

Comparative efficacy and immune responses induced by aluminium hydroxide gel (Algel) adjuvanted and non-adjuvanted formalin killed *Vibrio alginolyticus* vaccines in Asian seabass *Lates calcarifer* (Bloch, 1790)

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Abstract

Vibriosis caused by *Vibrio alginolyticus* is an important bacterial disease affecting Asian seabass *Lates calcarifer* (Bloch, 1790). This study evaluated the protective efficacy and immune responses induced by two intraperitoneally administered formalin killed *V. alginolyticus* vaccine formulations: a non-adjuvanted bacterin (T1) and an aluminium hydroxide gel (Algel) adjuvanted bacterin (T2). Phosphate buffered saline (PBS) injected fish served as the challenged control (T0), while a non-challenged naive group (T3) was maintained to monitor background mortality. Each vaccine was administered at a dose of 0.2 ml containing 1×10^8 CFU-equivalent per fish. Protection was assessed three weeks after vaccination following intraperitoneal challenge with live *V. alginolyticus* at 1×10^8 CFU per fish. Serum agglutination titre, serum bactericidal activity, serum lysozyme activity, and the expression of complement factor H (CFH), Cystatin B, HSP70, and HSP90α in the spleen and head kidney were evaluated at 7, 14 and 21 days post-vaccination (DPV). T2 showed the highest survival (90%) and relative percent survival (RPS; 86.4%), followed by T1 (80% survival; 72.7% RPS), whereas the T0 challenged control showed 26.6% survival and no mortality was observed in the non-challenged naive group (T3). T2 produced higher serum agglutination titres and lysozyme activity, particularly at 14 DPV. At 21 DPV, bacterial survival percentage was similar between T1 (77.2%) and T2 (77.9%; $p > 0.05$), but both were lower than the T0 control (92.1%). Immune gene expression showed tissue- and time-dependent patterns without a uniform response across the four genes. Overall, both formalin killed vaccine formulations provided substantial protection under experimental challenge conditions, with the Algel adjuvanted formulation demonstrating enhanced survival, RPS, and early humoral and innate immune responses. The practical advantages of Algel over the non-adjuvanted formulation requires further evaluation through analysis of cost-effectiveness, assessment of duration of protective immunity, booster vaccination studies and field studies.

Introduction

Vibrio alginolyticus is a halophilic Gram negative bacterium associated with disease in marine animals and occasional human infection (Schmidt *et al.*, 1979; Manchanayake *et al.*, 2023). In Asian seabass *Lates calcarifer* (Bloch, 1790), a widely farmed euryhaline carnivorous fish in South and South-east

Asia, *V. alginolyticus* infection can cause mass mortality in warm water culture systems (Krupesha Sharma *et al.*, 2012); the same pathogen has also caused epizootic mortality in other cage-farmed marine finfish in India, including cobia (Rameshkumar *et al.*, 2014, 2017). Bacterial pathogens are important causes of disease and production losses in aquaculture, and increasing concern regarding antimicrobial resistance has strengthened

the need for preventive approaches such as vaccination (Nathan *et al.*, 2021; Kumareswaran and Jayasinghe, 2022). Antibiotics have been used to control bacterial diseases in aquaculture, but repeated or indiscriminate use favours antimicrobial resistance, alters beneficial microbiota, and raises concerns about drug residues in seafood (Serwecińska, 2020; Pepi and Focardi, 2021). Vaccination is therefore a preferred preventive approach for reducing bacterial disease losses in cultured fish (Ma *et al.*, 2019; Rosini *et al.*, 2020; Mkulo *et al.*, 2024).

Formalin inactivated whole cell vaccines, or bacterins, are widely used because they are relatively simple to prepare and provide a safe approach for stimulating protective immunity (Dadar *et al.*, 2017; Ma *et al.*, 2019). However, their immunogenicity and duration of protection may be improved by incorporating suitable adjuvants. Aluminium hydroxide gel (Algel) is an aluminium based adjuvant that has been widely investigated for its ability to enhance antigen retention and immune stimulation and has also been evaluated in fish vaccine formulations (Jiao *et al.*, 2010; Tammam *et al.*, 2024). This study assessed vaccine responses using serum assays and immune related gene expression analyses. Serum agglutination titres were used to evaluate antibody response (Dhasarathan *et al.*, 2014). Serum bactericidal activity was assessed based on the serum mediated killing of *V. alginolyticus*, while lysozyme activity was measured as an indicator of the innate antibacterial response (Saurabh and Sahoo, 2008; Biller *et al.*, 2021; Miccoli *et al.*, 2021). Quantitative real-time PCR (RT-qPCR) was used to quantify expression of CFH, Cystatin B, HSP70, and HSP90α in spleen and head kidney, two key lymphoid tissues in teleost fish (Nguyen *et al.*, 2017; Rebl and Goldammer, 2018; Du *et al.*, 2022). Intraperitoneal injection was selected because it ensures uniform antigen delivery and dose control in experimental fish vaccine trials (Bromage and Owens, 2002; Mutoloki *et al.*, 2015; Meza *et al.*, 2019). HSP70 and HSP90α were included as stress responsive molecular chaperones to assess whether vaccination and adjuvant administration elicited cellular stress associated and innate response signatures in lymphoid tissues; they were not intended as direct markers of protective immunity (Tort *et al.*, 2003; Celi *et al.*, 2012).

The objective of the present study was to compare the protective efficacy and selected immune responses induced by non-adjuvanted (T1) and Algel adjuvanted (T2) formalin killed *V. alginolyticus* whole cell vaccines in Asian seabass. The two formulations were evaluated under a defined intraperitoneal vaccination and challenge model using survival, serum humoral and innate immune parameters, and transcriptional responses in the head kidney and spleen.

Materials and methods

All experimental procedures involving live fish were conducted in accordance with the ARRIVE guidelines 2.0 (Percie du Sert *et al.*, 2020) and were approved by the Institute Research Council of ICAR-Central Marine Fisheries Research Institute (ICAR-CMFRI), Kochi.

Experimental fish

A total of 450 clinically healthy *L. calcarifer*, with a mean body weight of 15±3 g and length of 10±2 cm, were procured from the Rajiv Gandhi Centre for Aquaculture (RGCA) Seabass Hatchery, Nagapattinam, Tamil Nadu, India. Fish were transported to the

Laboratory of the Marine Biotechnology, Fish Nutrition and Health Division (MBFNHD), ICAR-Central Marine Fisheries Research Institute (ICAR-CMFRI), Kochi, Kerala, India and acclimatised for two weeks in ten 1,000 l capacity, aerated recirculating tanks maintained at 28±3°C. Fish were screened before experimental use by bacteriological culture on Thiosulfate Citrate Bile Salts Sucrose (TCBS) agar to confirm the absence of detectable *V. alginolyticus*. Following acclimation, fish were randomly allocated to the experimental tanks and treatment groups. The allocation of fish for vaccination, immune response sampling, and challenge experiments was predetermined to maintain the specified biological and tank replicates.

Bacterial strain and culture conditions

A pathogenic strain of *Vibrio alginolyticus* (CMFRI-JF260912; ICAR-CMFRI, Kochi, India) was used throughout this study. The strain was preserved in Tryptone Soy Broth (TSB; HiMedia) supplemented with 2% NaCl and 20% glycerol at -80°C. Working cultures were revived in TSB supplemented with 2% NaCl for 24 h at 30°C with gentle shaking and adjusted to 1×10⁸ colony forming units per millilitre (CFU ml⁻¹) prior to use.

Determination of lethal dosage (LD50)

The dose required to cause 50% mortality was estimated using 10³ to 10⁸ CFU fish⁻¹. Asian seabass were maintained in 250 l glass tanks with filtered seawater and continuous aeration. Each dose was tested in duplicate tanks with 10 fish per tank (20 fish dose⁻¹). Fish were injected intraperitoneally (i.p.) with 0.1 ml bacterial suspension containing 10³, 10⁴, 10⁵, 10⁶, 10⁷, or 10⁸ CFU fish⁻¹, respectively. Control fish received 0.1 ml sterile PBS. Fish were observed for 7 days, and mortality was recorded daily. Dead or moribund fish were examined bacteriologically immediately after death or humane removal. LD50 was calculated from cumulative mortality following Reed and Muench (1938).

Formalin killed vaccine preparation and vaccination

Bacterial cells were cultured in TSB with 2% NaCl, harvested by centrifugation (6,000 g, 5 min), and resuspended in sterile PBS. The bacterial concentration was adjusted during formulation so that the final T1 and T2 vaccine preparations each contained 5×10⁸ CFU equivalent ml⁻¹, corresponding to 1 × 10⁸ CFU equivalent per 0.2 ml dose. Inactivation was achieved by adding formalin to a final concentration of 0.5% (v/v) (Li *et al.*, 2016; Silvaraj *et al.*, 2020), followed by overnight incubation at 30°C with gentle agitation and further incubation at 4°C overnight. Complete inactivation was confirmed by plating on TSA supplemented with 2% NaCl and incubating for 48 h at 30°C, showing no visible bacterial growth. The inactivated cells were washed three times in PBS by centrifugation to remove residual formalin. Two vaccine formulations were prepared: T1: Formalin killed cells in PBS without adjuvant and T2: Formalin killed cells mixed with aluminium hydroxide gel (Algel; Sigma-Aldrich, USA). A sterile 2% (w/v; 20 mg ml⁻¹) stock suspension of hydrated aluminium hydroxide gel was blended with the bacterin suspension in a 1:3 (v/v) ratio (2.5 ml stock gel plus 7.5 ml antigen suspension per 10 ml total volume) to

deliver a final concentration of 5.0 mg ml⁻¹ (1.0 mg Algel per 0.2 ml dose per fish) following formulation matrices. The adjuvant antigen matrix was agitated slowly overnight at 4°C to ensure complete adsorption.

A PBS injected challenged group served as the mock vaccinated control (T0), and an un-injected/non-challenged group served as the naive control (T3). Fish were anaesthetised with MS-222 (100 mg l⁻¹) and vaccinated by intraperitoneal injection of 0.2 ml formulation containing 1×10⁸ CFU-equivalent per fish. Control fish received 0.2 ml sterile PBS. Fish were maintained in 300 l recirculating tanks with continuous aeration at 28±3°C and salinity of 25-30 ppt. Fish were fed a commercial pelleted diet at 5% body weight per day, distributed equally across multiple daily feeding sessions to optimise feed utilisation, support immune competence, and prevent size variation or aggression. Water pH, dissolved oxygen, and ammonia were monitored daily.

Immunological parameters

At each sampling time (7, 14, and 21 DPV), blood was collected from three fish from each of three replicate tanks per treatment group, giving nine sampled fish per treatment at each time point (n=9). Fish were anaesthetised before blood collection, and blood was obtained by caudal vein puncture using a 23-gauge needle following Tancredo *et al.* (2015). Blood was allowed to clot for 30 min at room temperature and then centrifuged at 1,500 *g* for 15 min at 4°C. The resulting serum was collected and stored at -20°C until analysis.

Serum agglutination assay

Antigen-specific agglutination titres were determined using the modified method of Dhasarathan *et al.* (2014). Serum samples (50 µl) were serially diluted twofold in 0.85% saline in U-bottom 96-well microtitre plates. An equal volume (50 µl) of *V. alginolyticus* suspension (1×10⁸ CFU ml⁻¹) was added to each well. Plates were incubated at 25°C for 2 h followed by overnight incubation at 4°C. The endpoint titre was defined as the highest serum dilution showing visible agglutination or diffuse mat formation, whereas compact button formation was considered negative. Titres were expressed as log₂ values.

Serum bactericidal activity

Bactericidal activity was assessed following Vasudeva Rao *et al.* (2006) with modifications. A mixture of 2 µl *V. alginolyticus* suspension (OD₆₀₀ = 1) and 20 µl fish serum was incubated at 25°C for 1 h in 96 well plates. The mixture was plated on TCBS agar and incubated at 30°C for 18 h. Bacterial survival percentage (BSP) was calculated as: BSP = (log₁₀ CFU in serum sample / log₁₀ CFU in initial culture)×100, following Byadgi *et al.* (2018). All assays were performed in triplicate.

Serum lysozyme activity

Lysozyme activity was measured turbidimetrically using lyophilised *Micrococcus lysodeikticus* (Sigma-Aldrich, USA) following Biller *et al.* (2021) with modifications. Fish serum (20 µl) was added in duplicate

to a U-bottom 96-well plate; 180 µl of *M. lysodeikticus* (0.02% w/v in 66 mM potassium phosphate buffer, pH 6.24) was added to each well. Absorbance at 450 nm was read immediately and after 30 min at 25°C. Results were expressed in µg ml⁻¹.

Pathogenic challenge and relative percent survival

Three weeks after vaccination, vaccinated and control fish were challenged intraperitoneally with live *V. alginolyticus*. Ten fish from each replicate tank were challenged, giving 30 fish per treatment group. Fish were anaesthetised with MS-222 and injected intraperitoneally with 0.1 ml of live bacterial suspension containing 1×10⁸ CFU fish⁻¹. The estimated LD50 of the isolate was 10^{4.05} CFU fish⁻¹; therefore, the challenge dose used in the present study was approximately 8.9×10³ fold higher than the estimated LD50. This higher challenge dose was selected to produce substantial infection pressure and mortality in the challenged control while retaining sufficient survival in vaccinated groups to permit statistical discrimination of protective efficacy. T0 group received sterile PBS before challenge and served as the challenged control. T3 fish remained non-vaccinated and non-challenged and were maintained to monitor background mortality. Challenged fish were maintained under the same water quality and husbandry conditions and monitored twice daily for 14 days. Mortality and clinical signs were recorded throughout the observation period. *V. alginolyticus* was re-isolated from heart blood and kidney samples of dead or moribund fish and confirmed by PCR followed by Sanger sequencing.

Relative percent survival (RPS) was calculated as:

RPS (%) = [1 - (% Mortality in vaccinated group / % Mortality in T0 challenged control)] × 100 (Amend, 1981).

Quantitative RT-PCR (RT-qPCR) gene expression analysis

Spleen and head kidney tissues were collected at 7, 14, and 21 DPV. Total RNA was extracted using TRIzol reagent (Invitrogen, USA), quantified by spectrophotometry (A260/A280), and verified by agarose gel electrophoresis. Residual genomic DNA was eliminated by DNase I treatment (New England Biolabs, UK). First-strand cDNA was synthesised from 1 µg of gDNA-free total RNA using the iScript™ cDNA Synthesis Kit (Bio-Rad, USA) containing pre-blended oligo(dT) and random hexamer primers.

Real-time PCR amplifications were coordinated in technical and biological triplicates on a Roche LightCycler 96 platform using iTaq™ Universal SYBR® Green Supermix (Bio-Rad, USA). Each 20 µl reaction contained 10.0 µl of 2× iTaq Universal SYBR Green Supermix (1× final), 0.8 µl of forward primer (0.4 µM final), 0.8 µl of reverse primer (0.4 µM final), 2.0 µl of diluted cDNA template (50–100 ng total input), and 6.4 µl of nuclease-free water. The thermal cycling parameters consisted of an initial enzyme activation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 10 s, primer annealing at 60–62°C for 30 s, and extension/fluorescence acquisition at 72°C for 30 s. A dissociation melting curve analysis (65°C to 95°C with a heating rate of 0.5°C per 10 s) was performed at the end of each run to verify reaction specificity and single product amplicon formation.

Standard curves generated from 5 fold serial dilutions of pooled cDNA demonstrated amplification efficiencies (E) between 93.2 and 102.8% with linear correlation coefficients ($R^2 > 0.99$) across all target and reference genes. Relative expression was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001; Rao *et al.*, 2013) using elongation factor (EF; GenBank: GQ507427) (Mohd-Shaharuddin *et al.*, 2013) as the constitutive reference gene. Primers for CFH, Cystatin B, HSP70, and HSP90 α were designed using NCBI Primer-BLAST (Table 1).

Statistical analysis

Bacterial survival percentage, lysozyme activity, and agglutination titres are expressed as mean $\pm$ standard error (SE). Gene expression values are expressed as fold change relative to the PBS control at the corresponding sampling time (mean $\pm$ SE; $n=3$ biological replicates). Statistical analyses were performed in GraphPad Prism version 10.0 (GraphPad Software, San Diego, USA). Normality and homogeneity of variance were assessed using Shapiro-Wilk and Levene's tests. Two-way ANOVA was used to analyse immune parameters measured across treatment groups and sampling

times, followed by Tukey's multiple-comparison test. Survival after bacterial challenge was analysed by Kaplan-Meier curves and compared using the log-rank (Mantel-Cox) test. Differences were considered statistically significant at $p < 0.05$.

Results

Determination of LD50

The LD50 of *V. alginolyticus* in Asian seabass was calculated as $10^{4.05}$ CFU fish $^{-1}$ (approximately 1.12×10^4 CFU fish $^{-1}$) via the Reed and Muench (1938) method. The challenge confirmed that the CMFRI-JF260912 isolate was virulent to Asian seabass.

Protective efficacy of formalin killed vaccines

After *i.p.* challenge with *V. alginolyticus* (1×10^8 CFU fish $^{-1}$) at three weeks post-vaccination, T2 showed 90% survival and 86.4% RPS. T1 showed 80% survival and 72.7% RPS. The PBS challenged control (T0) showed 26.6% survival (73.4% cumulative mortality), while the unchallenged naive group (T3) showed no mortality. *V. alginolyticus*

Table 1. Primer sequences, amplicon characteristics, and functional classifications used for RT-qPCR analysis of immune related genes in *L. calcarifer*

Gene target	Accession No.	Primer sequence (5'-3')	Product size (bp)	T _m (°C)	Functional pathway / Category
Complement factor H (CFH)	PZ727114	F: CTGTATCAGAGGAAGACGACAT R: TGGCAAGAAGGCAGGTTTCAT	84	64	Complement and coagulation cascades
Cystatin B (CTB)	PZ727115	F: GAGGTATGTGTGGGGTCTCT R: CTTTTTGCTCTGCATGGGGC	93	69	Protease inhibitor / Macrophage killing
HSP70	PZ727116	F: TACCTCGGCCAAAAGGTGTC R: TCTTTAGTCGCCTGTCGCTG	80	68	Cellular stress / Innate defence
HSP90 α	PZ723671	F: ATGACCCACAGACGCACTCC R: CATGTCCTCAGTGGGAGCAGA	113	70	Chaperone / Cytoskeleton support
Elongation factor (EF)	GQ507427	F: AAATTGGCGGTATTGGAAC R: GGGAGCAAAGGTGACGAC	91	61	Housekeeping reference control

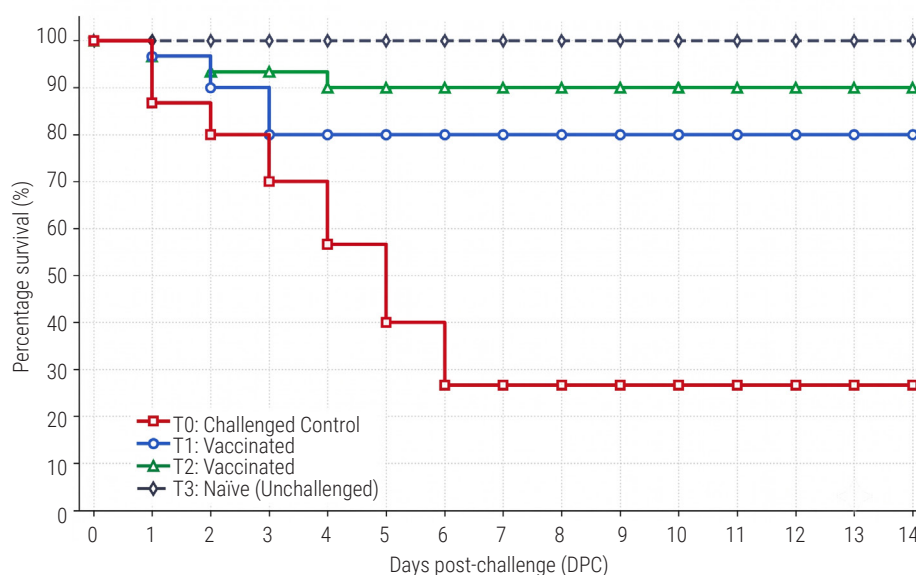


Fig. 1. Kaplan-Meier survival curves of Asian seabass (*L. calcarifer*) vaccinated with formalin killed *V. alginolyticus* without adjuvant (T1) or with Algel adjuvant (T2), together with the PBS challenged control (T0), following intraperitoneal challenge with 1×10^8 CFU per fish at three weeks post-vaccination. The non-challenged naive group (T3) was maintained separately and showed no mortality. Relative percent survival (RPS) values were calculated relative to T0

was re-isolated from all dead and moribund fish and confirmed by PCR and sequencing (Fig. 1).

Serum agglutination response

Both vaccinated groups showed significantly higher serum agglutination titres than the PBS control (T1 vs. PBS, $p < 0.05$; T2 vs. PBS, $p < 0.01$), which exhibited no detectable agglutination (Fig. 2). T2 titres reached 4, 5, and 4 $\log_2$ at 7, 14, and 21 DPV, respectively. T1 titres were 3, 4, and 3 $\log_2$ at the same time points. Agglutination titres in T2 were significantly higher than T1 at all sampled time points.

Serum bactericidal activity

Serum bactericidal activity increased from 7 to 21 DPV in both vaccinated groups (Fig. 3). At 21 DPV, bacterial survival percentage

was 77.2% in T1 and 77.9% in T2, compared with 92.1% in the PBS control. T1 and T2 did not differ significantly at 21 DPV ($p > 0.05$).

Serum lysozyme activity

Serum lysozyme activity increased in both vaccinated groups by 7 DPV, peaked at 14 DPV, and declined by 21 DPV (Fig. 4). At 14 DPV, T2 showed significantly higher lysozyme activity than T1 ($p < 0.05$). The PBS control remained low throughout the sampling period.

Immune gene expression

Complement factor H (CFH)

CFH expression varied by tissue, treatment, and sampling time (Fig. 5). In head kidney, T1 was lower than control at 7 and 14 DPV and elevated

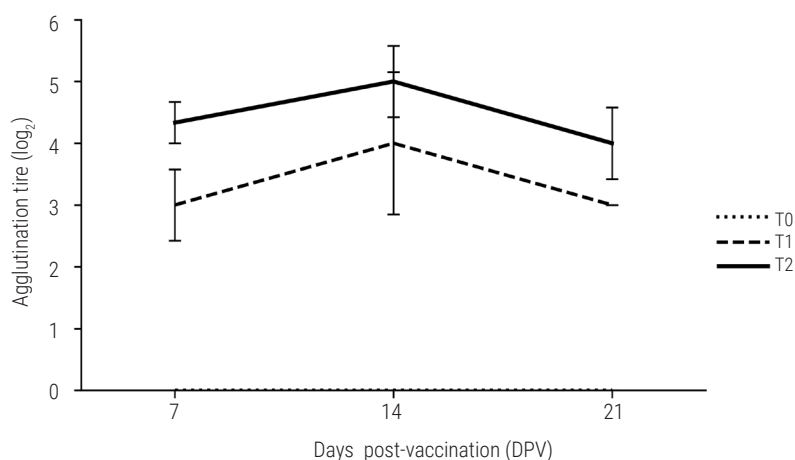


Fig. 2. Serum agglutination antibody titres in Asian seabass vaccinated with formalin killed *V. alginolyticus* without adjuvant (T1), with Algel adjuvant (T2), or PBS control (T0) at 7, 14, and 21 days post-vaccination (DPV). Values are presented as mean $\pm$ SE (n=9 per group)

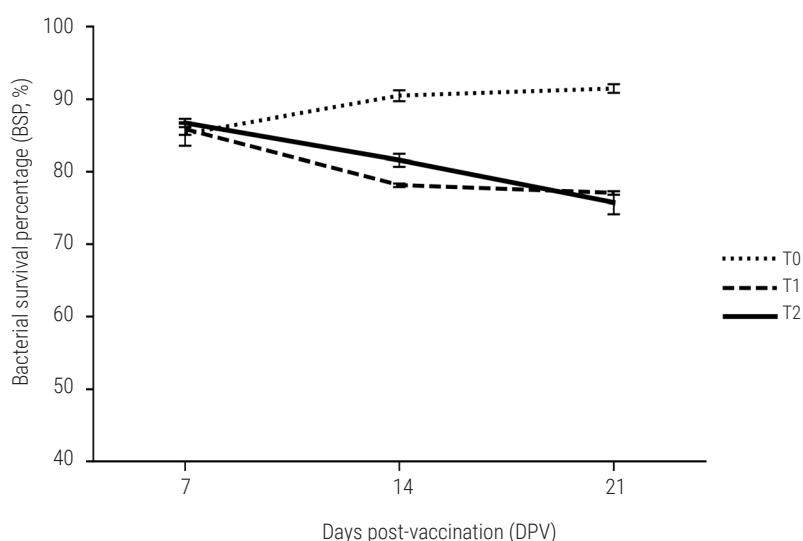


Fig. 3. Bacterial survival percentage (BSP) of *V. alginolyticus* incubated with sera from PBS control (T0), T1, and T2 groups at 7, 14, and 21 DPV. Values are presented as mean $\pm$ SE (n=9 per group). Lower BSP indicates stronger serum bactericidal effect

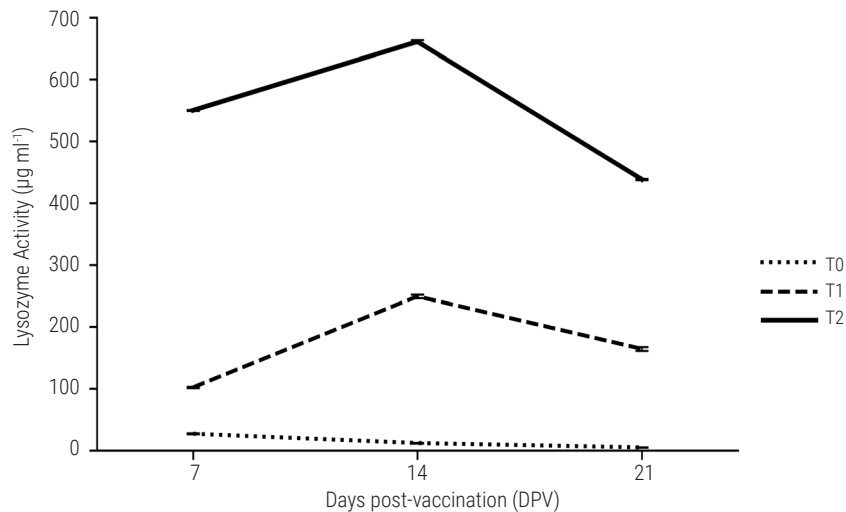


Fig. 4. Serum lysozyme activity in PBS control (T0), T1, and T2 groups at 7, 14, and 21 DPV. Values are presented as mean±SE (n=9 per group)

above control at 21 DPV, whereas T2 remained below or near control levels across the sampled time points. In spleen, T1 was significantly up-regulated relative to the control at 7 DPV (0.88 log₂ fold change) and 21 DPV (0.84 log₂ fold change), but down-regulated at 14 DPV (-0.37 log₂ fold change). In contrast, T2 in the spleen remained down-regulated at 7 DPV (-0.26 log₂ fold change) and 21 DPV (-0.23 log₂ fold change), showing only a slight elevation near baseline at 14 DPV (0.08 log₂ fold change). Thus, CFH exhibited tissue and time dependent modulation without consistent up-regulation across vaccine groups.

Cystatin B

Cystatin B expression also varied by tissue and time (Fig. 6). In head kidney, T2 showed its highest relative expression at 7 DPV

and declined at later time points, while T1 tended to increase over the sampling period. In spleen, both groups were low at 7 DPV and increased by 14 and 21 DPV. These results indicate modulation of Cystatin B expression after vaccination, but they do not by themselves demonstrate a direct functional role in protection.

HSP70

HSP70 expression differed between head kidney and spleen (Fig. 7). In head kidney, T1 showed higher relative expression at 14 and 21 DPV than at 7 DPV, whereas T2 remained lower at the later time points. In spleen, T2 showed higher relative expression at 14 and 21 DPV, while T1 was lower or near control depending on the time point. These patterns indicate tissue specific

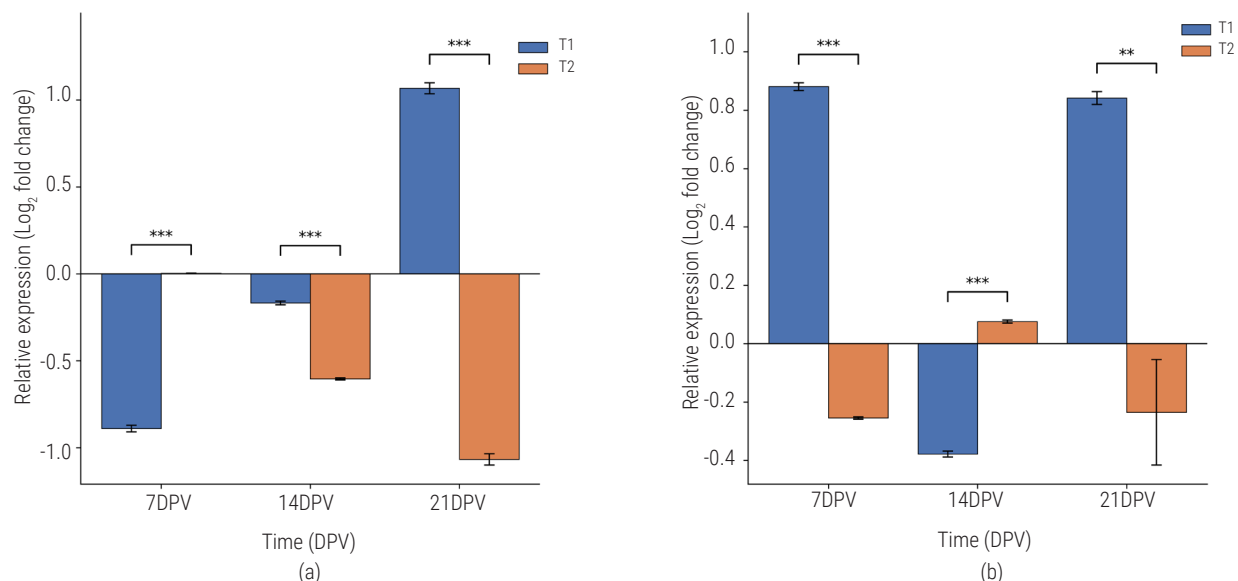


Fig. 5. Relative mRNA expression of complement factor H (CFH) in (a) Head kidney and (b) Spleen of Asian seabass vaccinated with T1 and T2 at 7, 14, and 21 DPV. Results are presented as log₂ fold change relative to the PBS control at the corresponding time point (mean±SE; n=3 biological replicates). Asterisks denote significant differences between groups (*p<0.05; ** p<0.01, *** p<0.001)

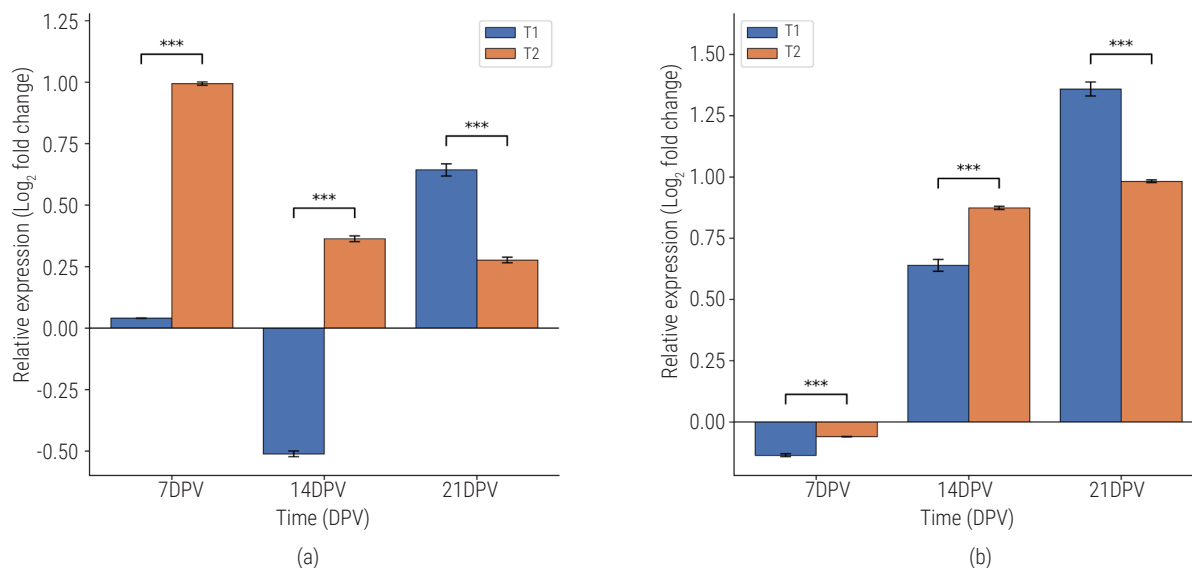


Fig. 6. Relative mRNA expression of Cystatin B in (a) Head kidney and (b) Spleen of Asian seabass vaccinated with T1 and T2 at 7, 14, and 21 DPV. Results are presented as log₂ fold change relative to the PBS control at the corresponding time point (mean±SE; n=3 biological replicates). Asterisks denote significant differences between groups (*p<0.05; ** p<0.01; *** p<0.001)

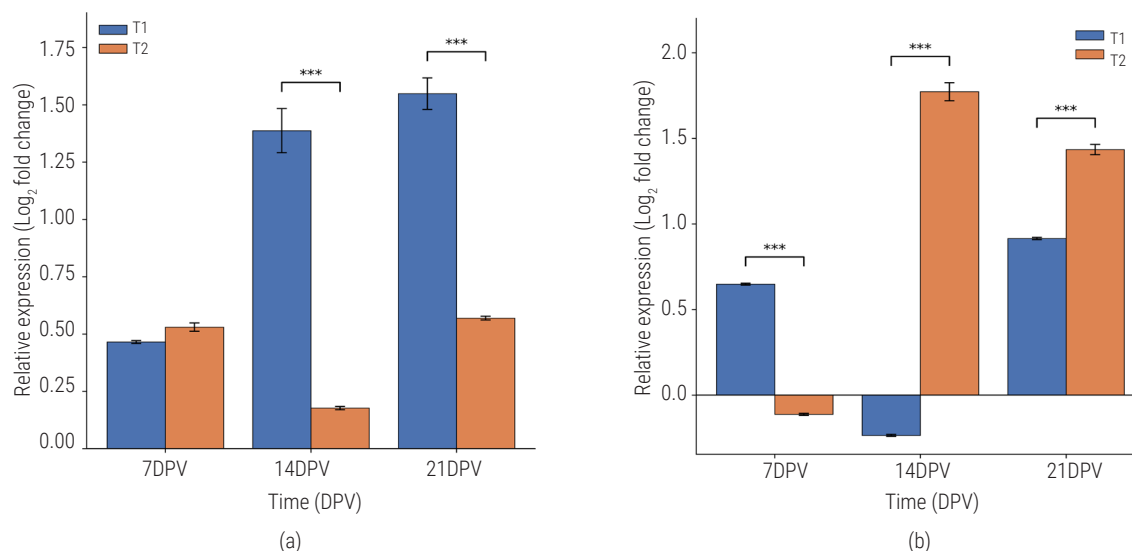


Fig. 7. Relative mRNA expression of HSP70 in (a) Head kidney and (b) Spleen of Asian seabass vaccinated with T1 and T2 at 7, 14, and 21 DPV. Results are presented as log₂ fold change relative to the PBS control at the corresponding time point (mean±SE; n=3 biological replicates). Asterisks denote significant differences between groups (*p<0.05; ** p<0.01, *** p<0.001)

vaccination associated stress/immune responses rather than a single protection marker.

HSP90α

HSP90α expression also differed by tissue and time (Fig. 8). In spleen, T2 showed higher relative expression than T1 at the later time points, whereas head kidney expression was small, reduced, or near control depending on treatment and time. These data support a tissue specific stress/immune response after vaccination but should not be interpreted as direct evidence that HSP90α mediated

protection. Overall, T2 produced the highest protection and stronger agglutination and lysozyme responses. Bactericidal activity at 21 DPV was similar between T1 and T2. Gene expression responses varied by tissue and time rather than showing a single uniform pattern across all four genes.

Discussion

The present study demonstrated that both formalin killed *V. alginolyticus* vaccine formulations provided substantial protection against experimental challenge in Asian seabass. T1 produced 80%

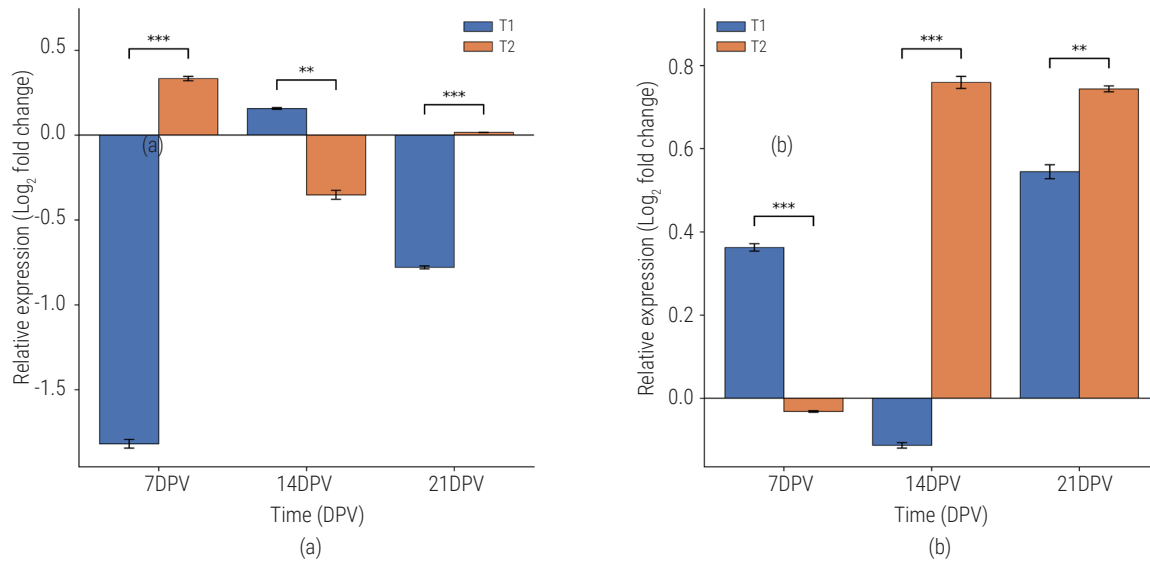


Fig. 8. Relative mRNA expression of HSP90α in (a) Head kidney and (b) Spleen of Asian seabass vaccinated with T1 and T2 at 7, 14, and 21 DPV. Results are presented as log₂ fold change relative to the PBS control at the corresponding time point (mean±SE; n=3 biological replicates). Asterisks denote significant differences between groups (* p<0.05; ** p<0.01, *** p<0.001)

survival and 72.7% RPS, whereas T2 produced 90% survival and 86.4% RPS. Thus, the Algel containing formulation showed higher protective response under the challenge conditions used in this study. The difference between the formulations should, however, be interpreted in the context of the severe challenge model and the absence of an Algel only control.

The challenge dose used in the present study was approximately 8.9×10^3 -fold higher than the estimated LD50. Such a high challenge to LD50 ratio represents a severe experimental challenge and was intended to generate substantial mortality in the unvaccinated control while retaining sufficient survival in vaccinated fish to discriminate protective efficacy. However, the severity of the challenge should be considered when interpreting RPS values, because protection measured under an extreme experimental challenge may not directly predict protection under natural farm exposure. Future studies using challenge doses closer to those encountered during natural outbreaks would help establish the robustness and field relevance of the observed protection. The 86.4% RPS recorded for T2 and 72.7% RPS recorded for T1 indicate that both formalin killed formulations provided protection under experimental conditions, with the Algel adjuvanted vaccine performing better. Comparisons with other inactivated bacterial vaccines should be interpreted cautiously because host species, the pathogen, vaccine route, challenge dose, observation period, and RPS calculation differ among studies. Formalin inactivated *Vibrio harveyi* protected orange spotted grouper (Li *et al.*, 2016; Nguyen *et al.*, 2017), and an inactivated *Streptococcus iniae* vaccine gave high RPS in grouper (Huang *et al.*, 2014). In Asian seabass, Ahangarzadeh *et al.* (2023) reported $85.71 \pm 3.57\%$ survival after vaccination with a killed polyvalent vaccine against *V. harveyi*, *V. alginolyticus*, and *S. iniae*, comparable to the 90% survival observed in the T2 group in the present study. Other Asian seabass vaccine studies have reported protection using different routes and platforms (Silvaraj *et al.*, 2020; Lan *et al.*, 2024, 2025).

The route of administration significantly influences immune response and RPS values; the i.p. route ensures consistent delivery and serves as the reference standard for vaccine efficacy trials in fish (Bromage and Owens, 2002; Mutoloki and Evensen, 2015; Meza *et al.*, 2019; Ohtani *et al.*, 2019). While ideal for formulation comparison, practical deployment in commercial aquaculture will require exploring immersion or oral booster regimens (Amir-Danial *et al.*, 2023; Lan *et al.*, 2024).

Humoral antibody response to Algel adjuvant

The mechanism of aluminium adjuvants involves antigen depot formation, enhanced innate immune stimulation, improved antigen uptake by antigen presenting cells, and complement activation (Pellegrino *et al.*, 2015; Tammam *et al.*, 2024). In the present study, an Algel-only control group (PBS+Algel) was not included. Consequently, the experimental design does not allow the non-specific effects of Algel alone to be completely separated from the effects arising from the interaction between the bacterin and the adjuvant. This is acknowledged as a limitation of the present study. Inclusion of an Algel-only control group in future experiments would permit direct assessment of adjuvant associated non-specific immune stimulation and would strengthen the interpretation of the additional protection observed with the Algel adjuvanted formulation.

Although T2 produced higher survival and RPS than T1, the results do not establish that Algel is indispensable for practical vaccine deployment. The non-adjuvanted formulation also provided substantial protection (72.7% RPS). Therefore, the choice between formulations must consider formulation cost, ease of manufacture, duration of immunity, booster requirements, and performance under farm conditions. Field based efficacy and economic studies will be required before recommending Algel as essential for commercial seabass vaccine application. The antibody response followed a distinct kinetic profile, peaking at 14 DPV in both groups before

declining by 21 DPV. T2 maintained significantly higher agglutination titres throughout. This is consistent with the capacity of aluminium adjuvants to enhance antigen presentation, B-cell activation and differentiation, and antibody responses (Tammam *et al.*, 2024). Studies utilising bacterial ghost vaccines (Cao *et al.*, 2018) and alternative inactivation protocols (Gu *et al.*, 2021) similarly demonstrate that antigen presentation directly influences humoral magnitude.

Lysozyme and bactericidal responses

Serum bactericidal activity demonstrated that sera from vaccinated fish significantly reduced *V. alginolyticus* survival relative to the PBS control. At 21 DPV, bacterial survival was nearly identical in T1 (77.2%) and T2 (77.9%), despite higher agglutination titres in T2. This indicates that agglutination titres and serum bactericidal activity did not increase strictly in parallel at the final sampling point. Lysozyme activity peaked at 14 DPV in both vaccinated groups, with T2 demonstrating significantly higher activity. Similar lysozyme kinetics have been documented in Asian seabass and other teleosts following bacterial immunisation (Du *et al.*, 2022; Erfanmanesh *et al.*, 2024; Uchuwittayakul *et al.*, 2025).

Tissue specific gene expression

CFH and Cystatin B exhibited tissue and time dependent regulation after vaccination. CFH serves as an essential negative regulator of the alternative complement pathway, maintaining homeostasis and preventing host tissue destruction (Nakao *et al.*, 2011; Najafpour *et al.*, 2020). Cystatin B acts as an intracellular cysteine protease inhibitor, modulating macrophage activity and preventing excessive proteolytic damage during inflammation (Zavasník-Bergant, 2008; Ahn *et al.*, 2013). HSP70 and HSP90α responded as molecular chaperones associated with physiological and handling stress following intraperitoneal injection rather than as direct correlates of protective immunity (Tort *et al.*, 2003; Celi *et al.*, 2012). Future studies should incorporate canonical adaptive immune markers such as IgM, MHC-I, and MHC-II to directly map lymphocyte differentiation.

The present study demonstrated that formalin killed *V. alginolyticus* vaccines can protect Asian seabass against experimental vibriosis. Both vaccine formulations conferred protection, but the Algel adjuvanted vaccine showed better efficacy, producing an RPS of 86.4% compared with 72.7% in the non-adjuvanted group. The adjuvanted formulation also induced higher agglutination titre and lysozyme activity at 14 DPV, indicating stronger early humoral and innate immune responses. However, serum bactericidal activity was comparable between the two vaccinated groups at 21 DPV, and the expression of CFH, Cystatin B, HSP70, and HSP90α showed tissue and time dependent regulation rather than a uniform pattern of immune gene up-regulation. Overall, the findings support the protective value of the Algel adjuvanted formalin killed vaccine, while also indicating that vaccine induced protection is associated with multiple immune responses rather than a single marker. Further studies should evaluate booster schedules, duration of immunity, and practical delivery routes such as oral or immersion vaccination under farm conditions.

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