–490. * https://doi.org/10.1590/S0100-879X1998000400002

Chettri, J. K., Jaafar, R. M., Skov, J., Kania, P. W., Dalsgaard, I., & Buchmann, K. (2015). Booster immersion vaccination using diluted *Yersinia ruckeri* bacterin confers protection against ERM in rainbow trout. Aquaculture, 440, 1–5.

* https://doi.org/10.1016/j.aquaculture.2015.01.027

Citarasu, T., Sivaram, V., Immanuel, G., Rout, N., & Murugan, V. (2006). Influence of selected Indian immunostimulant herbs against white spot syndrome virus (WSSV) infection in black tiger shrimp *Penaeus monodon* with reference to haematological, biochemical and immunological changes. Fish & Shellfish Immunology, 21(4), 372–384. * https://doi.org/10.1016/j.fsi.2006.01.002

Cui, H., Hao, S., & Arous, E. (2007). A distinct cause of necrotizing fasciitis: *Aeromonas veronii* biovar sobria. Surgical Infections, 8(5), 523–528. * https://doi.org/10.1089/sur.2006.04

Dong, C., He, G., Mai, K., Zhou, H., & Xu, W. (2016). Palatability of water-soluble extracts and fishmeal replacement for juvenile turbot (*Scophthalmus maximus*). Journal of Ocean University of China, 15(3), 561–567. * https://doi.10.1007/s11802-016-2898-8

Doumas, B. T., Watson, W., & Biggs, H. G. (1971). Albumin standards and measurement of serum albumin with bromocresol green. Clinica Chimica Acta, 31(1), 87–96. * https://doi.10.1016/0009-8981(71)90365-2

Duncan, D. B. (1955). Multiple range and multiple F tests. Biometrics, 11(1), 1–42. * https://doi.org/10.2307/3001478

Farias, T. H., Arijo, S., Medina, A., Pala, G., da Rosa Prado, E. J., Montassier, H. J., & de Andrade Belo, M. A. (2020). Immune responses induced by inactivated vaccine against *Aeromonas hydrophila* in pacu (*Piaractus mesopotamicus*). Fish & Shellfish Immunology, 101, 186–191. * https://doi.10.1016/j.fsi.2020.03.059

Firdaus Nawi, M., Yusoff, S. M., Yusof, H., Abdullah, S. Z., & Zamri Saad, M. (2014). Efficacy of feed-based adjuvant vaccine against *Streptococcus agalactiae* in *Oreochromis* spp. in Malaysia. Aquaculture Research, 45(1), 87–96. *https://doi.10.1111/j.1365-2109.2012.03207.x

Fraser, T. W., Hansen, T., Mayer, I., Skjæraasen, J. E., Glover, K. A., Sambraus, F., & Fjelldal, P. G. (2014). The effect of triploidy on vaccine side-effects in Atlantic salmon. Aquaculture, 433, 481–490. * https://doi.org/10.1016/j.aquaculture.2014.07.009

Gencer, Ö. (2025). Dietary innovation in blue crab aquaculture: Effects on performance and product quality. Aquaculture International, 33(3), 1–12. * https://doi. 10.1007/s10499-025-01899-9

Han, B., Xu, K., Liu, Z., Ge, W., Shao, S., Li, P., Yan, N., Li, X., & Zhang, Z. (2018). Oral yeast-based DNA vaccine confers effective protection from *Aeromonas hydrophila* infection on *Carassius auratus*. Fish & Shellfish Immunology, 84, 948–954. *https://doi.org/10.1016/j.fsi.2018.10.065

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

Jomova, K., Raptova, R., Alomar, S. Y., Alwasel, S. H., Nepovimova, E., Kuca, K., & Valko, M. (2023). Reactive oxygen species, toxicity, oxidative stress, and antioxidants: Chronic diseases and aging. Archives of Toxicology, 97(10), 2499–2574. * https:/doi.org/ 10.1007/s00204-023-03562-9

Kahiesh Esfandiari, M., Ina-Salwany, M., Aslah, M., Soltani, M., Sabili, A., Karimi, M., & Muthukrishnan, S. (2019). Growth performance, palatability and water stability of oral feed-based vaccines against *Streptococcus agalactiae* in red tilapia (*Oreochromis* sp.). Journal of Biochemical Technology, 10(2), 106–115.

Kalita, B., Pokhrel, H., Hussain, I. A., & Mohan, C. V. (2018). Kinetics of antibody titers and protection in Indian major carps to *Aeromonas hydrophila* antigen. International Journal of Current Microbiology and Applied Sciences, 7(9), 1461–1469. * https://doi.org/10.20546/ijcmas.2018.709.175

Kaur, A., Holeyappa, S. A., Bansal, N., Kaur, V. I., & Tyagi, A. (2020). Ameliorative effect of turmeric supplementation in feed of *Labeo rohita* challenged with pathogenic *Aeromonas veronii*. Aquaculture International, 28(3), 1169–1182. * https://doi.org/10.1007/s10499-020-00518-z

Kumar, V., Sahu, N. P., Pal, A. K., & Kumar, S. (2007). Immunomodulation of *Labeo rohita* juveniles due to dietary gelatinized and non-gelatinized starch. Fish & Shellfish Immunology, 23*(*2), 341–353. * https://doi.org/10.1016/j.fsi.2006.11.008

Legario, F. S., Choresca, C. H., Jr., Turnbull, J. F., & Crumlish, M. (2020). Isolation and molecular characterization of streptococcal species from farmed Nile tilapia in the Philippines. Journal of Fish Diseases, 43(12), 1431–1442. * https://doi.org/10.1111/jfd.13247

Magnadóttir, B. (2006). Innate immunity of fish: Overview. Fish & Shellfish Immunology, 20(2), 137–151. * https://doi.org/10.1016/j.fsi.2004.09.006

Mahmood, S., Rasool, F., Parveen, S., Ayub, A., Manzoor, K., Latif, M. D., Hussain, T., Younas, A., Rabbani, G., Hidait, N., & Anjum, K. M. (2024). Prevalence, antimicrobial profiling and molecular characterization of antimicrobial resistant genes of pathogenic bacteria detected in *Channa marulius* of the Indus riverine system in Pakistan. Pakistan Veterinary Journal, 44(4). . * https://doi.org/ 10.29261/pakvetj/2024.xxx

Mamun, M., Nasren, S., Abhiman, P., Rathore, S., Sowndarya, N., Ramesh, K., & Shankar, K. (2020). Effect of biofilm of *Aeromonas hydrophila* oral vaccine on growth and tissue histopathology in striped catfish. Indian Journal of Animal Research, 54(4), 563–569. * https://doi. 10.18805/ijar.B-3814

Manzoor, K., Rasool, F., Khan, N., Anjum, K. M., & Parveen, S. (2023). Prevalence and molecular detection of *Edwardsiella tarda* in cultured tilapia species of fish farms of Punjab in Pakistan and their postmortem examination. Pakistan Veterinary Journal, 43(2), 309–314. * https://doi.10.29261/pakvetj/2023.015

Maqsood, S., Samoon, M. H., & Singh, P. (2009). Immunomodulatory and growth promoting effect of dietary levamisole in *Cyprinus carpio* fingerlings against the challenge of *Aeromonas hydrophila*. Turkish Journal of Fisheries and Aquatic Sciences, 9(1), 111–120.

Matsuura, Y., Terashima, S., Takano, T., & Matsuyama, T. (2019). Current status of fish vaccines in Japan. Fish & Shellfish Immunology, 95, 236–247. * https://doi.org/10.1016/j.fsi.2019.09.031

Mohamad, A., Zamri-Saad, M., Amal, M. N. A., Al-Saari, N., Monir, M. S., Chin, Y. K., & Md Yasin, I. S. (2021). Vaccine efficacy of a feed-based polyvalent vaccine against vibriosis, streptococcosis and MAS in Asian seabass (*Lates calcarifer*). Vaccines, 9(4), 368. * https://doi.10.3390/vaccines9040368

Mohd Ali, N. S., Ngalimat, M. S., Zamri-Saad, M., Azmai, M. N. A., Salleh, A., Zulperi, Z., & Md Yasin, I. S. (2024). Stability characterizations of feed-based bivalent vaccine containing inactivated *Streptococcus agalactiae* and *Aeromonas hydrophila* against streptococcosis and *Aeromonas* infections in red hybrid tilapia (*Oreochromis* sp.). Archives of Microbiology, 206(11):444. *https:// https://doi.10.1007/s00203-024-04166-2

Monir, M. S., Yusoff, S. B., Zulperi, Z. B., Hassim, H. B., Mohamad, A., Ngoo, M. S., & Ina-Salwany, M. Y. (2020). Haemato-immunological responses and feed-based bivalent vaccine effectiveness against *Streptococcus iniae* and *Aeromonas hydrophila* in hybrid red tilapia. BMC Veterinary Research, 16, 1–4. * https://doi.10.1186/s12917-020-02443-y

Mubeen, N., Abbas, F., Hafeez-ur-Rehman, M., Amin, I., Jalil, S., & Can, E. (2025a). Immunopotential induced in culturable carps fed on edible bivalent vaccine against *Aeromonas veronii* and *Aeromonas hydrophila*. Aquaculture Research, 2025(1), 3462669. * https://doi.org/10.1155/are/3462669

Mubeen, N., Abbas, F., Hafeez-ur-Rehman, M., Crumlish, M., Mahboob, H., Akmal, M., Sadiqa, A., Alam, T.M. & Jalil, S. (2025b). Effectiveness of feed-based monovalent *Aeromonas* vaccine in farmed carp. Microorganisms, 13(8), 1903. * https://doi.org/10.3390/microorganisms13081903

Nasr-Eldahan, S., Attia Shreadah, M., Maher, A. M., El-Sayed Ali, T., & Nabil-Adam, A. (2024). New vaccination approach using formalin-killed *Streptococcus pyogenes* vaccine on the liver of *Oreochromis niloticus* fingerlings. Scientific Reports, 14(1), 18341. * https://doi.org/10.1038/s41598-024-67198-0

Nayak, D. K., Mishra, B. K., & Mishra, K. P. (2004). Effects of some pesticides on carbohydrate metabolism of the air-breathing fish *Clarias batrachus*. Journal of Applied Sciences and Environmental Management, 8(1), 59–62.

Obaldo, L. G., Divakaran, S., & Tacon, A. G. (2002). Method for determining physical stability of shrimp feeds in water. Aquaculture Research, 33(5), 369–377. * https://doi.org/10.1046/j.1365-2109.2002.00681.x

Paul, P., Adikesavalu, H., Banerjee, S., & Abraham, T. J. (2015). Antibiotic resistant motile aeromonads induced septicemia in Philippine catfish *Clarias batrachus* (Linnaeus, 1758). Croatian Journal of Fisheries, 73(4), 170–175. * https://doi.org/10.14798/73.4.844

Peepim, T., Dong, H., Senapin, S., Khunrae, P., & Rattanarojpong, T. (2016). Epr3: Conserved immunogenic protein among *Aeromonas* spp. inducing antibody response in Nile tilapia. Aquaculture, 464, 399–409. * https://doi.org/10.1016/j.aquaculture.2016.07.022

Poline, M. E., Babila Jasmine, J., Annalakshmi, T., Xavier Innocent, B., & Krishnamoorthy, S. (2023). Efficacy of *Aeromonas hydrophila* vaccine on biochemical parameters of freshwater fish *Channa striatus*. International Journal of Zoological Investigations, 9(2), 810–820. * https://doi.10.33745/ijzi.2023.v09i02.091

Pridgeon, J. W., Klesius, P. H., & Yildirim-Aksoy, M. (2013). Development of live attenuated bacterial vaccines via resistance selection. Vaccine, 31(18), 2222–2230. * https://doi.org/10.1016/j.vaccine.2013.03.004

Queipo-Ortuño, M. I., De Dios Colmenero, J., Macias, M., Bravo, M. J., & Morata, P. (2008). Boiling method for bacterial DNA template preparation and PCR inhibition by IgG. Clinical and Vaccine Immunology, 15(2), 293–296. * https://doi.org/10.1128/CVI.00270-07

Rahman, M. M., Somsiri, T., Tajima, K., & Ezura, Y. (2004). Distribution of *Aeromonas* spp. in aquatic animals in Southeast Asia. Pakistan Journal of Biological Sciences, 7(2), 258–268. * http://doi.org/10.3923/pjbs.2004.258.268

Rasmussen-Ivey, C. R., Hossain, M. J., Odom, S. E., Terhune, J. S., Hemstreet, W. G., Shoemaker, C. A., Zhang, D., Xu, D. H., Griffin, M. J., Liu, Y. J., Figueras, M. J., Santos, S. R., Newton,

J. C., & Liles, M. R. (2016). Classification of a hypervirulent *Aeromonas hydrophila* pathotype responsible for epidemic outbreaks in warm-water fishes. Frontiers in Microbiology, 7, 1615. *https:// doi: 10.3389/fmicb.2016.01615

Reinhold, J. G. (1953). Manual for determination of serum total protein, albumin and globulin fractions by Biuret method. In M. Reiner (Ed.), Standard Methods of Clinical Chemistry (Vol. 1, pp. 88–89). Academic Press.

Reyes, M., Ramírez, C., Ñancucheo, I., Villegas, R., Schaffeld, G., Kriman, L., Gonzalez, J., & Oyarzun, P. (2017). Oral DNA vaccination against IPNV using in-feed delivery provides protection in Atlantic salmon. Vaccine, 35(4), 626–632. * https://doi.org/10.1016/j.vaccine.2016.12.013

Rohani, M. F., Islam, S. M., Hossain, M. K., Ferdous, Z., Siddik, M. A., Nuruzzaman, M., Padeniya, U., Brown, C., & Shahjahan, M. (2022). Probiotics, prebiotics and synbiotics improve aquafeed functionality and fish performance. Fish & Shellfish Immunology, 120, 569–589. * https://doi.org/10.1016/j.fsi.2021.12.037

Ruckenstein, E., & Zeng, X. (1997). Macroporous chitin affinity membranes for lysozyme separation. Biotechnology and Bioengineering, 56(6), 610–617. * https://doi.org/10.1002/(SICI)1097-0290(19971220)56:6<610::AID-BIT3>3.0.CO;2-Q

Salas-Leiton, E., Anguis, V., Martín-Antonio, B., Crespo, D., Planas, J. V., Infante, C., Cañavate, J. P., & Manchado, M. (2010). Effects of stocking density and feeding on growth and immunity gene expression in Senegalese sole. Fish & Shellfish Immunology, 28(2), 296–302. * https://doi.org/10.1016/j.fsi.2009.11.006

Singh, V., Chaudhary, D. K., & Mani, I. (2012). Molecular characterization and modeling of 16S rRNA secondary structure from *Aeromonas veronii*. International Journal of Applied Biology and Pharmaceutical Technology, 3(1), 253–260.

Sotomayor-Gerding, D., Troncoso, J. M., Díaz-Riquelme, K., Torres-Obreque, K. M., Cumilaf, J., Yañez, A. J., & Rubilar, M. (2022). Microencapsulation of *Piscirickettsia salmonis* antigens for fish oral immunization: optimization and stability studies. Polymers, 14(23), 5115. * https://doi.org/10.3390/polym14235115

Sughra, F., Hafeez-ur-Rehman, M., Abbas, F., Altaf, I., Aslam, S., Ali, A., Khalid, M., Mustafa, G., & Azam, S. M. (2021). Evaluation of oil-based inactivated *Aeromonas hydrophila* vaccine in Indian major carps: Immune response, challenge, growth and histopathology. Aquaculture Reports, 21, 100885. * https://doi.org/10.1016/j.aqrep.2021.100885

Sukenda, Carman, O., Rahman, R., Hidayatullah, D., & Yumaidawati, N. (2017). Vaccination of Nile tilapia broodstock with whole cell vaccine and fry resistance to *Aeromonas hydrophila*. Indonesian Journal of Aquaculture, 16(2), 268–276. *https://doi.org/10.19027/jai.16.2.268-276

Sun, Y. I., Oberley, L. W., & Li, Y. (1988). A simple method for clinical assay of superoxide dismutase. Clinical Chemistry, 34(3), 497–500. *https://doi.org/10.1093/clinchem/34.3.497

Swain, P., Dash, S., Sahoo, P. K., Routray, P., Sahoo, S. K., Gupta, S. D., Meher, P. K., & Sarangi, N. (2007). Non-specific immune parameters of brood *Labeo rohita* and seasonal variation. Fish & Shellfish Immunology, 22(1–2), 38–43. * https://doi.org/10.1016/j.fsi.2006.03.010

Thirumalaikumar, E., Lelin, C., Sathishkumar, R., Vimal, S., Anand, S. B., Babu, M. M., & Citarasu, T. (2021). Oral delivery of pVAX-OMP and pVAX-hly DNA vaccine using Cs-TPP nanoparticles in *Labeo rohita* against *Aeromonas hydrophila.* Fish & Shellfish Immunology, 115*,* 189–197. * https://doi.org/10.1016/j.fsi.2021.06.004

Tkachenko, H., Kurhaluk, N., Grudniewska, J., & Andriichuk, A. (2014). Tissue-specific responses of oxidative stress biomarkers and antioxidant defenses in rainbow trout *Oncorhynchus mykiss* during a vaccination against furunculosis. Fish Physiology and Biochemistry, 40(4), 1289–1300. * https://doi. 10.1007/s10695-014-9924-9

Ullah, M., Rasool, F., Khan, N., Ali, S., & Sheikh, A. A. (2023). Antibiotic resistance and its gene profile in *Escherichia coli* isolated from diseased farm-raised carps in Punjab, Pakistan. Pakistan Veterinary Journal, 43(3), 470–476. * https://doi.10.29261/pakvetj/2023.041

Wang, Q. C., Ji, W., & Xu, Z. (2020). Current use and development of fish vaccines in China. Fish & Shellfish Immunology, 96*,* 223–234. * https://doi.org/10.1016/j.fsi.2019.12.010

Watts, J. E., Schreier, H. J., Lanska, L., & Hale, M. S. (2017). Rising antimicrobial resistance in aquaculture: Sources, sinks and solutions. Marine Drugs, 15(6), 158. * https://doi.org/10.3390/md15060158

Wendel, A. (1981). Glutathione peroxidase. *In* Methods in Enzymology (Vol. 77, pp. 325–333). Academic Press.