

Article

Protective Efficacy of Recombinant Glyceraldehyde-3-Phosphate Dehydrogenase Vaccine Against *Vibrio alginolyticus* in Asian Seabass (*Lates calcarifer*)

Mangottil Ayyappan Pradeep ^{1,*} , Thevanattil Sairanksha Azhar Shahansha ¹ , Cherammpillil Sukumaran Subin ¹ , Gokhlesh Kumar ^{1,*} , Chalil George Joshy ² , Nikathil Raveendranathan Dhanutha ¹, Thaliyil Veetil Arun Kumar ¹ , Esha Arshad ¹, Nidhiya Kottayi ¹, Nivya Attadappa ¹ and Nadiyath Karayi Sanil ¹

¹ Marine Biotechnology, Fish Nutrition and Health Division, Indian Council of Agricultural Research—Central Marine Fisheries Research Institute, Kochi 682018, India

² ICAR-Central Institute of Fisheries Technology, Matsyapuri, Kochi 682029, India

* Correspondence: pradeep.ma@icar.org.in (M.A.P.); gokhlesh.kumar@icar.org.in (G.K.)

Abstract

Vibriosis caused by *Vibrio alginolyticus* is a major bacterial disease in Asian seabass (*Lates calcarifer*) farming and leads to significant economic losses. The overuse of antibiotics has contributed to antimicrobial resistance, so there is a need for an effective prevention and control method, such as a vaccine against *V. alginolyticus*. In this study, we developed and evaluated formulations of a recombinant glyceraldehyde-3-phosphate dehydrogenase (rGAPDH) subunit vaccine against *V. alginolyticus* in Asian seabass. The GAPDH gene of *V. alginolyticus* was expressed in ClearColi BL21(DE3) *Escherichia coli* cells, and the 37 kDa rGAPDH protein was purified. Fish in the V1–V4 groups were intraperitoneally immunized with purified 37 kDa rGAPDH protein formulations comprising 5 µg rGAPDH without Algel (V1), 5 µg rGAPDH with Algel (V2), 10 µg rGAPDH without Algel (V3), and 10 µg rGAPDH with Algel (V4). Vaccinated fish exhibited significantly enhanced immune responses compared to the control group. The 10 µg adjuvanted formulation induced the highest serum agglutination titer at 14 days post-vaccination (DPV), which was sustained through 21 DPV, together with the greatest serum lysozyme and bactericidal activities. Expression analysis of complement factor H, cystatin B, HSP70, and HSP90α revealed tissue-specific and temporal transcriptional changes following vaccination, with distinct response patterns across vaccine formulations. Following challenge with *V. alginolyticus*, the V4 group achieved 100% relative percent survival (RPS), whereas V1, V2, and V3 groups provided RPS values of 39.47%, 63.16%, and 63.16%, respectively. These findings demonstrate that the 10 µg rGAPDH formulation containing aluminum hydroxide conferred complete protection following experimental challenge with *V. alginolyticus* under the conditions tested. The developed 37 kDa rGAPDH vaccine could serve as a promising and effective vaccine candidate for preventing vibriosis in Asian seabass farming.

Keywords: vibriosis; recombinant GAPDH vaccine; aquaculture



Academic Editor: Hasan Tekedar

Received: 24 August 2026

Revised: 20 October 2026

Accepted: 23 October 2026

Published: 25 October 2026

Copyright: © 2026 by the authors.

Licensee MDPI, Basel, Switzerland.

This article is an open access article distributed under the terms and conditions of the [Creative Commons Attribution \(CC BY\)](https://creativecommons.org/licenses/by/4.0/) license.

1. Introduction

Asian seabass (*Lates calcarifer*, Bloch 1790) is a commercially important marine finfish extensively cultured in Southeast Asia, South Asia, and Australia [1]. Asian seabass is the flagship candidate species for open-sea cage culture in India. Asian seabass mariculture

is a key pillar of the Government of India's Blue Economy. Open cage mariculture is a common method in the farming of fish, which involves the use of net cages placed in coastal, inshore, or offshore waters so that there is a constant exchange of water between the farming area and the surrounding environment. This system allows for the efficient use of marine resources and is now being used more and more for the production of marine finfish, such as Asian seabass. The fish kept in open cages, however, are constantly exposed to variations in the environment and to naturally occurring microorganisms, which may raise the risk of infectious diseases and, as a result, demand effective health management and preventive actions [2,3]. Bacterial diseases are a major constraint on the productivity and profitability of Asian seabass aquaculture. Among them, vibriosis caused by *Vibrio alginolyticus* is one of the most economically damaging infections. Infected fish show ulcerative skin lesions, exophthalmia, hemorrhagic septicemia, and high mortality rates. This bacterial infection causes substantial economic losses for marine fish farmers and the aquaculture industry [1,4,5]. Historically, vibriosis management in intensive aquaculture operations has depended heavily on broad-spectrum antibiotic treatment [6]. However, the use of antibiotics has generated multiple concerns such as dissemination of antibiotic-resistant bacteria, environmental contamination of aquatic ecosystems, and the accumulation of antibiotic residues in fish tissues, all of which present a threat to consumers and public health [7]. The rapid global spread of multidrug-resistant *Vibrio* strains has been documented from aquaculture environments. Given this, there is an urgent need to look into safer alternatives [8].

Vaccination is one of the most sustainable and biosafe methods for the management of infectious diseases in aquaculture [9]. Of the different vaccine technologies that have been developed, recombinant subunit vaccines have drawn a great deal of attention because of their good safety record and the high specificity of the immune response they trigger. Unlike other types, recombinant vaccines do not revert to a virulent form and are suitable for use with modern adjuvant systems [10]. On the other hand, inactivated whole-cell bacterins remain the main method used in commercial fish vaccination, although their performance varies by host species and between production batches, and they include extraneous microbial components that may contribute to reactogenicity [11]. Recent studies have shown that recombinant aquaculture vaccines with immunostimulatory compounds work better than those without them. They lead to higher antibody levels and better survival rates after exposure to pathogens [12].

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a highly conserved, multi-functional glycolytic enzyme that also functions as an extracellular moonlighting protein involved in host cell adhesion, plasminogen binding, and host immune modulation [13]. Over the past two decades, GAPDH has been extensively validated as a cross-protective candidate antigen against multiple teleost bacterial infections. Following the demonstration by Liu et al. [14] of GAPDH-mediated protection in Japanese flounder (*Paralichthys olivaceus*) against *Edwardsiella tarda*, numerous investigations have confirmed its protective value against other aquatic pathogens, including *Vibrio harveyi* in large yellow croaker (*Pseudosciaena crocea*) [15] and grouper (*Epinephelus coioides*) [16], *E. ictaluri* in tilapia [17], and *V. alginolyticus* in model organisms such as zebrafish (*Danio rerio*) [18]. Although these studies clearly show that GAPDH serves as an effective immunogenic target in teleosts, the effectiveness of subunit vaccines in aquaculture still depends greatly on the specific immune response of the host, the proper dosage of the antigen, and the use of adjuvant delivery systems tailored to the species [10,11]. To date, there has been no systematic attempt to optimize the formulation and test a recombinant GAPDH subunit vaccine against *V. alginolyticus* in Asian seabass, a species of great economic importance in marine cage farming. For this reason, rather than concentrating on the discovery of new

antigens, the present study was undertaken to optimize the vaccine formulation and to evaluate the species-specific protective efficacy of a recombinant GAPDH (rGAPDH) vaccine against *V. alginolyticus* in Asian seabass. More specifically, the aims of the study were to (i) produce and purify the full-length rGAPDH protein from a virulent strain of *V. alginolyticus*; (ii) optimize the vaccine formulations at two antigen concentrations (5 µg and 10 µg) with or without the use of aluminum hydroxide as an adjuvant; (iii) examine the protective efficacy and survival rates after challenging the fish experimentally with *V. alginolyticus*. Additionally, four immune-related genes were selected to provide complementary information on distinct components of the host response to vaccination. Complement factor H (CFH) was examined as a regulator of complement activation and innate antibacterial defence; cystatin B was selected because of its involvement in cysteine protease regulation, antigen processing, and immune-cell function; HSP70 and HSP90α were selected as molecular chaperones associated with cellular stress responses, antigen processing, and immune signalling.

2. Materials and Methods

2.1. Asian Seabass

Pathogen-free healthy Asian seabass (weight 15 ± 3 gm and length 10 ± 2 cm) were obtained from Rajiv Gandhi Centre for Aquaculture Seabass Hatchery, Nagapattinam, Tamil Nadu, India. Upon arrival at the laboratory, fish were disinfected with potassium permanganate to prevent disease transmission. After that, fish were distributed into 1000 L recirculating aerated tanks and acclimatized for four weeks at 28 ± 3 °C. Fish were maintained with continuous aeration, with pH recorded at 7.4 ± 0.52 , salinity at 33 ± 0.5 ‰, and dissolved oxygen at 6.6 ± 0.3 mg/L. Throughout the duration of the experiment, the fish were fed 5% of body weight with a commercial fish diet (Growel Nutrila Pvt Ltd., Andhra Pradesh, India). During the acclimatization period, the fish were maintained in sodium hypochlorite-treated filtered seawater, with regular water exchange and routine cleaning and disinfection of the tanks. Fish were regularly monitored for their swimming behaviour, feeding activity, external appearance, and any signs of disease and abnormality. In addition, fish were sampled at the end of acclimatization and examined externally and internally for gross lesions and infectious agents. Wet mounts of gill and skin were screened by light microscopy for ectoparasites, and the internal organs (liver, kidney, and spleen) were screened for bacterial and viral pathogens. Tissue homogenates were plated on thiosulfate citrate bile salts sucrose (TCBS) agar and tryptone soy agar supplemented with 2% NaCl and incubated at 30 °C for 48 h. Nucleic acids were extracted and assayed by PCR for *V. alginolyticus* and *V. harveyi* and by RT-PCR for betanodavirus. All fish were negative for the presence of pathogens. A description of the experimental design and vaccine development process is presented in a flowchart (Figure 1).

2.2. Bacterial Strain and Culture Conditions

A pathogenic strain of *V. alginolyticus* CMFRI-JF260912, previously isolated and preserved in our laboratory at ICAR-CMFRI, Kochi, India, was used in this study. The strain was cultivated in tryptone soy broth (HiMedia, Thane, India) supplemented with 2% NaCl overnight at 30 °C with gentle agitation (150 rpm). Bacterial cells were determined by optical density measurement at 600 nm and validated by standard plate count on TCBS.

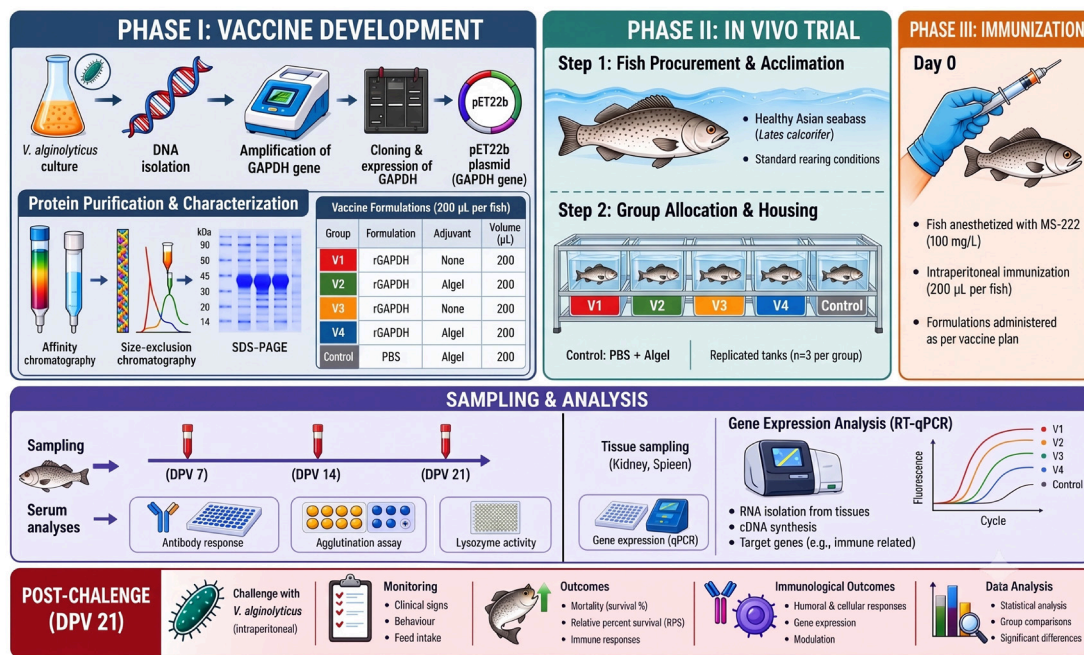


Figure 1. Schematic representation of experiment design and development of rGAPDH vaccine of *Vibrio alginolyticus*. Figure 1 is an original schematic illustration created and designed entirely by the authors using Microsoft PowerPoint and vector graphics software, with conceptual and layout assistance from Gemini Pro 3.1.

2.3. Determination of Lethal Dosage

A preliminary dose–response challenge was conducted to determine the median lethal dosage (LD_{50}) of *V. alginolyticus* CMFRI-JF260912 in healthy Asian seabass. A dose range of 10^3 to 10^8 colony-forming units (CFUs) was prepared to determine the dose required to cause 50% mortality in fish. Fish were distributed into glass tanks containing 250 L of filtered, aerated seawater (10 fish per tank) in duplicate. Following light anesthesia with MS-222, fish were inoculated by intraperitoneal injection with 0.1 mL of serial tenfold bacterial dilutions ranging from 10^3 – 10^8 CFU/fish. Control fish were injected with 0.1 mL of sterile phosphate-buffered saline (PBS) + Alginate. Fish were monitored twice daily for 7 days post-injection, and daily cumulative mortalities were documented. Dead fish were immediately necropsied to confirm *V. alginolyticus* infection and subjected to bacteriological examination. The LD_{50} value was calculated using the method of Reed and Muench [19].

2.4. Construction of Recombinant GAPDH

Genomic DNA was isolated from overnight cultures of *V. alginolyticus* CMFRI-JF260912 by phenol/chloroform extraction, followed by precipitation with isopropanol. For the recombinant expression of *V. alginolyticus* GAPDH, the complete GAPDH-encoding open reading frame (1002 bp) was amplified from genomic DNA using specifically designed primers (Table 1). PCR reaction was performed in a final volume of 25 µL, which contained 2 µL of DNA (2 ng / µL), 0.4 µM of each primer, 1X PCR master mix, and nuclease-free water. PCR consisted of an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 60 s. A final elongation step was performed at 72 °C for 7 min. PCR amplicons of GAPDH were run on 1% agarose gels and purified using the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Purified products were ligated into the pJET1.2 blunt-end cloning vector (Thermo Fisher Scientific, Waltham, MA, USA) and transformed into chemically competent *E. coli* Top10 cells. Positive clones were screened by colony PCR using GAPDH cloning primers and confirmed by sequencing.

Table 1. Primers used for cloning and expression of the GAPDH gene of *V. alginolyticus*.

Gene	Sequence (5'–3')	Product Size (bp)	Annealing Temp (°C)	Target
GAPDH (cloning)	F: 5' ATGGCAATTAAAGTCGGAATTAACGG 3' R: 5' TTATGCCAAACCGACTTTTGCG 3'	1002	59	Amplification of the GAPDH gene for cloning
GAPDH (Expression; NdeI/XhoI)	F: 5' TTATATACATATGGCAATTAAAGTCGAATT 3' R: 5' ATATTACTCGAGTGCCAAACCGACTTGCCTACT 3'	1022	59	Amplification of GAPDH gene for recombinant expression

For directional subcloning into the expression vector, a second primer pair was designed to incorporate NdeI and XhoI restriction sites at the 5' ends of the forward and reverse primers, respectively (Table 1). PCR reactions and conditions were performed as described above. The resulting amplicon was digested with NdeI and XhoI (New England Biolabs, Ipswich, MA, USA), purified using the MinElute PCR Purification Kit (Qiagen, Hilden, Germany), and ligated into a pET22b(+) expression vector (Novagen, Madison, WI, USA) (Figure 2). The resulting construct (pET22b-VibAlg.GAPDH) was transformed into ClearColi BL21 (DE3) competent cells, and transformants were selected on Luria–Bertani (LB) agar plates supplemented with 50 µg/mL ampicillin.

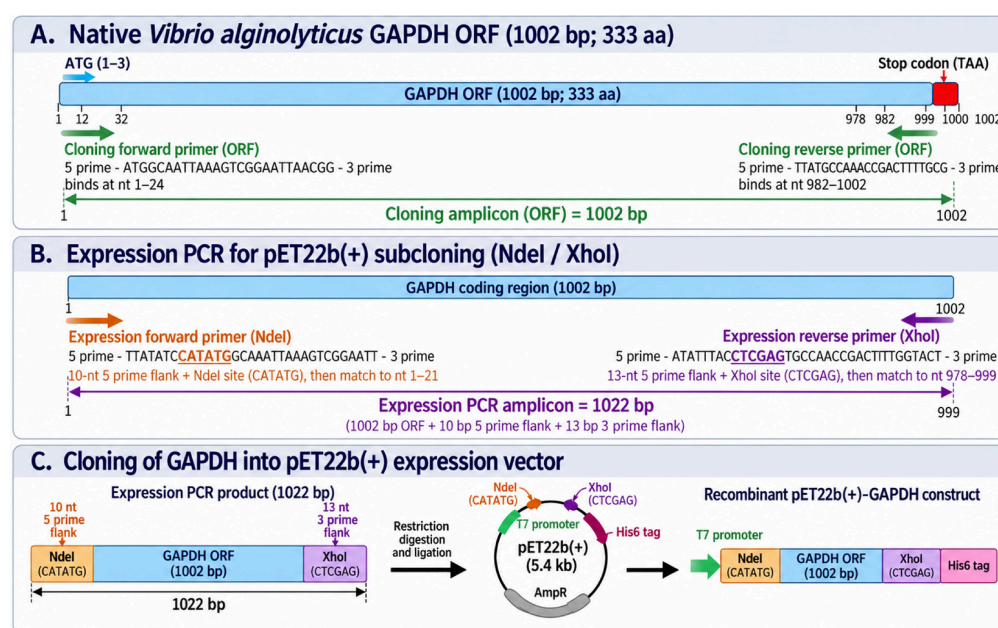


Figure 2. Schematic representation of GAPDH gene amplification and cloning into pET22b(+). (A) Native *Vibrio alginolyticus* GAPDH ORF showing primer-binding sites and the 1002 bp amplicon. (B) Expression PCR using primers incorporating NdeI and XhoI restriction sites to generate a 1022 bp product. (C) Directional cloning of GAPDH into pET22b(+) for recombinant protein expression with a His₆ tag.

2.5. Recombinant Protein Expression and Purification of GAPDH

ClearColi BL21(DE3) *E. coli* cells, an engineered low-endotoxin *E. coli* expression strain, harbouring pET22b-VibAlg.GAPDH were grown at 37 °C in LB broth (50 µg/mL ampicillin) with shaking at 200 rpm until the culture optical density at 600 nm (OD₆₀₀) reached approximately 0.6. Protein expression was induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 4 h at 37 °C. Cells were collected by centrifugation at 8000 rpm for 5 min. Total cell protein fractions (induced and uninduced) were prepared

in Laemmli sample buffer and resolved on 12% glycine SDS-PAGE. Gels were stained with Coomassie Brilliant Blue R-250 for 30 min and destained overnight.

rGAPDH protein was purified by Ni-NTA affinity chromatography (GE Healthcare, Chicago, IL, USA) under denaturing conditions. Briefly, the cell pellet obtained after IPTG induction was resuspended in denaturing lysis buffer (100 mM NaH_2PO_4 , 10 mM Tris-HCl, 8 M urea, pH 8.0) and disrupted by sonication on ice (30 s on/30 s off cycles for 15 min). The lysate was cleared by centrifugation at 12,000 rpm for 30 min at 4 °C, and the resulting supernatant containing solubilized inclusion bodies was incubated with Ni-NTA agarose resin equilibrated with lysis buffer. The resin was washed thoroughly with denaturing wash buffer (100 mM NaH_2PO_4 , 10 mM Tris-HCl, 8 M urea, pH 6.3) to remove non-specifically bound host proteins. Bound rGAPDH was eluted using denaturing elution buffer (100 mM NaH_2PO_4 , 10 mM Tris-HCl, 8 M urea, 250 mM imidazole, pH 4.5).

To refold the purified denatured protein into its native conformation and remove the chaotropic agent, the eluate was subjected to stepwise dialysis at 4 °C against refolding buffer (PBS, pH 7.4, 5% glycerol, 1 mM reduced glutathione and 0.2 mM oxidized glutathione) containing sequentially decreasing concentrations of urea (6 M, 4 M, 2 M, 1 M, 0.5 M, and 0 M urea), with each dialysis step performed for 6 h with continuous gentle stirring. The refolded protein solution was subsequently concentrated using Centricon ultrafiltration centrifugal filter units (10 kDa molecular weight cutoff; Millipore) and desalted into sterile PBS (pH 7.4) using a HiPrep 26/10 desalting FPLC column (Bio-Rad, Hercules, CA, USA). Protein concentration was determined by the Lowry method [20], and purity was verified by 12% SDS-PAGE. Densitometry was analyzed using ImageJ software 1.54, and the percentage of the target 37 kDa peak area relative to total density was calculated. Because the expression host, ClearColiBL21 (DE3), is a low-endotoxin *E. coli* strain, a separate endotoxin-removal step was not required for the purified rGAPDH.

2.6. rGAPDH Vaccine Preparation

Purified 37 kDa rGAPDH was emulsified with aluminum hydroxide (Algel; Sigma-Aldrich, Burlington, MA, USA) as an adjuvant. Briefly, a 2% aluminum hydroxide stock (20 mg per mL) was prepared in ultra-pure molecular biology-grade water using continuous magnetic stirring to prevent powder clumping and stored at 4 °C. The aluminum hydroxide stock solution was vortexed immediately before use to re-suspend the settled colloidal particles. Four vaccine formulations and an adjuvanted control were prepared under aseptic conditions (Table 2). For all adjuvanted preparations (V2, V4, and the PBS + Alginate control group), the stock 2% aluminum hydroxide solution was mixed with the respective protein solutions or sterile PBS in a 1:1 (v/v) ratio to achieve an identical final Alginate concentration of 1% (10 mg/mL). The unadjuvanted formulations (V1 and V3) were prepared in sterile PBS without adjuvant. The Alginate-protein mixture was incubated at 4 °C overnight with slow, continuous agitation to achieve maximum electrostatic binding efficiency. To test for sterility, 100 µL of each formulation was spread onto non-selective nutrient agar plates and then incubated at 37 °C for 24 to 48 h; since no microbial growth was seen, the preparations were confirmed to be sterile. The pH values of the formulations were between 7.2 and 7.5. The emulsified rGAPDH-prepared vaccines looked like uniform, milky-white suspensions with no visible clumping. The emulsified vaccine formulations were incubated at 4 °C until use.

Table 2. Vaccine formulations prepared for immunization of Asian seabass. All groups (V1–V4 and PBS + Algel control) received an identical administration volume of 200 μ L per fish, containing the respective vaccine formulation or PBS + Algel control preparation.

Group	Formulation	Protein Dose (μ g)	Adjuvant	Volume (μ L)
V1	rGAPDH	5	None	200
V2	rGAPDH	5	Algel	200
V3	rGAPDH	10	None	200
V4	rGAPDH	10	Algel	200
Control	Sterile PBS	0	Algel	200

2.7. Fish Vaccination

Asian seabass were randomly allocated into five experimental groups (V1–V4 and PBS + Algel Control). Each group was maintained in three separate replicate tanks with 27 fish per tank (81 fish per group). Prior to immunization, fish were anesthetized using tricaine methanesulfonate (MS-222; Sigma-Aldrich, Burlington, MA, USA). Fish in groups V1–V4 were immunized by intraperitoneal injection with 200 μ L of their respective rGAPDH formulations (Table 2) using a sterile 23-gauge syringe, while fish in the PBS + Algel control group were injected with an identical volume (200 μ L) of sterile PBS containing 1% Algel adjuvant. Fish were observed daily for behavioural changes, mortality, and clinical signs of disease. Blood and tissue samples (spleen and head kidney) were collected from nine fish per group (3 fish per tank from 3 tanks) at 7, 14, and 21 days post-vaccination (DPV). Following sampling across the three time points, the remaining 54 fish per group (18 fish per replicate tank) were subsequently used for the pathogenic challenge.

For serum-based immunological assays (agglutination, bactericidal activity, and lysozyme activity), blood from the three fish within each tank was pooled, processed, and analyzed at each sampling time point. A statistical power analysis performed using G*Power 3.1 indicated an estimated power of approximately 50%, assuming a large effect size (Cohen's $f = 0.40$), a significance level of $\alpha = 0.05$, and five experimental groups. Although the estimated statistical power was below the conventionally recommended threshold of 80%, the sample size of nine fish per group was retained, as it provided biological replication and sufficient observations to perform the planned ANOVA, while considering experimental feasibility and ethical principles aimed at minimizing animal use.

2.8. Agglutinating Antibody Titer

Serum agglutinating antibody titers were determined according to the method of Roberson [21] with minor modifications. Briefly, 50 μ L of two-fold diluted serum from all the groups, including the control, were transferred into U-bottom 96-well microtiter plates. To each well, an equal volume (50 μ L) of *V. alginolyticus* bacterial suspension (1×10^5 CFU/mL) was added. The plates were incubated at 25 $^{\circ}$ C for 2 h, then stored at 4 $^{\circ}$ C overnight, and positive agglutination was defined by visible precipitation.

2.9. Serum Bactericidal Activity

The serum bactericidal activity assay was performed according to the method described by Vasudeva Rao et al. [22], with minor modifications. An overnight culture of *V. alginolyticus* was harvested, washed, and resuspended in PBS, and the bacterial suspension was adjusted to an optical density of 1.0 at 600 nm (OD₆₀₀). Briefly, 2 μ L of the bacterial suspension was mixed with 20 μ L of serum collected from fish belonging to the different experimental groups, including the PBS + Algel-injected control group. The mixtures were incubated in 96-well microtiter plates at 25 $^{\circ}$ C for 1 h. Following incubation, viable bacteria

were enumerated by plating onto TCBS agar and incubating at 30 °C for 18 h. Serum bactericidal activity was expressed as bacterial survival percentage (BSP), calculated from viable colony counts recovered following serum exposure relative to the mean bacterial count obtained with serum from PBS + Algel-injected control fish at the corresponding sampling time point, using the following equation:

$$\text{BSP (\%)} = [\text{CFU recovered following incubation with test serum} / \text{Mean CFU recovered following incubation with serum from PBS + Algel-injected control fish}] \times 100.$$

Bacterial survival percentages were determined at 7, 14, and 21 days DPV. Lower BSP values indicated greater bactericidal activity relative to serum from the PBS-Algel-injected control group.

2.10. Serum Lysozyme Activity

Serum lysozyme activity was determined using the turbidimetric method with lyophilized *Micrococcus lysodeikticus* (Sigma-Aldrich, Burlington, MA, USA), following the procedure described by Ellis [23], with minor modifications. Briefly, 20 µL of fish serum was added in duplicate to U-bottom microtiter plates, followed by 180 µL of a 0.02% (*w/v*) suspension of *M. lysodeikticus* prepared in 66 mM potassium phosphate buffer (pH 6.24). Absorbance at 450 nm was measured immediately after mixing (T_0) and after 30 min of incubation at 25 °C (T_{30}). One unit (U) of lysozyme activity was operationally defined as a decrease in absorbance of 0.001 per minute under the specified assay conditions. Lysozyme activity was calculated from the blank-corrected decrease in absorbance using the following equation:

$$\text{Lysozyme activity (U/mL)} = [(A_0 - A_{30}) \times D] / (0.001 \times 30 \times 0.020)$$

where A_0 and A_{30} represent the blank-corrected absorbance values at 0 and 30 min, respectively; D represents the serum dilution factor ($D = 1$ for undiluted serum); 30 represents the incubation period (min); and 0.020 represents the serum volume (mL). Results were expressed as U/mL of serum.

2.11. Pathogenic Challenge

At 21 DPV, Asian seabass across all experimental groups (V1–V4 and PBS + Algel control, $n = 54$ fish per group) were distributed across triplicate tanks (18 fish per tank). The calculated median lethal dose (LD_{50}) of *V. alginolyticus* CMFRI-JF260912 in Asian seabass was 1.12×10^4 CFU/fish ($\log_{10} LD_{50} = 4.05$; Table S1). Fish were anesthetized with MS-222 and experimentally challenged by intraperitoneal (i.p.) injection of 0.1 mL of virulent *V. alginolyticus* CMFRI-JF260912 suspension adjusted to 1×10^5 CFU/fish. Challenged fish were monitored twice daily over a 30-day post-challenge observation period for feed intake, behavioural abnormalities, clinical signs of vibriosis, and mortality. Humane endpoints were strictly enforced: fish exhibiting clinical moribund criteria, such as persistent loss of equilibrium, spiralling swimming behaviour, lethargy, or severe skin ulcerations, were humanely euthanized by immersion in an overdose of MS-222 and tallied as challenge mortalities. Relative percent survival (RPS) was calculated as $\text{RPS} = [1 - (\% \text{ mortality in vaccinated group} / \% \text{ mortality in PBS + Algel control group})] \times 100$. At necropsy, bacteria were re-isolated from the heart blood and kidney on TCBS agar, and identity was confirmed by PCR. Survival distributions were analyzed using the Kaplan–Meier method and compared using the log-rank (Mantel–Cox) test.

2.12. Quantitative RT-PCR

Total RNA was extracted from spleen and head kidney ($n = 9$ biological replicates per tissue per time point per group) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, including DNase I treatment (New England Biolabs, Hitchin, UK). RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized from total RNA (1 μ g) using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The designed primer sequences, annealing temperatures, and amplicon sizes of tested genes are given in Table 3. Each of the 20 μ L qPCR reactions included 10 μ L of 2x iQTM SYBR[®] Green Supermix 2X (Bio-Rad, Hercules, CA, USA), 2 μ L of diluted cDNA (1:20), 0.4 μ M of each primer, and nuclease-free water. Reactions were performed on a LightCycler 96 (Roche, Basel, Switzerland), using the following thermal profile: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 s, annealing at 61–70 °C for 15 s, and elongation at 72 °C for 30 s. A melting-point curve analysis was carried out from 61 to 95 °C with an increment of 0.5 °C per 10 s to verify amplicon specificity. Each sample was amplified in biological and technical triplicates. qRT-PCR data of each gene were normalized to the reference gene, EF1 α [24], and relative gene expression was calculated using the $2^{-\Delta\Delta C_T}$ method [25]. To achieve symmetrical representation of both upregulated and downregulated genes, data were converted to log₂ fold change ($\log_2 FC = -\Delta\Delta C_T$) for graphical presentation and statistical comparisons.

Table 3. Primers used for RT-qPCR analysis of immune-related gene expression in Asian seabass.

Accession Number	Gene	Sequence (5'–3')	Product Size (bp)	Temp (°C)
PZ727114	Complement Factor H	F: 5' CTGTATCAGAGGAAGACGACAT 3' R: 5' TGGCAAGAAGGCAGGTTCAT 3'	84	64
PZ727115	Cystatin B	F: 5' GAGGTATGTGTGGGGGTCCT 3' R: 5' CTTTTGCTCTGCATGGGGC 3'	93	69
PZ727116	HSP70	F: 5' TACCTCGGCCAAAAGGTGTC 3' R: 5' TCTTTAGTCGCCTGTCGCTG 3'	80	68
PZ723671	HSP90 α	F: 5' ATGACCCACAGACGCACTCC 3' R: 5' CATGTCCTCAGTGGGAGCAGA 3'	113	70
GQ507427	Elongation Factor 1-alpha (reference)	F: 5' AAATTGGCGGTATTGGAAC 3' R: 5' GGGAGCAAAGGTGACGAC 3'	83	61

2.13. Statistical Analysis

The experimental design evaluated two primary explanatory factors: treatment formulation (five groups: V1, V2, V3, V4, and PBS + Algel Control) and time (observation/sampling periods: 7, 14, and 21 DPV). The experimental design was not formulated as a multiple conventional full-factorial experiment encompassing all treatment groups, time points, immunological parameters, and gene expression targets simultaneously. Instead, each immunological parameter and each gene expression target in the head kidney and spleen was analyzed independently with treatment and time specified as explanatory factors.

For each outcome variable, a two-way analysis of variance (ANOVA) was performed to evaluate the main effects of Treatment and Time and their Treatment $\times$ Time interaction. Wherever significant main effects were detected, marginal means were compared using Tukey's honestly significant difference (HSD) test. Treatment $\times$ Time interaction means were compared using pairwise least-squares-mean *t*-tests implemented in SAS. These comparisons were not adjusted for multiple testing and were therefore interpreted as exploratory. Marginal means were compared using Tukey's HSD test, where appropriate.

Statistical significance was set at $p < 0.05$. Statistical significance was set at $p < 0.05$. All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) and IBM SPSS Statistics version 26.0 (IBM Corp., Armonk, NY, USA). Uniform environmental conditions were maintained across replicate tanks. Tank-level samples were treated as independent experimental units to avoid pseudoreplication. However, tank effects were not separately estimated in the statistical model.

3. Results

3.1. Recombinant Expression and Purification of rGAPDH

The GAPDH-encoding open reading frame (1002 bp) was successfully amplified from *V. alginolyticus* (Figure 3A). Sequencing confirmed the identity and integrity of the insert in pJET1.2-GAPDH. SDS-PAGE analysis revealed a prominent protein band of approximately 37 kDa in the induced whole-cell lysate, which was absent in uninduced controls. This matched the expected size of the predicted molecular weight of the VibAlg.GAPDH-His fusion protein (Figure 3B). The recombinant protein was predominantly present in the insoluble fractions, and the purified rGAPDH was obtained with a purity of $92.4 \pm 1.8\%$ as determined by densitometric analysis of SDS-PAGE elution profiles (Figure 3B, Lanes 6–7). The final yield of purified rGAPDH was approximately 110 mg per litre of culture.

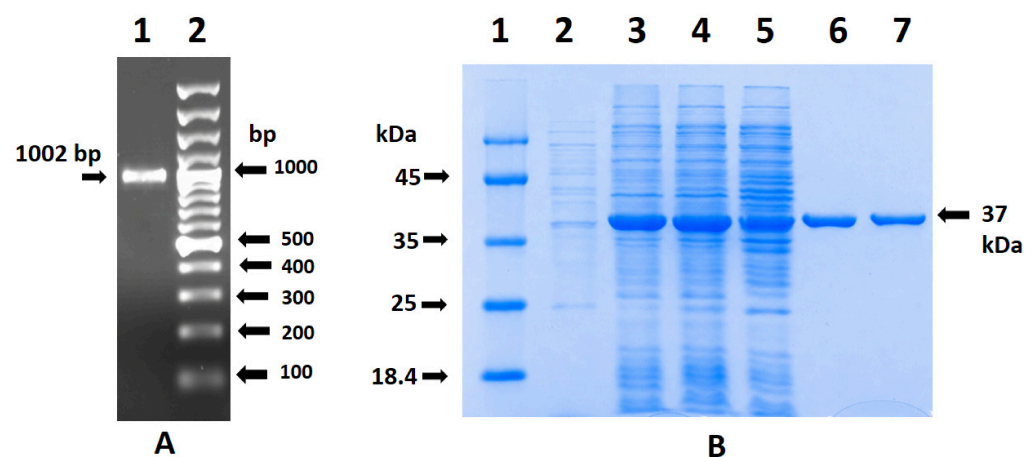


Figure 3. PCR and SDS-PAGE analysis. (A): A Gel image shows the GAPDH gene of *Vibrio alginolyticus*. Lane 1: amplified GAPDH gene, 1002 bp, and Lane 2: DNA ladder; (B): SDS-PAGE gel image shows IPTG-induced expression of GAPDH protein. Lane 1: Protein ladder, Lane 2: uninduced total cell protein, Lane 3: induced total cell protein, Lane 4: insoluble fraction, Lane 5: soluble fraction; Lanes 6 and 7: purified 37 kDa rGAPDH protein.

3.2. Protective Efficacy of rGAPDH

Protective efficacy was evaluated following an intraperitoneal challenge with 1×10^5 CFU/fish of virulent *V. alginolyticus* at 21 DPV, where measurable protection was defined as a significant reduction in cumulative mortality and quantified by RPS over a 30-day observation period. Following intraperitoneal challenge with *V. alginolyticus*, the non-vaccinated PBS + Alginate control cohort exhibited a cumulative mortality of 70.37% over the 30-day monitoring window (Figure 4). Group V4 (10 μ g rGAPDH + Alginate) exhibited no observed mortality during the 30-day experimental challenge period, corresponding to 100% RPS compared with the PBS + Alginate control group. Groups V2 (5 μ g rGAPDH + Alginate) and V3 (10 μ g rGAPDH alone) each experienced 25.93% cumulative mortality, corresponding to identical RPS values of 63.16%. Group V1 (5 μ g rGAPDH alone) exhibited 42.59% cumulative mortality, resulting in an RPS of 39.47% (Table S2).

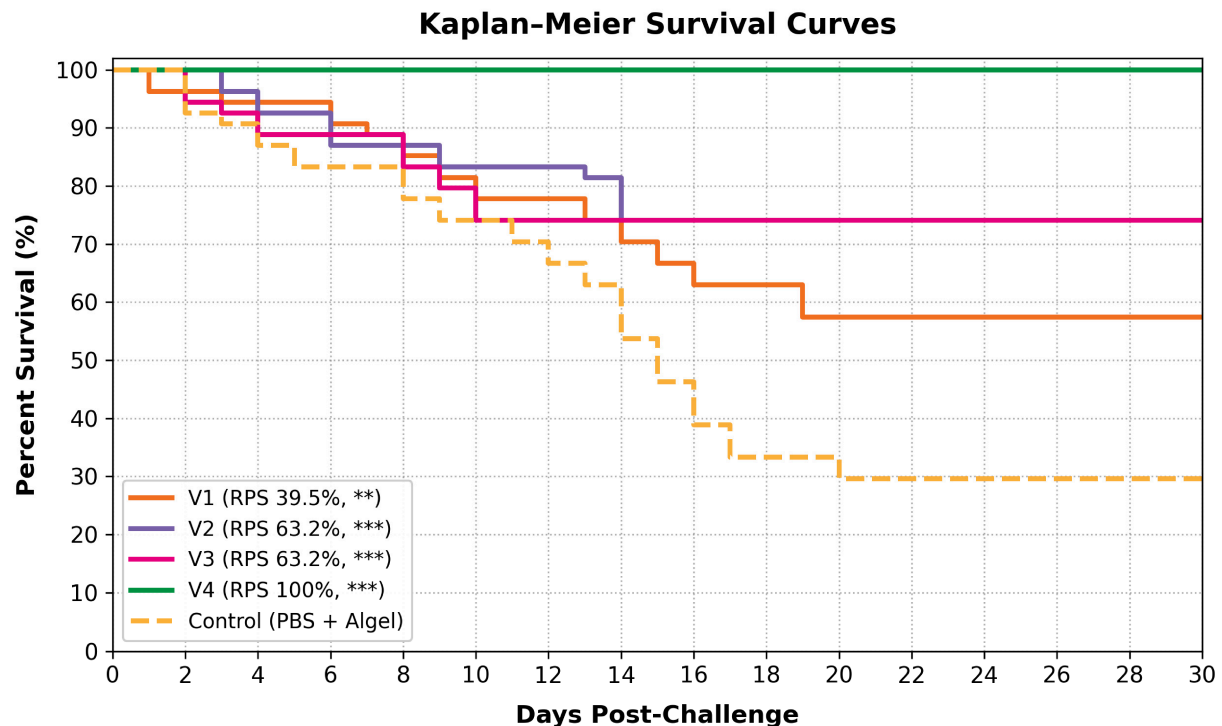


Figure 4. Kaplan–Meier curve showing the percent survival of Asian seabass challenged with *Vibrio alginolyticus* at 21 days post-vaccination and monitored for 30 days post-challenge. The vaccinated group and the non-vaccinated PBS + Algel control group received an intraperitoneal injection of *V. alginolyticus* (1×10^5 CFU/fish). Statistical annotations indicate pairwise comparisons against the PBS + Algel control cohort: ** $p < 0.01$, *** $p < 0.001$. Survival in group V4 was significantly higher than in all other vaccinated cohorts ($p < 0.01$).

3.3. Serum Agglutination Titers

Specific humoral responses were measured by a whole-cell agglutination assay in which two-fold serum dilutions were reacted against a suspension of *V. alginolyticus* CMFRI-JF260912 (1×10^5 CFU/mL); titres are expressed as log2 of the reciprocal of the highest dilution showing visible agglutination (Section 2.8). Serum agglutination titers were significantly increased in all vaccinated fish groups (V1–V4) against *V. alginolyticus* (Figure 5). At 7 DPV, all vaccinated groups showed moderate antibody responses against *V. alginolyticus*. At 14 DPV, the V4 group (10 µg rGAPDH with Algel) had the highest antibody titer (5 ± 1), followed by V3, V2, and V1 groups. At 21 DPV, the V4 group sustained its peak response (5 ± 2), while the V3 group remained stable (4 ± 2). In contrast, the V2 group showed a moderate decline (3 ± 1), and the V1 group declined to 2 ± 0 .

3.4. Serum Bactericidal Activity

Serum-associated bactericidal activity was evaluated by incubating serum with *V. alginolyticus* and enumerating surviving bacteria on TCBS agar. Results were expressed as BSP, with lower values indicating greater serum bactericidal activity (Figure 6). Two-way ANOVA identified significant effects of vaccine formulation ($F_{4,30} = 905.29$, $p < 0.0001$), sampling day ($F_{2,30} = 207.07$, $p < 0.0001$), and their interaction ($F_{8,30} = 101.77$, $p < 0.0001$). At 7 DPV, all vaccinated groups exhibited significantly lower BSP than the PBS + Algel control ($100.00 \pm 5.36\%$; $p < 0.05$). The greatest reduction in bacterial survival was observed in V2 ($19.82 \pm 1.39\%$), followed by V4 ($33.36 \pm 4.16\%$), V3 ($65.11 \pm 4.39\%$), and V1 ($88.28 \pm 2.38\%$). All four vaccinated groups differed significantly from one another ($p < 0.05$), indicating distinct early serum bactericidal responses among the formulations. At 14 DPV, all vaccinated groups continued to exhibit significantly lower BSP than the PBS + Algel control

($100.00 \pm 1.54\%$; $p < 0.05$). The lowest BSP was recorded in V4 ($13.92 \pm 1.42\%$), followed by V3 ($17.06 \pm 3.30\%$), V1 ($21.21 \pm 1.16\%$), and V2 ($29.37 \pm 3.77\%$). V4 exhibited significantly lower BSP than V1 and V2 ($p < 0.05$), but did not differ significantly from V3 ($p > 0.05$). Similarly, V1 and V3 were not significantly different from each other, whereas V2 exhibited significantly higher BSP than the other vaccinated groups. At 21 DPV, all vaccinated groups exhibited significantly lower BSP than the PBS + Algel control ($100.00 \pm 8.08\%$; $p < 0.05$). V4 had the lowest BSP ($8.31 \pm 0.72\%$), followed by V3 ($14.78 \pm 1.01\%$), V2 ($20.37 \pm 1.96\%$), and V1 ($82.15 \pm 0.61\%$). V4 differed significantly from V1 and V2, but not from V3 (V3 versus V4: $p = 0.0531$). V2 and V3 also did not differ significantly ($p = 0.0541$).

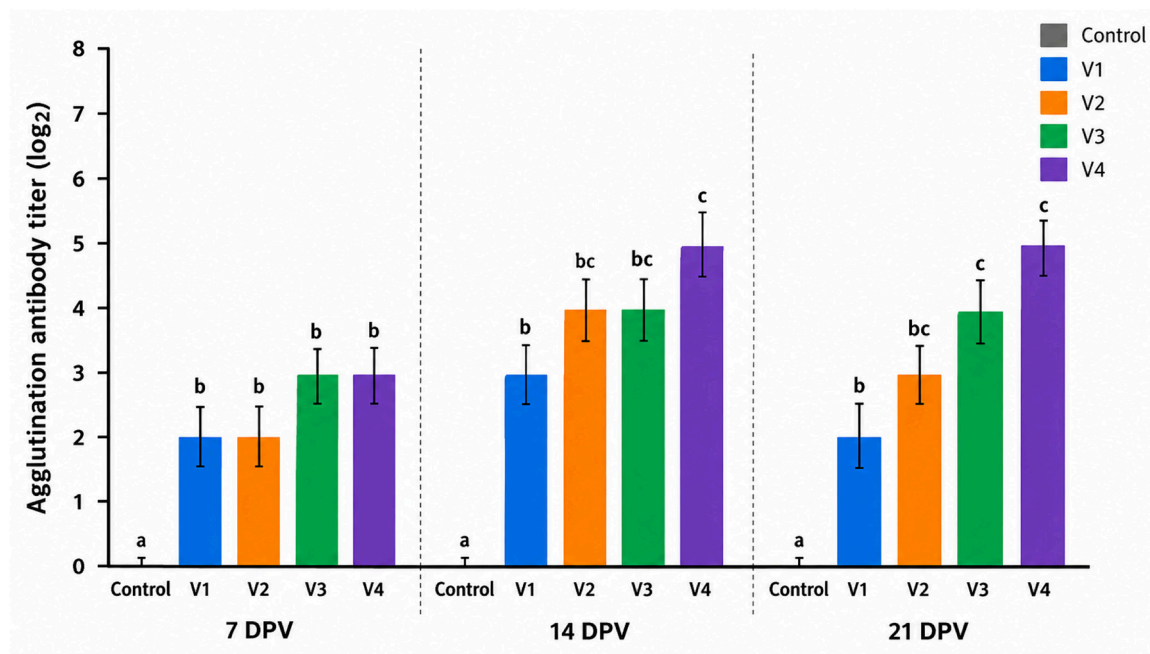


Figure 5. Serum agglutination antibody titres in Asian seabass following vaccination with recombinant GAPDH of *Vibrio alginolyticus*. Serum samples were collected at 7, 14, and 21 days post-vaccination, and antibody titres were determined by the serum agglutination assay. Data are presented as mean $\pm$ SD of three independent pooled serum samples from each tank per group at each time point ($n = 9$). Differences among treatment groups and sampling days were analyzed using two-way ANOVA followed by Tukey's HSD multiple-comparison test. Distinct lowercase letters above the bars indicate statistically significant differences among experimental treatment groups within each sampling day ($p < 0.05$).

3.5. Serum Lysozyme Activity

Serum lysozyme activity was quantified turbidimetrically against *Micrococcus lysodeikticus* and expressed as kinetic units (U/mL) based on the rate of decrease in absorbance at 450 nm. Serum lysozyme activity showed significant effects of treatment, sampling day and their interaction (Figure 7). Based on the observations, treatment group, sampling day and the group $\times$ day interaction were all significant (two-way ANOVA, $p < 0.0001$ for each effect). At 7 DPV, mean activity ranged from 78.6 U/mL in PBS + Algel controls to 417.1 U/mL in V4. At 14 DPV, activity ranged from 167.8 U/mL in PBS + Algel controls to 898.8 U/mL in V4. At 21 DPV, activity ranged from 124.7 U/mL in PBS + Algel controls to 1077.4 U/mL in V4. The PBS + Algel control group did not show noticeable change throughout the experimental period.

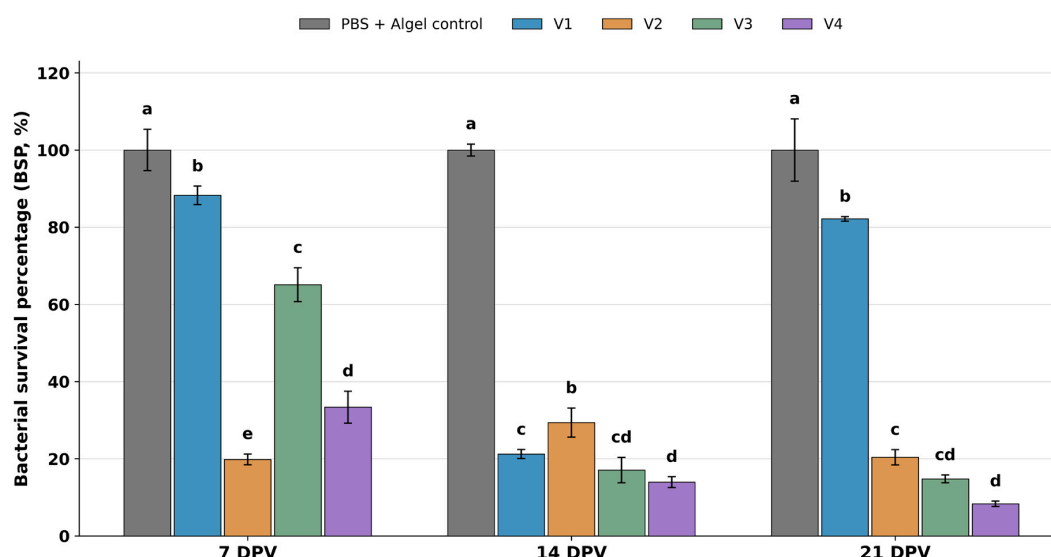


Figure 6. Serum bactericidal activity against *Vibrio alginolyticus* in Asian seabass at 7, 14, and 21 days post-vaccination, expressed as bacterial survival percentage (BSP). Data are presented as the mean $\pm$ SD of three independent pooled serum samples from each tank per group at each time point ($n = 9$). BSP was calculated relative to the corresponding PBS + Algel control mean (100% per day); lower BSP indicates greater serum bactericidal activity. Different lowercase letters indicate significant differences among treatments within the same sampling day ($p < 0.05$).

Serum Lysozyme activity

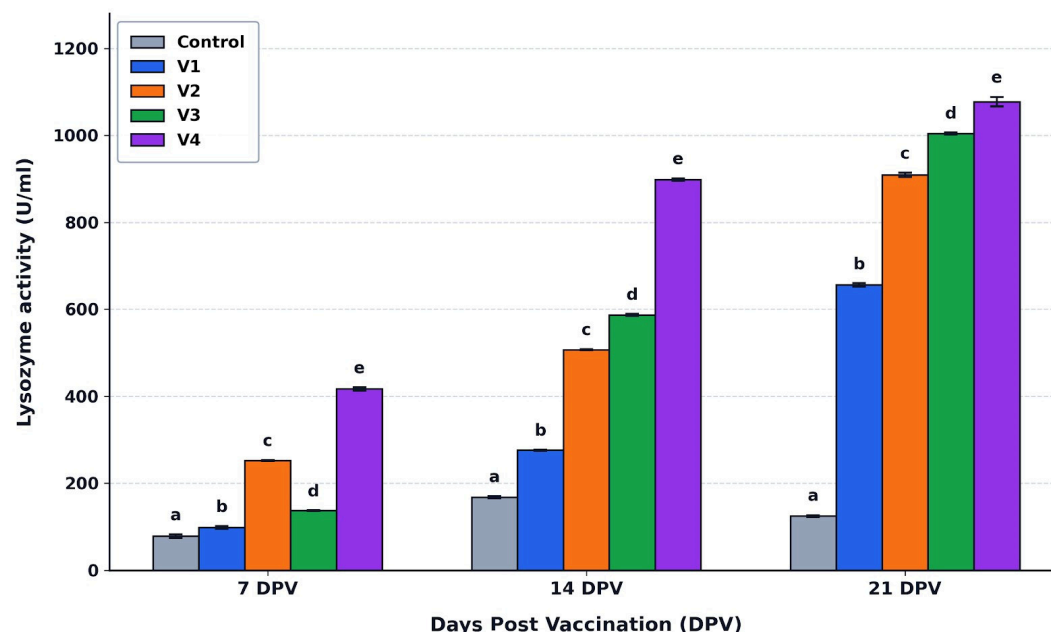


Figure 7. Serum lysozyme activity in Asian seabass at 7, 14, and 21 days post-vaccination. Data are presented as mean $\pm$ SD of three independent pooled serum samples from each tank per group at each time point ($n = 9$). Lysozyme activity was determined turbidimetrically from the decrease in absorbance at 450 nm over 30 min and expressed as kinetic units (U/mL). Statistical differences were assessed using two-way ANOVA followed by Tukey's HSD multiple-comparison test. Different lowercase letters above the bars indicate significant differences among treatment groups within the same sampling day ($p < 0.05$).

3.6. Gene Expression Profiling

3.6.1. Complement Factor H

Transcript abundance of the four target genes was measured by RT-qPCR in spleen and head kidney at 7, 14 and 21 DPV, normalized to *EF1a* and expressed as log₂ fold change relative to the time-matched PBS + Alginate control group (Section 2.12). In the spleen, CFH expression was numerically highest in the V4 group at 21 DPV, reaching a relative expression level of 1.34 ± 0.10 log₂ fold change. The expression of CFH was upregulated in V1 and V2 groups at 7 and 14 DPV but downregulated at 21 DPV. In contrast, the V3 group exhibited downregulation at 7 DPV with upregulation at 14 and 21 DPV that was maximal at 14 DPV (Figure 8A). In the head kidney, the V3 group showed peak CFH upregulation at 7 DPV, with expression again elevated at 21 DPV, while the V4 group demonstrated a steady progressive increase from 7 to 21 DPV. Groups V1 and V2 showed downregulation of CFH at 21 DPV and 14 DPV, respectively, in the head kidney (Figure 9A).

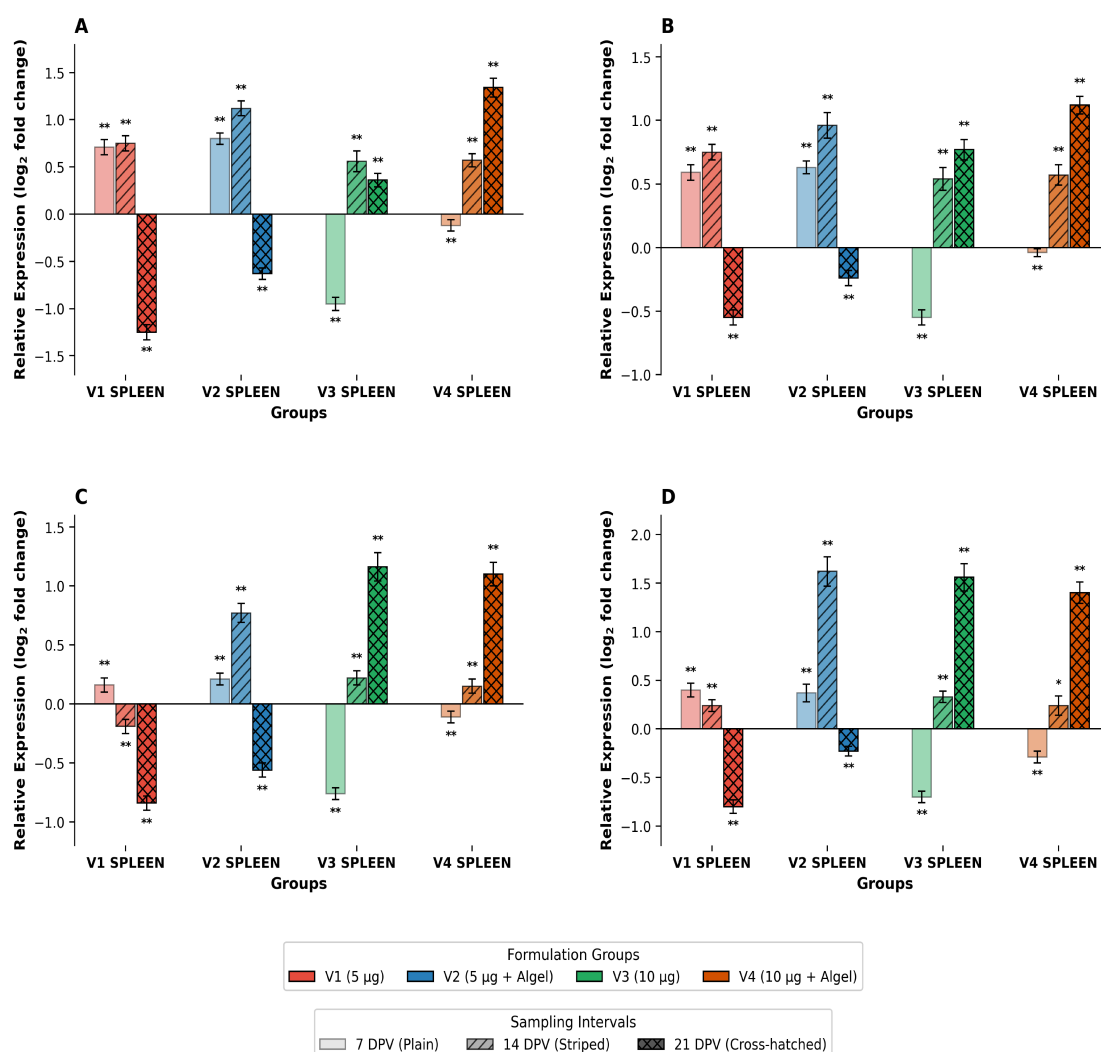


Figure 8. Relative mRNA expression of target immune genes in Asian seabass spleen at 7, 14, and 21 days post-vaccination. Complement factor H (A), Cystatin B (B), HSP70 (C), and HSP90α (D). Data are expressed as relative expression (log₂ fold change, mean ± SD, *n* = 9 biological replicates per group per time point) normalized to *EF1a* and relative to the time-matched PBS + Alginate control. Asterisks denote statistically significant differences compared to the PBS + Alginate control cohort (* *p* < 0.05, ** *p* < 0.01).

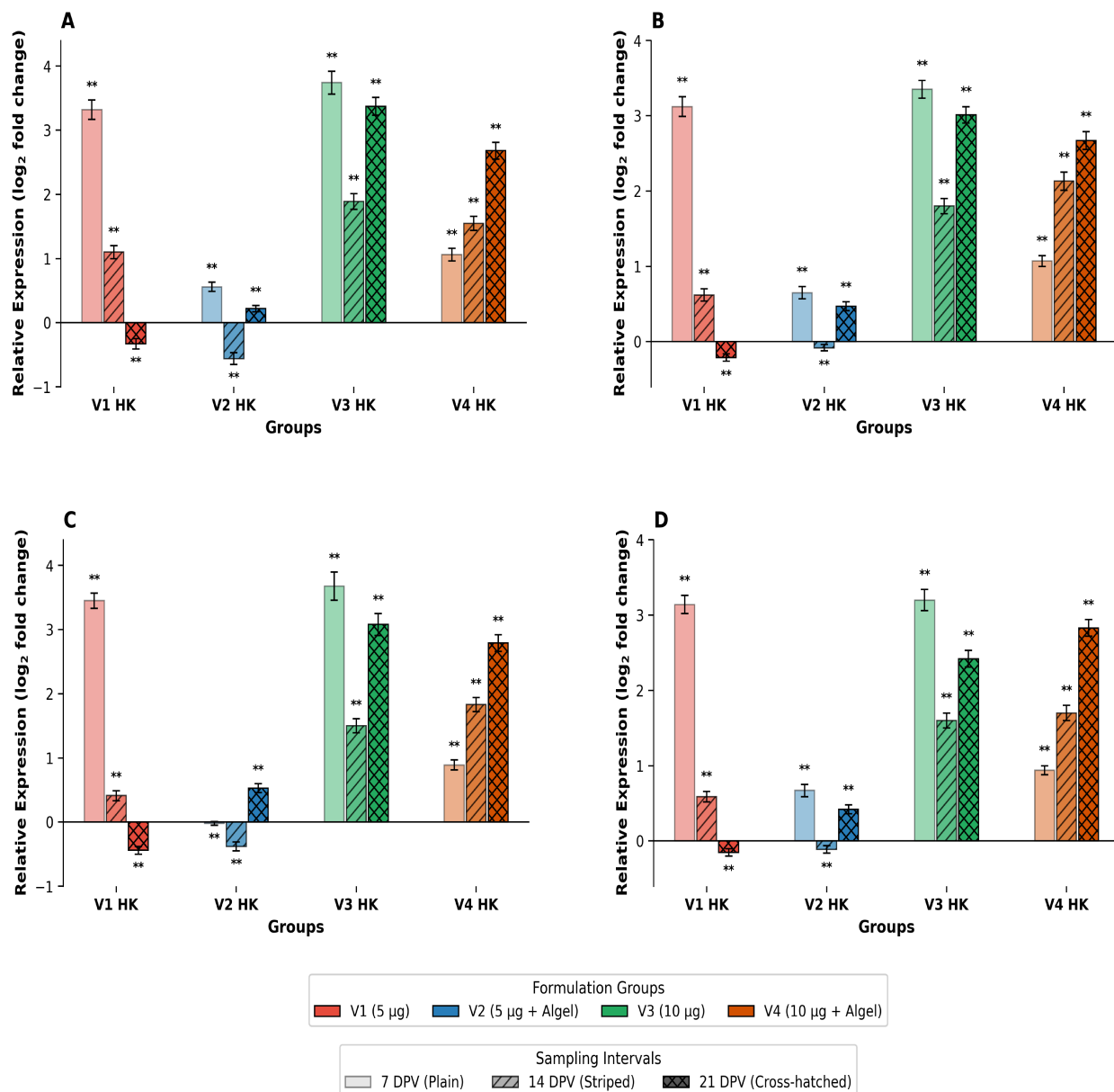


Figure 9. Relative mRNA expression of target immune genes in Asian seabass head kidneys at 7, 14, and 21 days post-vaccination. Complement factor H (A), Cystatin B (B), HSP70 (C), and HSP90α (D). Data are expressed as relative expression (log₂ fold change, mean ± SD, $n = 9$ biological replicates per group per time point) normalized to *EF1α* and relative to the time-matched PBS + Alginate control. Asterisks denote statistically significant differences compared to the PBS + Alginate control cohort (** $p < 0.01$).

3.6.2. Cystatin B

Cystatin B transcript levels were determined in the same tissue panel and expressed on a log₂ fold-change scale. Cystatin B expression in the head kidney peaked markedly in the V3 group at 7 DPV and remained elevated relative to other groups throughout the 21-day observation period (Figure 9B). In the V4 group, cystatin B was slightly downregulated in the spleen at 7 DPV (Figure 8B), followed by marked upregulation at 14 and 21 DPV in both the head kidney and spleen (Figures 8B and 9B). Conversely, downregulation was observed in the V1 group in the head kidney at 21 DPV and in the spleen of the V1, V2, and V3 groups at different sampling time points (Figure 8B).

3.6.3. HSP70 and HSP90 α

The expression profiles of the two heat shock protein genes, HSP70 and HSP90 α , were evaluated in parallel in the spleen and head kidney. At 7 DPV, HSP70 expression was significantly downregulated in the spleen of the V3 and V4 groups (Figure 8C), whereas significant upregulation was observed in the head kidney of both groups at the same time point (Figure 9C). By 21 DPV, HSP70 expression was significantly elevated in both the V3 and V4 groups in the spleen (Figure 8C) and head kidney (Figure 9C). A similar temporal expression pattern was observed for HSP90 α , with elevated transcript levels at 21 DPV in both tissues in the V3 and V4 groups (Figures 8D and 9D).

4. Discussion

This study describes the development of a rGAPDH subunit vaccine of *V. alginolyticus* for Asian seabass. The adjuvanted high-dose formulation (10 μ g) conferred complete protection against experimental *V. alginolyticus* infection. To our knowledge, this protection level is among the highest reported for recombinant *Vibrio* vaccines evaluated in teleost fish. The higher relative percent survival observed in the adjuvanted formulations compared with their corresponding unadjuvanted formulations suggests a beneficial association between aluminum hydroxide inclusion and protective efficacy. Group V4 (10 μ g rGAPDH formulated with Algel) obtained 100% RPS, while the unadjuvanted group V3 (10 μ g rGAPDH alone) achieved only 63.16% RPS. At the lower protein dose a similar dose–response relationship was observed in the V2 group (5 μ g rGAPDH with Algel) and V1 group (5 μ g rGAPDH alone), yielding 63.16% and 39.47% RPS. This is in agreement with the well-known role of aluminum hydroxide as a depot-forming adjuvant that extends the availability of antigen at the injection site and enhances humoral immune responses in teleost fish [11,26,27].

The level of protection observed is consistent with earlier studies that demonstrated immunogenic and cross-protective properties of GAPDH. Li et al. [18] reported that recombinant GAPDH induced protective immunity in zebrafish against *V. alginolyticus*, with RPS values over 60%. Similarly, Cao et al. [28] also observed RPS of 66% after vaccinating large yellow croaker (*Larimichthys crocea*) with a bacterial ghost vaccine against *V. alginolyticus*. Zhang et al. [29] reported that live-attenuated Δ cpxA and Δ cpxR vaccine candidates conferred over 70% RPS against *V. alginolyticus* infection in pearl gentian grouper, demonstrating the protective potential of attenuated vaccine strategies in marine fish. In our study, 100% RPS was obtained against *V. alginolyticus* in Asian seabass vaccinated with rGAPDH–Algel. This represents a high level of protection compared to previously reported vaccines against *V. alginolyticus*. However, direct comparisons between our developed rGAPDH–Algel vaccine and other available vaccines are difficult due to variations in fish species, adjuvants, and immunization protocols. A summary of representative studies to facilitate comparison is presented in Supplementary Table S3 [30–33].

The importance of adjuvant formulation is further supported by Dar et al. [34], who tested a VHSV vaccine adjuvanted with squalene–aluminum hydroxide in olive flounder (*Paralichthys olivaceus*). This vaccine gave high levels of protection, as shown by RPS values from 71.4% to 100% in the fish that were vaccinated intraperitoneally, indicating protective efficacy under the conditions tested of this adjuvant formulation against the pathogen. In the present study, the formulation (10 μ g rGAPDH with Algel) conferred 100% RPS in Asian seabass against *V. alginolyticus*. This finding demonstrates that the developed vaccine resulted in 100% RPS under the experimental challenge conditions against vibriosis under the experimental challenge conditions evaluated in this trial. The fact that aluminum hydroxide adjuvant may be associated with improved protective efficacy of subunit vaccine against *Vibrio* species in fish is in line with the findings of Nguyen et al. [35], who found

significantly higher specific IgM levels and improved survival rate in grouper that had been vaccinated with recombinant outer membrane proteins of *V. harveyi* adjuvanted with Algel. The V4 group achieved 100% RPS under the experimental challenge conditions against *V. alginolyticus*, which is comparable to that of the most advanced vibriosis subunit vaccines described in the recent literature [11,36].

The serum agglutination titer of Asian seabass, which was used to evaluate the humoral immune response, showed that all vaccinated groups induced robust antibody levels. The V4 group reached the highest agglutination titer ($5 \log_2$) at 14 DPV and sustained it at 21 DPV. Interestingly, the V1 and V2 groups had peaked at 14 DPV before titers declined, which may be associated with a possible adjuvant-associated antigen retention effect provided by the Algel adjuvant in the V4 for sustained agglutinating antibody responses. In studies involving recombinant *V. harveyi* vaccines in grouper [16], sustained antibody titers were observed for up to 8 weeks post-vaccination when PLG microparticle encapsulation was used. This suggests that formulation strategy is a major factor in determining immune persistence. Further study is required to compare Algel and polymer-based delivery systems for rGAPDH in Asian seabass.

Protection against bacterial pathogens is mediated by coordinated humoral and innate immune mechanisms. Functional assays demonstrated that vaccination enhanced both antibody response and serum bactericidal activity. Because whole-serum bactericidal assays measure the combined bactericidal action of complement activation, natural and specific antibodies, and other circulating soluble innate factors, this assay reflects collective serum antibacterial clearance rather than functionally differentiating antibody-mediated neutralization from complement-mediated lysis. Serum bactericidal activity varied according to vaccine formulation and sampling time, with V2 exhibiting the greatest activity at 7 DPV and V4 at 14 and 21 DPV. The differences were statistically significant and aligned with the bactericidal trends seen in similar studies involving recombinant subunit vaccines [27,30,35]. Lysozyme, a major component of non-specific innate immunity, has been shown to be increased in vaccinated fish and contributes to bacterial lysis and facilitates subsequent phagocytosis [37]. In the case of transient, non-specific immune responses, which generally fall off within 2 to 3 weeks after exposure to unadjuvanted soluble antigens (as observed in group V1), the observed changes in lysozyme activity and later HSP70/HSP90 α expression in groups V2 and V4 may be associated with formulation-dependent antigen persistence caused by the aluminum hydroxide adjuvant and the greater amount of antigen. Aluminum hydroxide may influence antigen availability and immune responses, thus allowing for continuous antigen presentation and the subsequent stimulation of the circulating innate effectors over the entire 21-day immunization period.

The marked upregulation of CFH in the head kidney and spleen of the high-dose cohorts, particularly the sustained induction observed in the V4 group through 21 DPV suggests a possible association with complement regulation in Asian seabass. CFH is the primary soluble regulatory cofactor of the alternative complement cascade, acting to accelerate the disassembly of alternative C3-convertase and promoting Factor I-mediated cleavage of C3b [38,39]. While activation of the alternative complement pathway is essential for the rapid deposition of opsonins and assembly of lytic membrane attack complexes on *Vibrio* cell surfaces, unconstrained complement activation can trigger destructive host tissue damage and systemic immunopathology [38,40]. The sustained transcriptional induction of CFH in group V4 at 21 DPV occurred alongside changes in serum bactericidal activity. This activity reached its peak killing capacity in this cohort (BSP $8.31 \pm 0.72\%$). This association is consistent with vaccine-related changes in immune responses and the observed 100% relative percent survival under the experimental challenge conditions. However, CFH transcript abundance does not directly demonstrate complement activation,

tissue protection, or complement-mediated bacterial clearance, as these mechanisms were not specifically evaluated in the present study.

Cystatin B, which is an inhibitor of cysteine proteases and plays a role in antigen processing and immune cell function, reached its highest level of expression in the head kidney of the V3 group at 7 DPV and remained elevated until 21 DPV. Persistent cystatin B expression may indicate prolonged regulation of antigen processing and immune cell activation, potentially supporting the development of protective adaptive immunity. This agrees with earlier reports which showed that cystatin B is induced during both bacterial and viral infections and plays a role in the innate immune responses of fish [41–43]. The transient downregulation of cystatin B in the spleen at 7 DPV, followed by sustained upregulation at 14 and 21 DPV in the V4 group, suggests a temporally regulated response involving intracellular cysteine protease control. Given the role of lysosomal cysteine proteases in protein turnover and antigen processing, this expression pattern may reflect dynamic modulation of intracellular proteolytic pathways in teleost immune tissues rather than persistent, generalized immune activation.

Heat shock proteins (HSPs) function as molecular chaperones and are involved in antigen processing and presentation, lymphocyte activation, and innate immune signalling [44]. In the present study, HSP70 and HSP90 α were upregulated at 21 DPV in both the spleen and head kidney of the V3 and V4 groups, suggesting the involvement of molecular chaperone-associated pathways during the later stages of the vaccine-induced immune response. The similar expression patterns of HSP70 and HSP90 α may indicate coordinated regulation of these stress-response genes following vaccination. Consistent with these observations, Huang et al. [45] identified HSP90AA1.2 as a central hub gene in protein–protein interaction networks associated with vaccine-induced immune responses in largemouth bass (*Micropterus salmoides*) following immunization with an inactivated viral hemorrhagic septicemia virus vaccine. These findings suggest that HSP90-associated pathways may contribute to immune regulation following vaccination. Furthermore, the elevated expression of HSP70 and HSP90 α in the vaccine groups exhibiting greater protective efficacy is consistent with previous observations linking heat-shock proteins to immune responses in fish [31,44–46]. Overall, the observed changes in serum immunological parameters and immune-related gene expression suggest that rGAPDH vaccination was associated with multiple components of the host immune response. Further functional investigations are required to establish the specific contributions of these proteins to vaccine-induced protection against *V. alginolyticus*.

Although the present study provides an effective vaccine development formulation against *V. alginolyticus*, it has certain limitations. The immune responses were examined up to 21 DPV, and the duration of long-term protection remains unknown. The challenge experiment used a single *V. alginolyticus* strain, and cross-protection against other *Vibrio* species or strains was not evaluated. No apparent adverse clinical signs or abnormal behaviour were observed in vaccinated fish during the study; however, longer-term safety remains to be evaluated. Previous human immunological studies have raised concerns regarding potential autoimmune cross-reactivity associated with conserved GAPDH epitopes [47]. Nevertheless, these findings cannot be directly extrapolated to teleost fish, and further studies are required to evaluate the long-term safety and potential cross-reactivity of the rGAPDH vaccine.

Additionally, in the present study, the intraperitoneal route was used to deliver a defined dose of rGAPDH and evaluate its protective efficacy in Asian seabass against *V. alginolyticus*. In practical terms, IP vaccination would be feasible during the hatchery or nursery phase, or immediately before transfer of the fish to sea cages. However, the practical application of IP vaccination in open-sea Asian seabass cage culture might present

some challenges, such as individual fish handling and carrying out the injection. Therefore, field-scale trials under commercial cage-farming conditions are necessary to assess vaccine efficacy and duration of protection under variable environmental conditions, as well as handling-associated stress, operational feasibility, and cost-effectiveness.

5. Conclusions

This study demonstrates, for the first time, that a 37 kDa rGAPDH subunit vaccine formulated with 10 µg of antigen and aluminum hydroxide adjuvant achieved 100% RPS in Asian seabass challenged with *V. alginolyticus* under the experimental conditions. Vaccination elicited substantial immune responses, as evidenced by serum agglutination titers, bactericidal activity, and lysozyme activity. Moreover, the tissue-specific and temporal modulation of complement factor H, cystatin B, HSP70, and HSP90α gene expression in vaccinated fish provides molecular evidence of alterations in immune-related pathways following rGAPDH vaccination, particularly in the V4 group. These findings suggest that vaccination may influence multiple immune-related processes, including complement regulation, protease inhibition, cellular stress responses, and immune signalling. Collectively, the results support the potential of rGAPDH as a protective vaccine antigen against *V. alginolyticus* in Asian seabass. The higher RPS observed in the adjuvanted formulations further suggests that aluminum hydroxide may enhance vaccine efficacy under the experimental conditions evaluated. Further studies are warranted to assess the duration of protective immunity, potential cross-protection against different *Vibrio* species and strains, and the efficacy of the rGAPDH subunit vaccine under commercial aquaculture conditions. Overall, this study provides a foundation for the further development of a recombinant subunit vaccine against *V. alginolyticus*, with the potential to improve fish health, support sustainable marine aquaculture, and reduce reliance on antibiotics for disease management.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/pathogens15111116/s1>: Table S1: Dose–response mortality and Reed–Muench estimation of the median lethal dose (LD₅₀) of *Vibrio alginolyticus* CMFRI-JF260912 in Asian seabass (*Lates calcarifer*). Table S2: Cumulative mortality and relative recent survival in Asian seabass challenged with *V. alginolyticus* at 21 DPV. Table S3: Comparison of the relative percent survival of the developed rGAPDH vaccine with previous vaccines against fish pathogens.

Author Contributions: Conceptualization, M.A.P.; methodology, M.A.P., T.S.A.S. and N.K.S.; investigation, M.A.P., C.S.S., E.A., N.K., N.A. and T.V.A.K.; data analysis, N.K.S., G.K. and C.G.J.; validation, M.A.P., C.S.S., N.R.D., T.S.A.S. and G.K.; writing—original draft preparation, M.A.P. and G.K.; writing—review and editing, M.A.P., G.K. and N.K.S. All authors have read and agreed to the published version of the manuscript.

Funding: This work was carried out under the project entitled “Consortium Research Platform on Vaccines and Diagnostics (project no: 1007832)” funded by the National Agricultural Innovation Project and “Health Monitoring and Management strategies for Marine Finfish and Shellfish” (MBFNHD/HM/23), funded by ICAR–Central Marine Fisheries Research Institute, Kochi.

Institutional Review Board Statement: The animal study protocol was approved by the Institute Research Committee of ICAR–Central Marine Fisheries Research Institute, Kochi (Approval No. MBT/HM/23; dated 30 April 2020).

Informed Consent Statement: Not applicable.

Data Availability Statement: The GAPDH sequence has been deposited in GenBank under the accession number PZ760168.

Acknowledgments: The authors would like to thank the Director, ICAR-CMFRI, Kochi, for providing the necessary facilities to undertake this work. Research was supported by the Indian Council of

Agricultural Research, Department of Agricultural Research and Education, Government of India. “During the preparation of the manuscript, the author(s) used Gemini Pro for the purpose of creating Figure 1. The authors have reviewed and edited the output and take full responsibility for the content of this Figure”.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

BSP	Bacterial survival percentage
DPV	Day post-vaccination
HSP	Heat shock protein
rGAPDH	recombinant glyceraldehyde-3-phosphate dehydrogenase
RPS	Relative percent survival
<i>V. alginolyticus</i>	<i>Vibrio alginolyticus</i>

References

1. Ina-Salwany, M.Y.; Al-Saari, N.; Mohamad, A.; Mursidi, F.A.; Mohd-Aris, A.; Amal, M.N.A.; Kasai, H.; Mino, S.; Sawabe, T.; Zamri-Saad, M. Vibriosis in Fish: A Review on Disease Development and Prevention. *J. Aquat. Anim. Health* **2019**, *31*, 3–22. [CrossRef] [PubMed]
2. Joseph, I.; Joseph, S.; Ignatius, B.; Rao, G.S.; Sobhana, K.S.; Prema, D.; Varghese, M. A pilot study on culture of Asian seabass *Lates calcarifer* (Bloch) in open sea cage at Munambam, Cochin coast, India. *Indian J. Fish.* **2010**, *57*, 29–33.
3. Philipose, K.K.; Sharma, S.R.K.; Loka, J.; Damodaran, D.; Narsimhulu, S.; Dube, P. Culture of Asian seabass (*Lates calcarifer*, Bloch) in open sea floating net cages off Karwar, South India. *Indian J. Fish.* **2013**, *60*, 67–70.
4. Sharma, S.R.K.; Rathore, G.; Verma, D.K.; Sadhu, N.; Philipose, K.K. *Vibrio alginolyticus* infection in Asian seabass (*Lates calcarifer*, Bloch) reared in open sea floating cages in India. *Aquac. Res.* **2012**, *44*, 86–92. [CrossRef]
5. Rameshkumar, P.; Nazar, A.K.A.; Pradeep, M.A.; Kalidas, C.; Jayakumar, R.; Tamilmani, G.; Sakthivel, M.; Samal, A.K.; Sirajudeen, S.; Venkatesan, V.; et al. Isolation and characterization of pathogenic *Vibrio alginolyticus* from sea cage cultured cobia (*Rachycentron canadum* (Linnaeus 1766)) in India. *Lett. Appl. Microbiol.* **2017**, *65*, 423–430. [CrossRef] [PubMed]
6. Raharjo, H.M.; Budiyanah, H.; Mursalin, M.F.; Chokmangmeepisarn, P.; Sakulworakan, R.; Madyod, S.; Sewaka, M.; Sonthi, M.; Debnath, P.P.; Elayaraja, S.; et al. Distribution of Vibrionaceae in farmed Asian sea bass, *Lates calcarifer* in Thailand and their high prevalence of antimicrobial resistance. *J. Fish Dis.* **2022**, *45*, 1355–1371. [CrossRef] [PubMed]
7. Pepi, M.; Focardi, S. Antibiotic-Resistant Bacteria in Aquaculture and Climate Change: A Challenge for Health in the Mediterranean Area. *Int. J. Environ. Res. Public Health* **2021**, *18*, 5723. [CrossRef] [PubMed]
8. Defoirdt, T. Interfering with the response of bacterial pathogens to host substances: An unexplored strategy to control bacterial diseases in aquaculture. *Microbiol. Res.* **2026**, *308*, 128492. [CrossRef] [PubMed]
9. Rathor, G.S.; Swain, B. Advancements in Fish Vaccination: Current Innovations and Future Horizons in Aquaculture Health Management. *Appl. Sci.* **2024**, *14*, 5672. [CrossRef]
10. Du, Y.; Hu, X.; Miao, L.; Chen, J. Current status and development prospects of aquatic vaccines. *Front. Immunol.* **2022**, *13*, 1040336. [CrossRef] [PubMed]
11. Tamas, I.; Bitchava, K.; Gelasakis, A.I. Advances in Vaccine Adjuvants for Teleost Fish: Implications for Aquatic Welfare and the Potential of Nanoparticle-Based Formulations. *Vaccines* **2024**, *12*, 1347. [CrossRef] [PubMed]
12. Priya, T.A.J.; Kappalli, S. Modern biotechnological strategies for vaccine development in aquaculture—Prospects and challenges. *Vaccine* **2022**, *40*, 5873–5881. [CrossRef] [PubMed]
13. Perez-Casal, J.; Potter, A.A. Glyceraldehyde-3-phosphate dehydrogenase as a suitable vaccine candidate for protection against bacterial and parasitic diseases. *Vaccine* **2016**, *34*, 1012–1017. [CrossRef] [PubMed]
14. Liu, Y.; Oshima, S.; Kurohara, K.; Ohnishi, K.; Kawai, K. Vaccine efficacy of recombinant GAPDH of *Edwardsiella tarda* against *Edwardsiellosis*. *Microbiol. Immunol.* **2005**, *49*, 605–612. [CrossRef] [PubMed]
15. Zhang, C.; Yu, L.; Qian, R. Characterization of OmpK, GAPDH and their fusion OmpK-GAPDH derived from *Vibrio harveyi* outer membrane proteins: Their immunoprotective ability against vibriosis in large yellow croaker (*Pseudosciaena crocea*). *J. Appl. Microbiol.* **2007**, *103*, 1587–1599. [CrossRef] [PubMed]
16. Liu, S.P.; Chuang, S.C.; Yang, C.D. Protective Immunity against *Vibrio harveyi* in Grouper Induced by Single Vaccination with Poly (Lactide-co-glycolide) Microparticles Releasing Pleurocidin Peptide and Recombinant Glyceraldehyde-3-phosphate Dehydrogenase. *Vaccines* **2020**, *8*, 33. [CrossRef] [PubMed]

17. Cao, T.T.; Tsai, M.A.; Yang, C.D.; Wang, P.C.; Kuo, T.Y.; Chen, H.C.G.; Chen, S.C. Vaccine efficacy of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from *Edwardsiella ictaluri* against *E. tarda* in tilapia. *J. Gen. Appl. Microbiol.* **2014**, *60*, 241–250. [[CrossRef](#)] [[PubMed](#)]
18. Li, X.; Wu, H.; Zhang, M.; Liang, S.; Xiao, J.; Wang, Q.; Liu, Q.; Zhang, Y. Secreted glyceraldehyde-3-phosphate dehydrogenase as a broad spectrum vaccine candidate against microbial infection in aquaculture. *Lett. Appl. Microbiol.* **2012**, *54*, 1–9. [[CrossRef](#)] [[PubMed](#)]
19. Reed, L.J.; Muench, H. A simple method of estimating fifty per cent endpoints. *Am. J. Epidemiol.* **1938**, *27*, 493–497. [[CrossRef](#)]
20. Lowry, O.H.; Rosebrough, N.J.; Farr, A.L.; Randall, R.J. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **1951**, *193*, 265–275. [[CrossRef](#)]
21. Roberson, B.S. Bacterial agglutination. In *Techniques in Fish Immunology*; Stolen, J.S., Fletcher, T.C., Anderson, D.P., Roberson, B.S., van Muiswinkel, W.B., Eds.; SOS Publications: Fair Haven, NJ, USA, 1990; pp. 81–86.
22. Vasudeva Rao, Y.; Das, B.K.; Jyotirmayee, P.; Chakrabarti, R. Effect of *Achyranthes aspera* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. *Fish Shellfish Immunol.* **2006**, *20*, 263–273. [[CrossRef](#)] [[PubMed](#)]
23. Ellis, A.E. Lysozyme assays. In *Techniques in Fish Immunology*; Fish Immunology Technical Communications, No. 1; Stolen, J.S., Fletcher, T.C., Anderson, D.P., Roberson, B.S., van Muiswinkel, W.B., Eds.; SOS Publications: Fair Haven, NJ, USA, 1990; pp. 101–103.
24. Mohd-Shaharuddin, N.; Mohd-Adnan, A.; Kua, B.C.; Nathan, S. Expression profile of immune-related genes in *Lates calcarifer* infected by *Cryptocaryon irritans*. *Fish Shellfish Immunol.* **2013**, *34*, 762–769. [[CrossRef](#)] [[PubMed](#)]
25. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* **2001**, *25*, 402–408. [[CrossRef](#)] [[PubMed](#)]
26. Reed, S.; Orr, M.; Fox, C. Key roles of adjuvants in modern vaccines. *Nat. Med.* **2013**, *19*, 1597–1608. [[CrossRef](#)] [[PubMed](#)]
27. Jiang, J.; Zheng, Z.; Wang, K.; Wang, J.; He, Y.; Wang, E.; Chen, D.; Ouyang, P.; Geng, Y.; Huang, X. Adjuvant Immune Enhancement of Subunit Vaccine Encoding pSCPI of *Streptococcus iniae* in Channel Catfish (*Ictalurus punctatus*). *Int. J. Mol. Sci.* **2015**, *16*, 28001–28013. [[CrossRef](#)] [[PubMed](#)]
28. Cao, J.; Zhang, J.; Ma, L.; Li, L.; Zhang, W.; Li, J. Identification of fish source *Vibrio alginolyticus* and evaluation of its bacterial ghosts vaccine immune effects. *Microbiologyopen* **2018**, *7*, e00576. [[CrossRef](#)] [[PubMed](#)]
29. Zhang, Y.; Ning, D.; Nie, J.; Hou, X.; Li, W.; Gan, Z.; Lu, Y. Evaluation of Protective Immune Response of Live-Attenuated Candidate Vaccines $\Delta cpxA$ and $\Delta cpxR$ against *Vibrio alginolyticus* in Pearl Gentian Grouper. *Fish Shellfish Immunol.* **2025**, *159*, 110183. [[CrossRef](#)] [[PubMed](#)]
30. Liang, S.; Wu, H.; Liu, B.; Xiao, J.; Wang, Q.; Zhang, Y. Immune response of turbot (*Scophthalmus maximus* L.) to a broad spectrum vaccine candidate, recombinant glyceraldehyde-3-phosphate dehydrogenase of *Edwardsiella tarda*. *Vet. Immunol. Immunopathol.* **2012**, *150*, 198–205. [[CrossRef](#)] [[PubMed](#)]
31. Pham, T.H.; Cheng, T.C.; Wang, P.C.; Chen, S.C. Protective efficacy of four heat-shock proteins as recombinant vaccines against photobacteriosis in Asian seabass (*Lates calcarifer*). *Fish Shellfish Immunol.* **2021**, *111*, 179–188. [[CrossRef](#)] [[PubMed](#)]
32. Li, F.; Ge, H.; Lin, P.; Wang, Y.; Jiang, Y.; Zhang, Z.; Chen, Y.; Feng, J. Genome-based screening and immunogenicity evaluation of outer membrane protein antigens for *Vibrio harveyi* subunit vaccine in pearl gentian grouper (*Epinephelus lanceolatus* × *Epinephelus fuscoguttatus*). *Fish Shellfish Immunol.* **2025**, *165*, 110440. [[CrossRef](#)] [[PubMed](#)]
33. Li, J.; Tang, L.; Li, S.; Li, G.; Mo, Z. The efficacy and side-effects of oil-based adjuvants emulsified *Vibrio anguillarum* bivalent inactivated vaccine in turbot (*Scophthalmus maximus*) under production mode. *Aquaculture* **2020**, *524*, 735259. [[CrossRef](#)]
34. Dar, S.A.; Kole, S.; Shin, S.M.; Jeong, H.J.; Jung, S.J. Comparative study on antigen persistence and immunoprotective efficacy of intramuscular and intraperitoneal injections of squalene-Aluminium hydroxide (Sq + Al) adjuvanted viral hemorrhagic septicaemia virus vaccine in olive flounder (*Paralichthys olivaceus*). *Vaccine* **2021**, *39*, 6866–6875. [[CrossRef](#)] [[PubMed](#)]
35. Nguyen, H.T.; Nguyen, T.T.T.; Chen, Y.C.; Vu-Khac, H.; Wang, P.C.; Chen, S.C. Enhanced immune responses and effectiveness of refined outer membrane protein vaccines against *Vibrio harveyi* in orange-spotted grouper (*Epinephelus coioides*). *J. Fish Dis.* **2018**, *41*, 1349–1358. [[CrossRef](#)] [[PubMed](#)]
36. Mondal, H.; Thomas, J. A review on the recent advances and application of vaccines against fish pathogens in aquaculture. *Aquac. Int.* **2022**, *30*, 1971–2000. [[CrossRef](#)] [[PubMed](#)]
37. Song, Q.; Xiao, Y.; Xiao, Z.; Liu, T.; Li, J.; Li, P.; Han, F. Lysozymes in Fish. *J. Agric. Food Chem.* **2021**, *69*, 15039–15051. [[CrossRef](#)] [[PubMed](#)]
38. Qi, P.; Wu, B.; Guo, B.; Zhang, C.; Xu, K. The complement factor H (CFH) and its related protein 2 (CFHR2) mediating immune response in large yellow croaker *Larimichthys crocea*. *Dev. Comp. Immunol.* **2018**, *84*, 241–249. [[CrossRef](#)] [[PubMed](#)]
39. Wei, X.; Wu, Z.; Zhang, T.; Lei, Y.; Chen, M.; Yang, Y.; Gao, A.; Guo, Z.; Ye, J. Functional characterization of complement factor H in host defense against bacterial pathogen in Nile tilapia (*Oreochromis niloticus*). *Fish Shellfish Immunol.* **2022**, *129*, 114–126. [[CrossRef](#)] [[PubMed](#)]

40. Ma, X.; Wei, M.; Chen, H.; Zhang, J.; Chen, Q.; Chen, S.; Zhou, Q. Immune and regulative characterization of complement-related gene *cfhl5* in response to *Vibrio harveyi* challenge in *Cynoglossus semilaevis*. *Fish Shellfish Immunol.* **2024**, *152*, 109755. [[CrossRef](#)] [[PubMed](#)]
41. Wei, S.; Han, H.; Xu, S.; Huang, Y.; Wei, J.; Qin, Q. Identification and functional characterization of Cystatin B in orange-spotted grouper, *Epinephelus coioides*. *Dev. Comp. Immunol.* **2022**, *132*, 104383. [[CrossRef](#)] [[PubMed](#)]
42. Xiao, P.P.; Hu, Y.H.; Sun, L. *Scophthalmus maximus* cystatin B enhances head kidney macrophage-mediated bacterial killing. *Dev. Comp. Immunol.* **2010**, *34*, 1237–1241. [[CrossRef](#)] [[PubMed](#)]
43. Kodagoda, Y.K.; Liyanage, D.S.; Omeke, W.K.M.; Kwon, H.; Hwang, S.D.; Lee, J. Molecular characterization, expression, and functional analysis of cystatin B in the big-belly seahorse (*Hippocampus abdominalis*). *Fish Shellfish Immunol.* **2022**, *124*, 442–453. [[CrossRef](#)] [[PubMed](#)]
44. Mosca, F.; Romano, N.; Malatesta, D.; Ceccarelli, G.; Brunetti, A.; Bulfon, C.; Volpatti, D.; Abelli, L.; Galeotti, M.; Falconi, A.; et al. Heat shock protein 70 kDa (HSP70) increase in sea bass (*Dicentrarchus labrax*, L 1758) thymus after vaccination against *Listonella anguillarum*. *Fish Physiol. Biochem.* **2013**, *39*, 615–626. [[CrossRef](#)] [[PubMed](#)]
45. Huang, H.; Lu, X.; Guo, J.; Chen, Y.; Yi, M.; Jia, K. Protective Efficacy and Immune Responses of Largemouth Bass (*Micropterus salmoides*) Immunized with an Inactivated Vaccine against the Viral Hemorrhagic Septicemia Virus Genotype IVa. *Fish Shellfish Immunol.* **2024**, *151*, 109691. [[CrossRef](#)] [[PubMed](#)]
46. Xie, Y.; Song, L.; Weng, Z.; Liu, S.; Liu, Z. Hsp90, Hsp60 and sHsp families of heat shock protein genes in channel catfish and their expression after bacterial infections. *Fish Shellfish Immunol.* **2015**, *44*, 642–651. [[CrossRef](#)] [[PubMed](#)]
47. Razim, A.; Pacyga, K.; Aptekorz, M.; Martirosian, G.; Szuba, A.; Pawlak-Adamska, E.; Brzychczy-Włoch, M.; Myc, A.; Gamian, A.; Górka, S. Epitopes identified in GAPDH from *Clostridium difficile* recognized as common antigens with potential autoimmunizing properties. *Sci. Rep.* **2018**, *8*, 13946. [[CrossRef](#)] [[PubMed](#)]

Disclaimer/Publisher’s Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.