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Integrated Field Vaccination and Laboratory Challenge Reveal Immune Gene Responses and Protective Efficacy in Asian Seabass (*Lates calcarifer*) Following Oral Administration of an Inactivated *Vibrio harveyi* Vaccine

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ABSTRACT

Vibriosis, caused by *Vibrio spp*., poses a significant challenge in marine aquaculture, leading to severe economic losses. Vaccination has emerged as a safer and more effective alternative to antibiotics for disease control, with oral vaccination offering advantages such as ease of administration and scalability for large-scale aquaculture operations. This study evaluated the efficacy of an oral inactivated *Vibrio harveyi* vaccine in a field environment with cage-cultured Asian seabass (*Lates calcarifer*) at Pulau Ketam, Klang, Selangor, Malaysia. Fish were divided into two groups: (1) a vaccinated group fed a commercial diet supplemented with formalin-killed *V. harveyi* (10^8 CFU/mL) and palm oil as an adjuvant and (2) a placebo control group fed a commercial diet supplemented with phosphate-buffered saline (PBS) and palm oil adjuvant. The vaccine was administered orally at weeks 0, 2, and 6 for three consecutive days at a dosage of 4% of the fish's body weight. Immune-related gene expression, including dendritic cells (DCs), complement factor 3 (C3), chemokine ligand 4 (CCL4), tumor necrosis factor-α (TNF-α), and immunoglobulin-T (IgT), was significantly upregulated ($p<0.05$) in vaccinated fish compared to controls, with peak expression observed following booster doses. At week 10, a laboratory challenge trial with *V. harveyi* (1×10^8 CFU/mL) demonstrated an 80% relative percentage survival (RPS) in the vaccinated group, whereas the unvaccinated group exhibited 100% mortality. In addition, vaccinated fish displayed reduced clinical signs following infection. These findings highlight the potential of oral inactivated *V. harveyi* vaccines to induce robust immune responses and confer effective protection against vibriosis in Asian seabass. This approach provides a practical and scalable solution for disease management in marine aquaculture, improving health outcomes and productivity for fish farmers.

1 | Introduction

Vibriosis is a severe bacterial disease in marine aquaculture caused by various *Vibrio* species, leading to significant economic losses worldwide. *Vibrio* spp. are ubiquitous in marine environments, making vibriosis particularly challenging due to its rapid onset, high mortality rates, and broad host range [1]. The disease primarily affects both captive and wild marine fish [2], posing a major threat to the aquaculture industry [3, 4]. Clinically, vibriosis is characterized by necrotizing enteritis, anal hemorrhaging, and abdominal distension with opaque fluid accumulation [5]. Infection typically occurs when fish are exposed to water containing high concentrations of *Vibrio* spp., with disease progression occurring within a day [6]. Transmission is predominantly horizontal, as bacteria are shed from infected fish, often through open wounds [7]. *Vibrio* spp. enter the host via the skin, gills, or gastrointestinal tract, with skin penetration being the most common route [8]. Damage to the mucosal layer increases susceptibility, as mucus and epidermal barriers serve as key defenses against infection [8].

Vaccination is the most cost-effective and reliable method for enhancing host immunity, providing long-term protection against specific diseases, including vibriosis [9, 10]. Oral vaccination is particularly suitable for aquaculture due to its stress-free administration, scalability, and applicability to fish of all sizes. It also reduces labor costs and enables booster doses during the culture period [11]. However, the efficacy of inactivated feed-based vaccines against bacterial infections is often inconsistent, necessitating the use of adjuvants to elicit a sufficient immune response [12, 13]. Oral vaccination has been widely explored in aquaculture, with numerous prototypes reported in previous studies; however, no consistently effective commercial oral vaccines are currently available for most fish species.

Environmental fluctuations can influence vaccine performance, and farmed fish may experience higher stress levels, altered growth parameters, and exposure to additional pathogens compared to laboratory settings [14]. Therefore, factors such as vaccination protocols, fish immunobiology, vaccine timing, and environmental conditions must be carefully considered in field trials [15, 16]. Optimizing vaccine formulations with appropriate antigenic components, supported by field data, is crucial for ensuring both efficacy and practical application in farming conditions [17, 18].

Our laboratory previously developed and assessed an oral inactivated *V. harveyi* vaccine, which demonstrated efficacy in stimulating immune responses, providing protection against vibriosis, and enhancing growth performance in cage-cultured Asian seabass (*Lates calcarifer*) [19]. Additionally, this vaccine has been shown to be effective in preventing *V. harveyi* infection and to offer cross-protection against *V. alginolyticus* and *V. parahaemolyticus* in laboratory studies [20].

Despite these promising results, there is limited information on immune gene profiling in field-based evaluations of vaccinated fish. To address this gap, our current study aims to investigate immediate changes in gene expression following vaccination, focusing on key immune-related genes critical to post-vaccination immune responses. Furthermore, studies that concurrently validate protective efficacy in both field and laboratory settings using pathogenic *Vibrio* challenges remain scarce. Thus, the present study bridges these gaps by integrating field vaccination with subsequent laboratory challenges using pathogenic *V. harveyi*, thereby providing dual validation of vaccine efficacy. This combined framework enables a thorough evaluation of the protective efficacy of the oral inactivated *V. harveyi* vaccine, thereby advancing the development of effective oral vaccines for aquaculture.

2 | Materials and Methods

2.1 | Bacterial Strain and Growth Conditions

Vibrio harveyi strain VH1 used in this study was sourced from tiger grouper (*Epinephelus fuscoguttus*) infected with vibriosis in deepwater cages in Langkawi, Malaysia. The strain elicited strong antigenic responses to its homologous outer membrane protein (OMP) antigens and cross-reacted with heterologous OMP antigens from *V. parahaemolyticus* strain VPK1, *V. alginolyticus* strain VA2, and *Photobacterium damselae* strain PDS1, specifically at a molecular weight of 32 kDa during cross-reaction assays [21]. *V. harveyi* strain VH1 was recovered from glycerol stock maintained in the laboratory culture collection and cultured on selective thiosulfate-citrate-bile-salts-sucrose (TCBS) agar (Oxoid, Hampshire, England) at 30°C for 24 h. The colonies obtained were subjected to DNA extraction, and species identification was performed by polymerase chain reaction (PCR) amplification with 16S rRNA primers. The PCR products were sequenced by Apical Scientific Sdn. Bhd. (Selangor, Malaysia). The resulting sequences were analyzed using the BLASTn program (http://blast.ncbi.nlm.nih.gov) and showed 98% similarity to *V. harveyi*, with the closest match corresponding to the GenBank accession number LC189353.1.

2.2 | Preparation of the Feed-Based Vaccine

A commercial marine fish feed pellet (Star Feed, Star Feed Mills, Malaysia) containing 43% protein was ground into a fine powder using a blender. *V. harveyi* strain VH1 was cultured in TSB broth with 1.5% NaCl and incubated at 30°C and 150 rpm for 24 h. The formalin-killed cells (FKCs) of *V. harveyi* strain VH1 were prepared as described by Mohamad et al. [20]. Briefly, after incubation, the bacterial concentration was determined using the spread plate method [22] and adjusted to 10^8 CFU/mL. A 10% buffered formalin was added to the bacterial suspension at a final concentration of 0.5%, followed by incubation at 4°C for 12 h. Complete inactivation was confirmed by streaking the treated suspension onto tryptic soy agar (TSA) supplemented with 1.5% NaCl and incubating at 30°C overnight to verify the absence of bacterial growth. The formalin-treated bacterial suspension was centrifuged at 6000 × *g* for 15 min, and the pellet was washed three times with sterile phosphate-buffered saline (PBS). Palm oil was added as an adjuvant to a final concentration of 10%. The FKCs vaccine was thoroughly mixed with the palm oil-adjuvanted fish feed powder using an industrial mixer (Golden Bull-B10-A Universal Mixers, Malaysia). The unvaccinated control group received fish feed mixed with PBS and 10% palm oil. The vaccine-laden feed was then pelleted using an automatic mini-pelleting machine (Golden Avill, Guangdong, China) to obtain pellets measuring 1 × 0.5 cm. The pellets were dried at

30°C for 12 h in a hot air oven (Memmert, Schwabach, Germany) and stored at 4°C until further use.

2.3 | Study Site and Experimental Design

Field vaccination and evaluation were conducted at a commercial floating marine cage facility operated by KS Aquaculture Farm, located in Pulau Ketam, Klang, Selangor, Malaysia. A total of 4815 Asian seabass (*Lates calcarifer*), with an average weight of 182 ± 31 g, were sourced from a local hatchery in Terengganu, Malaysia, and used in this study. To assess the fish's health status prior to the experiment, 15 fish were randomly selected and dissected to examine the kidney tissue for bacterial infection. Kidney samples were aseptically streaked onto TSA (Oxoid, Hampshire, UK) and incubated at 30°C for 24 h. No bacterial growth was observed, indicating that all examined fish were free from detectable bacterial infections and were in good health. Figure 1 shows the feeding schedule and a brief description of the experimental procedure.

At the start of the trial, the fish were divided into two groups, each with two floating cages. To enhance vaccine uptake, fish were fasted for at least 12 h prior to each vaccination event throughout the vaccination period. Group 1 received the oral inactivated *V. harveyi* vaccine, while Group 2 was fed the control (PBS) feed. Fish were fed at a rate of 4% body weight for three consecutive days at weeks 0, 2, and 6 with the respective vaccine feeds. On nonvaccination days, regular commercial feed pellets (Star Feed, Klang, Malaysia) at a rate of 4% body weight were provided to all fish until the end of the 10-week trial. Head kidney samples were collected from six fish per group at 2-week intervals to analyze immune-related gene expression. At week 10, 20 fish from each group were transferred from the cage-cultured farm to the laboratory for experimental challenge with the pathogenic *V. harveyi* strain VH1. An additional 10 fish from the control group were set as unchallenged controls. Survival rates and abnormal features post-challenge were recorded.

Throughout the trial, water quality parameters, including pH, temperature, salinity, dissolved oxygen, and ammonia–nitrogen, were measured using a spectrophotometer (HACH Company, CO, USA) and a YSI Pro Plus (Yellow Spring Instrument, OH, USA). All measured parameters across experimental cages remained within the acceptable limits defined by the Malaysia Marine Water Quality Criteria and Standard (MMWQS) Class 2 (https://www.doe.gov.my/), with recorded values of pH 8.00 ± 0.15, temperature 30.45 ± 0.62°C, salinity 30.12 ± 1.33 ppt, dissolved oxygen 5.71 ± 0.39 mg/L, and ammonia–nitrogen 0.03 ± 0.02 mg/L.

2.4 | RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR (RT-qPCR) Analysis

Head kidney samples from three fish per cage (six samples per group) were collected every 2 weeks until the completion of the vaccination phase (week 10). Total RNA was extracted from the samples using the TRIzol Reagent (Invitrogen, Thermo Fisher Scientific, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA purity was assessed by measuring the absorbance at 260/280 nm using a Genova Nano (Jenway, Staffordshire, UK). Single-stranded cDNA was synthesized from 1 μg of total RNA using a reverse transcription kit (QIAGEN, Hilden, Germany). RT-qPCR was performed using Rotor-Gene RT-qPCR (QIAGEN, Hilden, Germany) with the SYBR Green RT-qPCR mixture, according

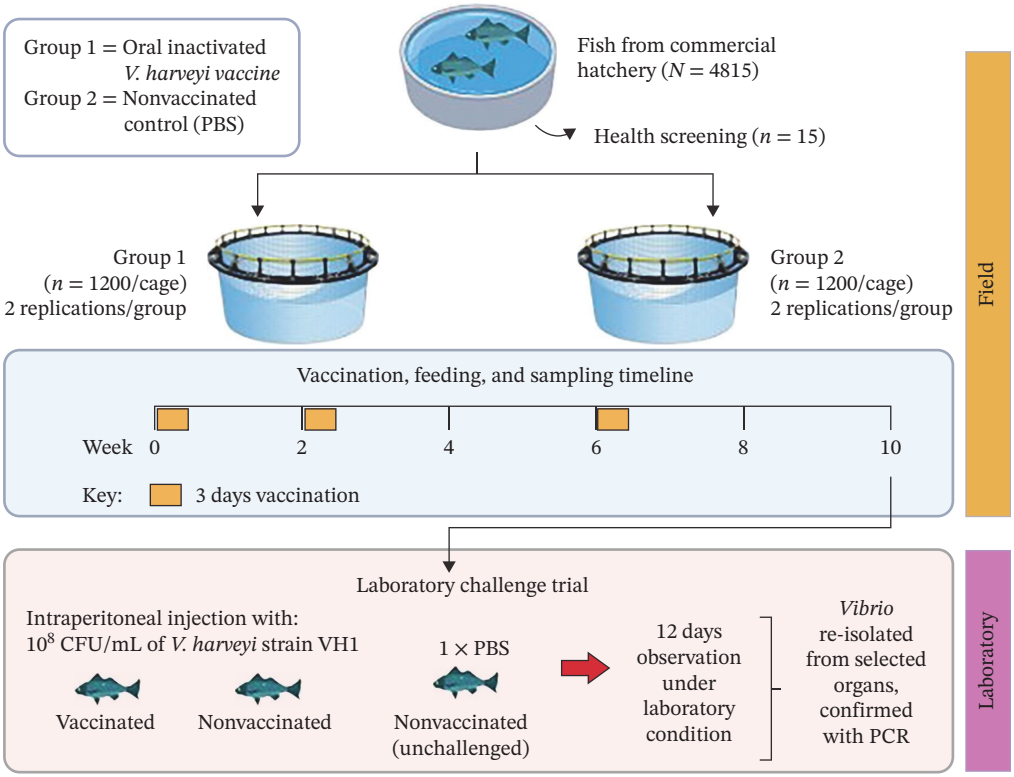

FIGURE 1 | Vaccination and challenge trial design. Fish were cultured and vaccinated under field conditions, followed by a laboratory-based bacterial challenge conducted at week 10 post-vaccination.

TABLE 1 | Primers used for gene expression analysis in Asian seabass by real-time PCR (RT-qPCR).

Target gene	Sequence (5′–3′)	Product size (bp)	Accession no.	Note	Reference
DC	F: AACAGCACACGCTCACTCAC R: CGATCATGTGAGCCTTGAGA	153	KM386621.1	Initiate an adaptive immune response	Bunnoy et al. [23]
C3-F	F: GCAATCCTCCACAACTACAG R: ACTCTGACCTCCTGACGATAC	93	XM_018679796.2	Innate defense against common pathogens	Mohd-Shaharuddin et al. [24]
CCL4	F: TCCTCGTCTCACTCTGTCTGT R: GACCTGCCACTGTCTTCAGC	301	AM490064.1	Chemokine attracts innate immune cells	Chin et al. [25]
TNF-α	F: CTGCTTCACGCTCCATAAGA R: CTGGTCCTGGTCATCTCTCC	150	AY427649.1	Pro-inflammatory cytokine gene	Hoseinifar et al. [26]
IgT	F: GCTGTCAAGGTGGCCCCAAAAG R: CAACATTCATGCGAGTTACCCTTGGC	110	XM_018703635.2	Generate B lymphocytes	Piazzon et al. [27]
β-actin	F: TACCACCGGTATCGTCATGGA R: CCACGCTCTGTCAGGATCTTC	126	XM_018667666.2	Reference gene	Chin et al. [25]

to the manufacturer's guidelines. Each sample was run in triplicate. Table 1 lists the primers used in the qPCR experiments for each gene. The selection of the immune-related genes was based on findings from our previous vaccination studies [25, 28, 29]. These genes are commonly used as markers to evaluate immunomodulatory responses in fish immunology and vaccine efficacy studies. PCR efficiencies ranged from 95% to 105%, with an annealing temperature of 59°C. The threshold cycle ($2^{-\Delta\Delta Ct}$) method was used to calculate the relative gene expression, with β-actin as the housekeeping gene [25].

2.5 | Challenge Trial With *V. harveyi*

To assess vaccine efficacy, a bacterial challenge was performed on day 70 (week 10) post-vaccination. Twenty fish from each group (vaccinated and unvaccinated) were transferred to 500 L fiberglass tanks (10 fish per group, in duplicate, for challenged groups and five fish per group, in duplicate, for nonchallenged controls). Fish from the challenged groups were intraperitoneally injected with 0.5 mL of live bacterial suspension containing 10^8 CFU/mL of *V. harveyi* strain VH1, following a previously reported challenge dose based on LD_{50} [30], while the unchallenged control groups were intraperitoneally injected with 0.5 mL of 1x PBS. Clinical signs, abnormalities, and mortality were recorded daily after the challenge.

2.6 | Relative Percentage Survival (RPS)

The cumulative percent mortality (CPM) and RPS were calculated as follows [31]:

$$\text{Cumulative percent mortality (CPM)} = \frac{\text{The number of fish mortality}}{\text{The total number of fish}} \times 100,$$

$$\text{Relative percentage survival (RPS)} = \left(1 - \frac{\text{Average CPM in the vaccinated group}}{\text{Average CPM in the control group}}\right) \times 100.$$

2.7 | Bacterial Isolation and Identification From Dead Fish During Challenge Trials

The livers, kidneys, spleens, and brains of each dead fish from the bacterial challenge groups were aseptically collected for bacterial isolation. The tissues were streaked onto TSA plates (Oxoid, Hampshire, UK) with 1.5% NaCl and incubated at 30°C for 24 h. Bacterial colonies were identified by PCR with 16S rRNA primers (Table 2).

2.8 | PCR for Detection of Bacterial Isolates

DNA was extracted from pure bacterial colonies using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. Colonies from the dead fish's kidney, spleen, brain, and liver were cultured on TSA plates at 30°C for 24 h. DNA was extracted and PCR was performed using 16S rRNA primers to identify the bacterial species (Table 2). PCR reactions were performed using REDiant 2× PCR Master Mix (FirstBase, Kuala Lumpur, Malaysia) in a final volume of 25 μL, containing 2× PCR master mix, 1 μM of each primer, and 100 ng of template DNA. The amplification conditions were as follows: initial denaturation at 95°C for 5 s, followed by 33 cycles of 95°C

TABLE 2 | Primers used in PCR amplification of 16S rRNA.

Primers	Primer Sequence (5′–3′)	Temperature (°C)	Expected size (bp)	Reference
16S rRNA_F	GGTTACCTTGTTACGACTT	54.0	1541	Yin et al. [32]
16S rRNA_R	AGAGTTTGATCCTGGCTCAG			

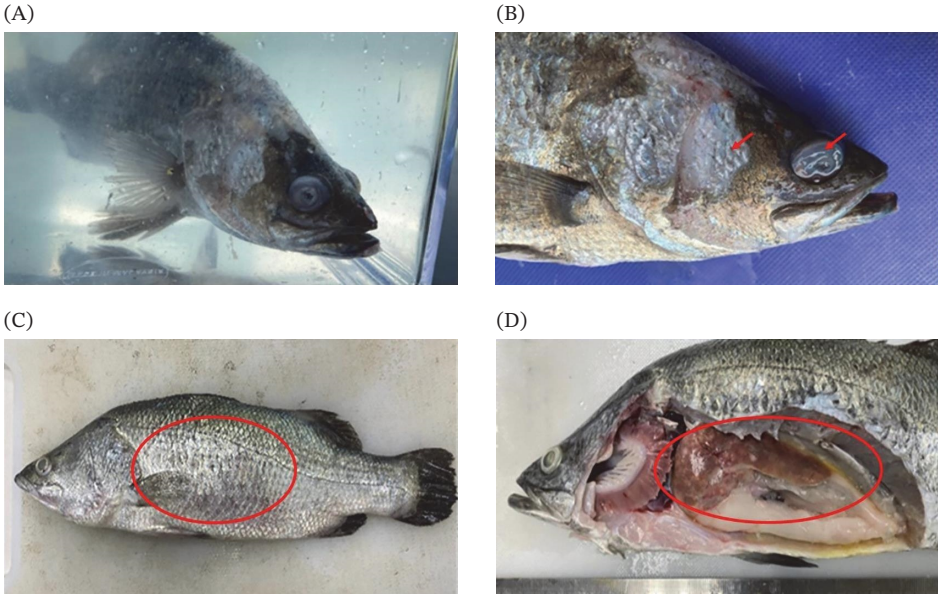

FIGURE 2 | Common clinical signs in the control group experimentally challenged with *Vibrio harveyi*. The fish exhibited (A) major scale loss, (B) eye opacity and loss of scale (red arrow), (C) swelling of the abdomen due to fluid accumulation (red circle), and (D) congested internal organs and hemorrhage with swollen intestine containing yellow fluid (gastroenteritis) (red circle).

for 1 min, 54°C for 2 min 15 s, 72°C for 1 min 15 s, and a final extension at 72°C for 10 s. PCR products were sequenced using Sanger sequencing (First Base Laboratories, Kuala Lumpur, Malaysia).

2.9 | Statistical Analysis

Data are presented as the mean ± standard deviation (SD) based on at least three replicate measurements. The number of replicates (n) is indicated in the corresponding figure and table captions. Data were assessed for normality using the Shapiro–Wilk test and for homogeneity of variance using the Levene's test. Treatment effects were evaluated using an independent sample t-test to determine significant differences between the vaccinated and control groups using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $p < 0.05$. Survival analysis was performed using the Kaplan–Meier method, and survival curves were compared using the log-rank test in GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA).

2.10 | Ethics Statement

All procedures involving Asian seabass (*Lates calcarifer*) in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Universiti Putra Malaysia, in compliance with the guidelines of the Department of Biosafety, Ministry of Natural Resources and Environment, Malaysia (Approval Number: UPM/IACUC/AUP-R078/2019). Fish were anesthetized with MS-222 (Sigma-Aldrich, Missouri, USA) at a concentration of 100 mg/L prior to sample collection and bacterial challenge procedures. After the experiment, all remaining fish were euthanized using an overdose of MS-222 (400 mg/L) for a minimum of 10 min. Following euthanasia, the fish were immersed in 25% sodium hypochlorite for 30 min and disposed of as clinical waste in accordance with the safety protocols.

3 | Results

3.1 | Clinical Signs and Gross Lesions Post-Challenge

In the bacterial challenge with virulent *V. harveyi* via intraperitoneal injection, all fish in the control group died within 8 days post-challenge. Post-mortem examinations revealed congestion in the liver, spleen, intestine, and stomach, along with gall bladder enlargement and hemorrhaging. The intestines were swollen and contained yellow fluid, indicative of gastroenteritis (Figure 2). Moribund fish displayed lethargy, swimming near the water's surface, and exhibiting abnormal swimming behavior.

3.2 | Mortality Pattern During Experimental Infection

Mortalities in the control group began on day 1 post-infection (dpi), while the vaccinated group showed mortalities primarily on days 6 and 7 dpi. At 6 dpi, 40% of the total number of fish in the control group had died. By 7 dpi, the control group reached 100% mortality, whereas the vaccinated group reached only 20% mortality at the end of the challenge period (12 dpi; Figure 3).

3.3 | Protective Efficacy of the Vaccinated Feed Against *V. harveyi*

The RPS is summarized in Table 3. Fish vaccinated with the monovalent *V. harveyi* feed vaccine exhibited an RPS of 80% after the challenge with virulent *V. harveyi*, compared to 0% in the control group.

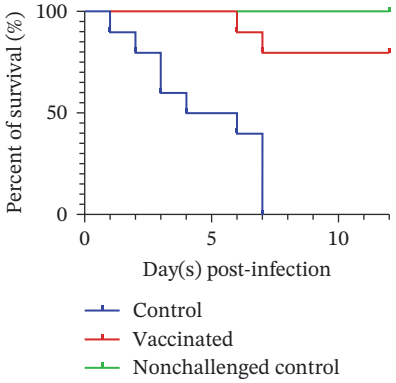

FIGURE 3 | Average percent survival of vaccinated groups compared to the nonvaccinated (control) group at 12 days post-infection with pathogenic *Vibrio harveyi*. Statistical analysis of cumulative survival between both vaccinated groups and the control was performed using a Kaplan–Meier curve with the log-rank test ($p < 0.0001$). Data are presented as mean ± SD ($n = 20$).

3.4 | Bacterial Isolation and PCR Detection in Recently Deceased Fish

Bacteria were re-isolated from the brain, kidney, liver, and spleen of moribund and recently deceased fish to confirm the causative pathogen. The recovered isolates were presumptively identified as *V. harveyi* based on their characteristic colony morphology on TCBS agar. Molecular confirmation was subsequently performed through PCR amplification targeting the 16S rRNA gene. The amplified PCR products were successfully sequenced, and bacterial identity was verified through nucleotide sequence comparison using the BLAST algorithm in the NCBI database. To further validate the identification and determine the genetic relationship among the isolates and reference strains, phylogenetic analysis was conducted based on the obtained 16S rRNA gene sequences (Figure 4). The results confirmed that the re-isolated bacteria clustered closely with reference *V. harveyi* strains, supporting the diagnosis of vibriosis in the infected fish.

3.5 | Immune Gene Expression in the Head Kidney of Asian Seabass

Following the 10-week vaccination trial, the expression levels of five immune-related genes (dendritic cells [DCs], chemokine ligand 4 [CCL4], complement factor 3 [C3], tumor necrosis factor-α [TNF-α], and immunoglobulin-T [IgT]) in the head kidney were measured using RT-qPCR. All genes exhibited significantly higher expression ($p < 0.05$) in the vaccinated groups compared to the control group (Figure 5). After the initial vaccination at week 0, DCs expression in the vaccinated group was significantly higher ($p < 0.05$) than in the control group as early as week 2. This elevated expression persisted and was significantly higher ($p < 0.05$) at week 4 following the first booster dose at week 2. After the second booster at week 6, DCs expression in the vaccinated group continued to increase significantly ($p < 0.05$) from week 6 to week 8 before declining

TABLE 3 | Protective efficacy of the feed-based vaccines against *Vibrio harveyi* in Asian seabass (*Lates calcarifer*) until days 12 post-challenge.

Fish group	Challenge dose (CFU/mL)	Dead/total	Mortality (%)	RPS (%)
Vaccinated	1×10^8	4/20	20	80 ± 0.0*
Control (PBS)	1×10^8	20/20	100	0 ± 0.0

Note: Asterisk (*) represents statistically significant differences between groups ($p < 0.05$). Data are expressed as mean ± SD ($n = 20$).

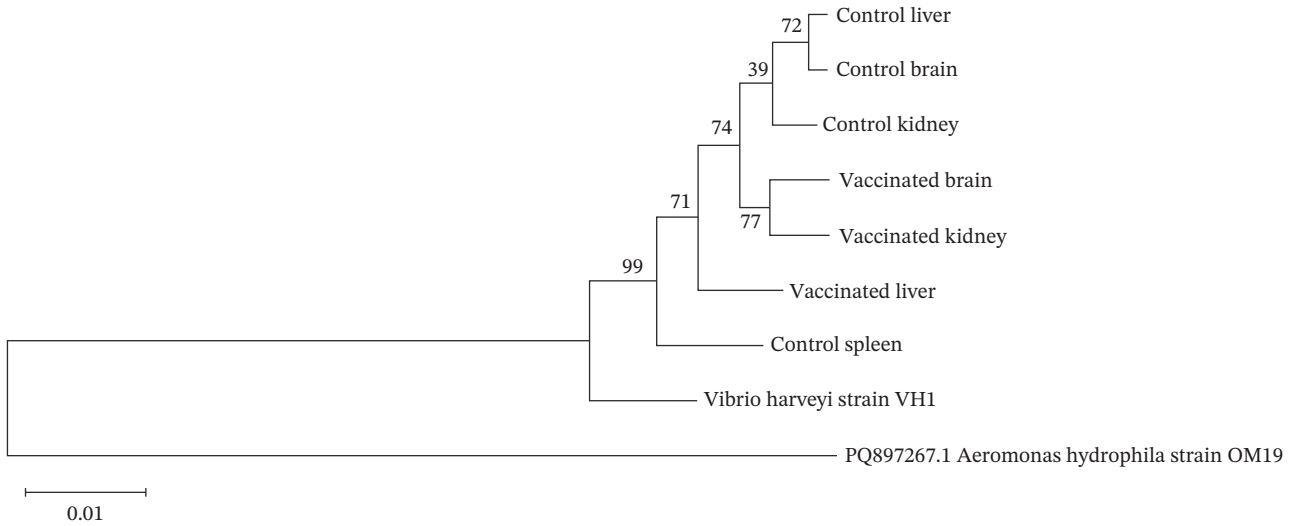

FIGURE 4 | Phylogenetic analysis of *Vibrio harveyi* isolates recovered from different organs of infected fish based on 16S rRNA gene sequences. The phylogenetic tree was constructed using the neighbor-Joining method to compare *V. harveyi* strain VH1 and isolates obtained from infected *Lates calcarifer*, with *Aeromonas hydrophila* strain OM19 as the outgroup. Bootstrap values, based on 1000 replicates, are shown next to the branches and indicate the percentage of replicate trees in which the associated taxa clustered together. Evolutionary distances were calculated using the maximum composite likelihood method and are expressed as the number of base substitutions per site. Phylogenetic analyses were performed using MEGA version 12.0 [33]. No bacterial growth was detected in the spleen of the vaccinated fish.

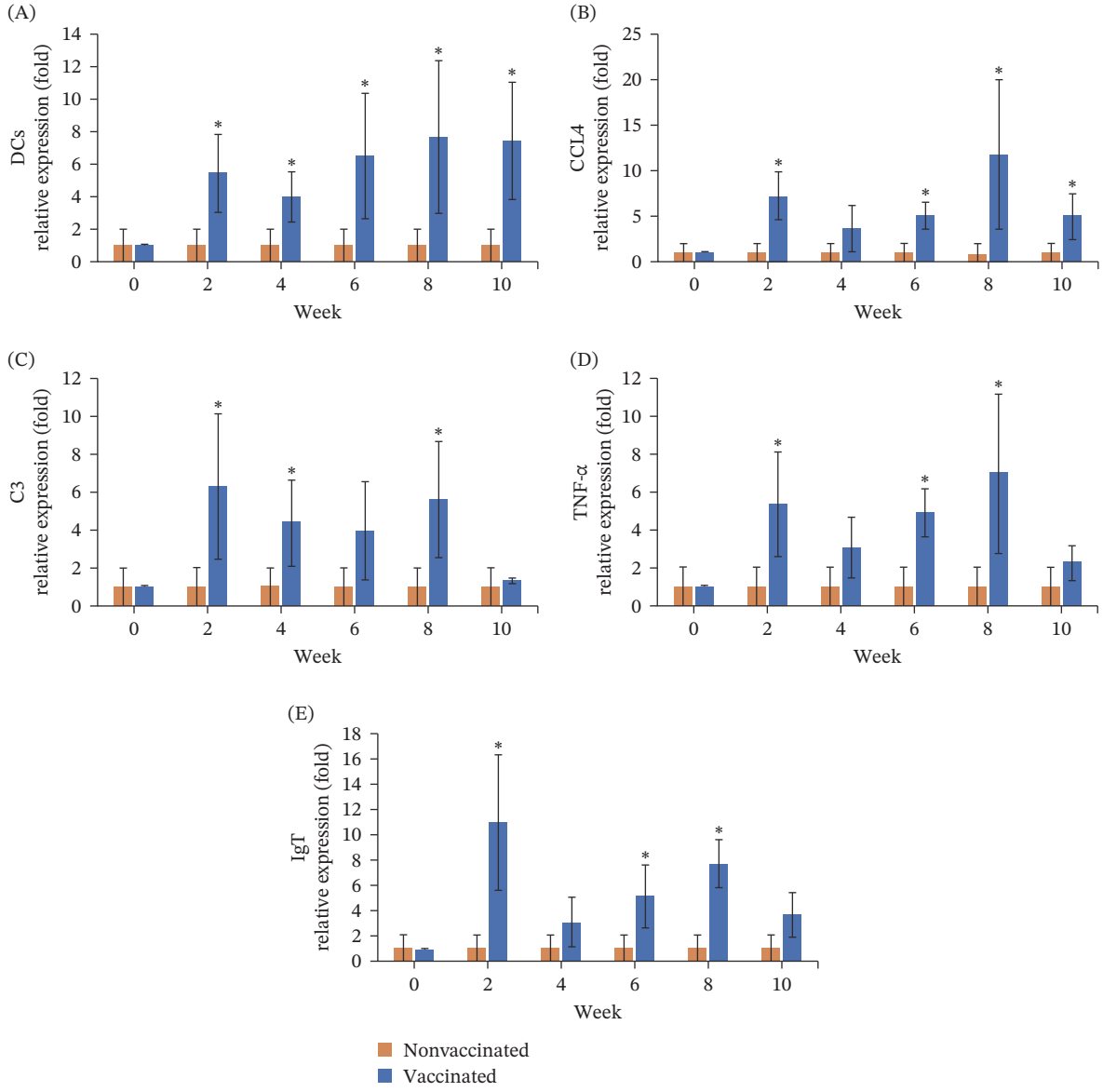

FIGURE 5 | Relative expression levels of (A) DCs, (B) CCL4, (C) C3, (D) TNF-α, and (E) IgT genes in the head kidney portion of Asian seabass at different time points after vaccination. Data are expressed as mean ± SD ($n = 6$). Asterisks (*) indicate statistically significant differences between groups within each sampling time point ($p < 0.05$).

at week 10, though it remained significantly higher ($p < 0.05$) than in the control group (Figure 5A).

Similarly, CCL4 expression in the vaccinated group was significantly higher ($p < 0.05$) than in the control group from week 2 post-vaccination onward. Following the first booster at week 2, CCL4 expression remained elevated in the vaccinated group at week 4, though the difference was not statistically significant ($p > 0.05$). After the second booster at week 6, CCL4 expression significantly increased ($p < 0.05$) in the vaccinated group from week 6 to week 8 but declined significantly ($p < 0.05$) by week 10, remaining significantly higher ($p < 0.05$) than in the control group (Figure 5B). For C3, expression was significantly higher ($p < 0.05$) in the vaccinated group at week 2 compared to the control group. Following the first booster at week 2, the expression of C3 remained significantly higher ($p < 0.05$) in the vaccinated group at week 4. At week 6, while expression was higher in the vaccinated group, the difference was not statistically significant ($p > 0.05$). Following the second booster administered at week 6, C3 expression in the vaccinated group was significantly higher ($p < 0.05$) than that in the control group at week 8. However, by week 10, no significant difference ($p > 0.05$) in C3 expression was observed between the vaccinated and control groups (Figure 5C).

TNF-α expression in the head kidney of the vaccinated groups was significantly higher ($p < 0.05$) compared to the control group and began to increase as early as week 2. Expression remained higher ($p < 0.05$) than in the control group after the first booster at week 2, but slightly decreased by week 3. After the second booster at week 6, TNF-α expression in the vaccinated group increased significantly ($p < 0.05$) compared to the control group at weeks 6 and 8. At week 10, TNF-α expression in the vaccinated group remained higher than in the control group, though the

difference was not statistically significant ($p > 0.05$) (Figure 5D). Before vaccination, there was no significant difference ($p > 0.05$) in IgT expression between the groups. However, after vaccination, IgT expression in the vaccinated group was significantly higher ($p < 0.05$) than in the control group as early as week 2. Expression remained significantly higher ($p < 0.05$) in the vaccinated group following the first booster at week 2, then declined at week 4, though it remained elevated compared to the control group. Following the second booster at week 6, IgT expression in the vaccinated group increased significantly ($p < 0.05$) at weeks 6 and 8. By week 10, IgT expression remained higher in the vaccinated group, but no significant difference was observed between the groups ($p > 0.05$) (Figure 5E).

4 | Discussion

Oral vaccination has been widely explored in aquaculture, with numerous prototypes being reported in previous studies. However, no consistently effective commercial oral vaccines are currently available for most fish species. Although the Chilean salmon industry briefly marketed one or two oral vaccines, they were discontinued due to their limited efficacy under field conditions [34]. In the present study, field trials of an oral vaccine demonstrated high efficacy against *V. harveyi* strain VH1. The unvaccinated control group exhibited 0% survival following the challenge with the pathogenic strain, whereas the vaccinated group achieved 80% survival. Most control fish died within 1 day of the challenge before clinical symptoms could manifest. The moribund fish exhibited lethargy and abnormal swimming behavior, consistent with previous reports of vibriosis by Ransangan et al. [35], Mohd-Aris et al. [30], and Chin et al. [25]. Chettri et al. [36] and Ismail et al. [37] suggested that a RPS between 70% and 80% should be achieved by a successful vaccination, which aligns with the results of this research, where the RPS of vaccinated fish post-challenge was 80%. Interestingly, our previous studies using smaller fish during primary vaccination (15–30 g) reported a comparable RPS of approximately 80% [20, 38]. These findings suggest that the vaccine is capable of providing comparable protective efficacy in both juvenile and grower-stage fish.

The high survival observed in the vaccinated group indicated that the oral inactivated *V. harveyi* fish vaccine provided sustained protection against *V. harveyi*, in contrast to 100% mortality in the unvaccinated control group. Therefore, the oral fish vaccine appears to provide an effective and reliable defense against *V. harveyi* infection, as suggested by these findings.

Numerous effective monovalent vaccines against vibriosis have been developed with different bacterial compositions and delivery methods. A study by Nguyen et al. [39] reported lower efficacy than this study, with 59.9% survival after challenge with *V. harveyi* in orange-spotted grouper (*Epinephelus coioides*) following post-injection vaccination with an inactivated *V. harveyi* vaccine. Meanwhile, another study by Liu et al. [40] with a recombinant *V. harveyi* vaccine reported a similar (80%) efficacy in orange-spotted grouper post-infection with *V. harveyi*. Not all vaccines provide sufficient protection for the host. For instance, research by Chakraborty et al. [41] on a commercial vaccine called vibrogen-2, which contained inactivated cultures of *V. anguillarum* serotypes O1 and O2 and *V. ordalii*, revealed that the vaccine conferred only 8% RPS in the vaccinated group. The authors suggested that factors such as temperature, fish species, and vaccine preparation must be considered, as they appear to influence vaccine efficacy against pathogenic infections.

Beyond the limited efficacy of previous oral vaccines under field conditions, several other major obstacles hinder the development of effective oral fish vaccines. These include an incomplete understanding of fish immunology, the high cost of vaccine delivery, and the physiological stress experienced by fish, which complicates the interpretation of immune responses to vaccination [42]. Therefore, gene expression changes were examined in the present study to better understand the consequences of fish immunization, with particular emphasis on events that occurred immediately after vaccination [39]. Several gene groups that are probably responsible for stimulating immunological responses after vaccination were examined in this study. The present research showed that the inactivated *V. harveyi* feed-based fish vaccine in Asian seabass caused significant alterations in immune-related gene expression. The findings were consistent with other studies on fish vaccinations, including one by Sun et al. [43], which revealed that immunization with VhhP2 increased the expression of many genes linked to the immune system. Following vaccination, most observed genes showed increased expression levels from week 2 to week 10. Specifically, DC expression was significantly elevated from week 2 onward, peaking at week 8 after the second booster dose. This study corroborated the findings of Nguyen et al. [39], who observed increased DC expression in orange-spotted groupers following antigen introduction. The higher expression of DCs in the vaccinated group might be due to their role in initiating adaptive immune responses by sensing foreign antigens and interacting with lymphocytes [44].

Comparable to a study by Chin et al. [5] on the effectiveness of bath immunization against vibriosis in Asian seabass using a live-attenuated *V. harveyi*, the present investigation demonstrated elevated expression of CCL4 genes in post-vaccinated fish compared to the control. CCL4 genes encode a chemotactic cytokine that controls immune cell migration in tissues (2020a). It contributes to innate immunity by binding to its cognate receptor, typically CCR5, which is expressed on the surface of target immune cells [45]. This interaction facilitates the recruitment and activation of various immune cell types, including macrophages and natural killer (NK) cells. Moreover, CCL4 coordinates cross-talk between innate and adaptive immune responses, particularly by promoting interactions between T cells and DCs [45].

In addition, the C3 gene, another gene responsible for innate immunity, was also increased post-vaccination with the oral vaccine. These findings are comparable to those of Bao et al. [46], who found that turbots (*Scophthalmus maximus* L.) that received combined live *V. anguillarum* and *Edwardsiella piscicida* vaccinations had significantly elevated C3 gene expression. A component of the complement system, the C3 gene is essential for phagocytosis promotion, activating inflammatory responses against pathogens, and regulating differentiation and maturation of B- and T-lymphocytes and DCs [47].

The classic pro-inflammatory cytokine gene, TNF-α regulates the equilibrium between humoral and cellular immune responses

and is produced by activated immune cells in response to various external antigens [48]. They are brought on by T-lymphocytes and mononuclear phagocytes, which are components of the innate and adaptive immune systems, respectively [49]. The current study found that the vaccinated group showed increased TNF-α expression in head kidney samples, suggesting that the oral inactivated *V. harveyi* vaccine may stimulate phagocyte and macrophage activity upon antigen introduction [50].

Moreover, the IgT gene produced by B cells in response to antigen stimulation [51] was significantly upregulated in the vaccinated group. Previous findings suggest that IgT may be involved in the immune response of Asian seabass (*Lates calcarifer*) against bacterial infections [52]. In the present study, high levels of IgT gene expression were observed in the head kidney of orally vaccinated fish, consistent with the findings of Thu Lan et al. [53], who reported elevated IgT expression in the head kidney following immersion vaccination. Notably, in the present study, IgT mRNA levels increased significantly before booster vaccinations at weeks 2 and 6 and again at week 8, indicating a potential role for IgT in protective immunity against vibriosis. Previous studies have shown that IgT is predominantly associated with mucosal immune responses in fish, particularly in the skin, gills, and gastrointestinal tract [54]. However, the role of IgT in mucosal immunity in Asian seabass post-oral vaccination with the vaccine from this study remains to be fully elucidated. Further research is needed to investigate IgT expression in mucosal tissues, such as the skin and gut, to clarify its involvement in immune responses induced by oral vaccination. These findings are further supported by our previous study, which demonstrated that specific IgM antibody levels in both serum and mucus of field-vaccinated *L. calcarifer* were significantly higher than those in the unvaccinated control group [19]. Elevated antibody responses were detected as early as week 2 post-primary vaccination and reached peak levels at week 8, indicating sustained humoral immune activation following vaccination with the feed-based vaccine.

Overall, the present vaccination strategy demonstrates strong potential for application in commercial aquaculture systems, as supported by both field vaccination and laboratory challenge outcomes. Oral vaccination can be readily implemented across production stages, from hatcheries to grow-out farms. This approach is practical and farmer-friendly and does not require additional vaccination infrastructure, specialized equipment, or highly trained personnel. In addition, oral immunization via vaccine incorporation into feed is particularly suitable for routine use during the grow-out phase in cage or earthen pond systems [55]. Feed-based vaccination offers several advantages over alternative delivery methods, including reduced handling stress, lower labor requirements, and scalability for both small- and large-scale farming operations. By providing a more accessible and operationally simple approach, this strategy may enhance disease control in aquaculture production systems. This is particularly relevant for low-income settings, where constraints in vaccine access, delivery logistics, and technical capacity often limit the adoption of conventional vaccination methods; oral vaccination therefore offers a practical means to overcome these barriers.

5 | Conclusion

This study highlights the potential of an oral inactivated *V. harveyi* vaccine in Asian seabass (*Lates calcarifer*) by examining immune-related gene expression and protective efficacy. The vaccine significantly enhanced the expression of key immune genes, including DCs, CCL4, C3, TNF-α, and IgT, from week 2 to week 10, with peak expression mostly observed after booster doses. These results indicate that the vaccine effectively stimulates both innate and adaptive immunity. Post-challenge trials with virulent *V. harveyi* showed an 80% RPS in vaccinated fish compared to 0% in unvaccinated controls, demonstrating robust protection. Further research should evaluate the vaccine's long-term immune memory, cross-protection against other pathogens, and performance under practical aquaculture conditions. Additionally, future studies should address the vaccine's economic viability and scalability for commercial applications.

Author Contributions

Aslah Mohamad: conceptualization, investigation, writing – original draft, writing – review and editing. **Zahaludin Amir-Danial:** conceptualization, investigation, visualization, validation, data curation, funding acquisition, writing – original draft, writing – review and editing. **Mohammad Noor Azmai Amal**, **Mohd Zamri-Saad, Annas Salleh, and Ina Salwany Md Yasin:** supervision, conceptualization, investigation, methodology, validation, data curation, funding acquisition, writing – original draft, writing – review and editing. **Mohamad Azzam-Sayuti and Chin Yong Kit**: writing – review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

1. Y. Li, Y. Chi, X. Yan, et al., "A QTL Associated With Vibriosis Resistance Highlights the Significant Role of jam2a Gene in Tongue Sole (*Cynoglossus semilaevis*)," *Aquaculture* 597 (2025): 741930.

2. G. M. M. Sanches-Fernandes, I. Sá-Correia, and R. Costa, "Vibriosis Outbreaks in Aquaculture: Addressing Environmental and Public Health Concerns and Preventive Therapies Using Gilthead Seabream Farming as a Model System," *Frontiers in Microbiology* 13 (2022): 904815.

3. V. Albert and J. Ransangan, "Effect of Water Temperature on Susceptibility of Culture Marine Fish Species to Vibriosis," *International Journal of Research in Pure and Applied Microbiology* 3 (2013): 48–52.

4. K. D. Lafferty, C. D. Harvell, J. M. Conrad, et al., "Infectious Diseases Affect Marine Fisheries and Aquaculture Economics," *Annual Review of Marine Science* 7, no. 1 (2015): 471–496.

5. Y. K. Chin, M. Y. Ina-Salwany, M. Zamri-Saad, et al., "Effects of Skin Abrasion in Immersion Challenge With *Vibrio harveyi* in Asian Seabass *Lates calcarifer* Fingerlings," *Diseases of Aquatic Organisms* 137, no. 3 (2020): 167–173.

6. I. K. M. Chiew, A. M. Salter, and Y. S. Lim, "The Significance of Major Viral and Bacterial Diseases in Malaysian Aquaculture Industry," *Pertanika Journal of Tropical Agricultural Science* 42 (2019): 1023–1047.

7. P. Rameshkumar, C. Kalidas, G. Tamilmani, et al., "Microbiological and Histopathological Investigations of *Vibrio Alginolyticus* Infection in Cobia *Rachycentron canadum* (Linnaeus, 1766) Cultured in Sea Cage," *Indian Journal of Fisheries* 61 (2013): 124–127.

8. T. Manchanayake, A. Salleh, M. N. A. Amal, I. S. M. Yasin, and M. Zamri-Saad, "Pathology and Pathogenesis of *Vibrio* Infection in Fish: A Review," *Aquaculture Reports* 28 (2023): 101459.

9. B. Peng, X.-P. Lin, S.-N. Wang, M.-J. Yang, X.-X. Peng, and H. Li, "Polyvalent Protective Immunogens Identified from Outer Membrane Proteins of *Vibrio parahaemolyticus* and Their Induced Innate Immune Response," *Fish & Shellfish Immunology* 72 (2018): 104–110.

10. I. S. M. Yasin, A. Mohamad, and M. Azzam-Sayuti, "Control of Fish Diseases Using Antibiotics and Other Antimicrobial Agents," in *Recent Advances in Aquaculture Microbial Technology*, (Elsevier Inc., 2023).

11. N. S. Mohd Ali, M. Z. Saad, M. N. A. Azmai, et al., "Immunogenicity and Efficacy of a Feed-Based Bivalent Vaccine against Streptococcosis and Motile Aeromonad Septicemia in Red Hybrid Tilapia (*Oreochromis* sp.)," *Animals* 13, no. 8 (2023): 1346.

12. H. T. Nguyen, T. T. Thu Nguyen, M.-A. Tsai, E. Ya-Zhen, P.-C. Wang, and S.-C. Chen, "A Formalin-Inactivated Vaccine Provides Good Protection Against *Vibrio harveyi* Infection in Orange-Spotted Grouper (*Epinephelus coioides*)," *Fish & Shellfish Immunology* 65 (2017): 118–126.

13. F. Samsing, W. Zhang, R. N. Zadoks, et al., "Cold Temperature Stress and Damaged Skin Induced High Mortality in Barramundi (*Lates calcarifer*) Challenged With *Vibrio harveyi*," *Journal of Fish Diseases* 46, no. 7 (2023): 751–766.

14. Y. J. Silva, L. Costa, C. Pereira, et al., "Phage Therapy as an Approach to Prevent *Vibrio anguillarum* Infections in Fish Larvae Production," *PLoS One* 9, no. 12 (2014): e114197–23.

15. N. Lorenzen and S. E. LaPatra, "DNA Vaccines for Aquacultured Fish," *OIE Revue Scientifique et Technique* 24, no. 1 (2005): 201–213.

16. I. Tobar, S. Arancibia, C. Torres, et al., "Successive Oral Immunizations against *Piscirickettsia salmonis* and Infectious Salmon Anemia Virus Are Required to Maintain a Long-Term Protection in Farmed Salmonids," *Frontiers in Immunology* 6 (2015): 1–7.

17. A. Marsella, F. Pascoli, T. Pretto, et al., "Efficacy of DNA Vaccines in Protecting Rainbow Trout Against VHS and IHN under Intensive Farming Conditions," *Vaccines* 10, no. 12 (2022): 1–20.

18. S. K. Nayak, "Current Prospects and Challenges in Fish Vaccine Development in India with Special Reference to *Aeromonas hydrophila* Vaccine," *Fish & Shellfish Immunology* 100 (2020): 283–299.

19. Z. Amir-Danial, M. Zamri-Saad, M. N. A. Amal, et al., "Field Efficacy of a Feed-Based Inactivated Vaccine Against Vibriosis in Cage-Cultured Asian Seabass, *Lates calcarifer*, in Malaysia," *Vaccines* 11, no. 1 (2023): 9–13.

20. A. Mohamad, F.-A. A. Mursidi, M. Zamri-Saad, et al., "Laboratory and Field Assessments of Oral Vibrio Vaccine Indicate the Potential for Protection Against Vibriosis in Cultured Marine Fishes," *Animals* 12, no. 2 (2022): 1–15.

21. F. Mursidi, *Antigenic Analysis of Outer Membrane Protein of Vibrio Species and Development of Versatile Recombinant vhDnaJ Vaccine against Vibriosis* Universiti Putra Malaysia, 2018).

22. T. Wohlsen, J. Bates, G. Vesey, W. A. Robinson, and M. Katouli, "Evaluation of the Methods for Enumerating Coliform Bacteria From Water Samples Using Precise Reference Standards," *Letters in Applied Microbiology* 42, no. 4 (2006): 350–356.

23. A. Bunnoy, P. Thangsunan, P. Chokmangmeepisarn, et al., "Mucoadhesive Cationic Lipid-Based Flavobacterium Oreochromis Nanoencapsulation Enhanced the Efficacy of Mucoadhesive Immersion Vaccination Against Columnaris Disease and Strengthened Immunity in Asian Sea Bass (*Lates calcarifer*)," *Fish & Shellfish Immunology* 127 (2022): 633–646.

24. N. Mohd-Shaharuddin, A. Mohd-Adnan, B. C. Kua, and S. Nathan, "Expression Profile of Immune-Related Genes in *Lates calcarifer* Infected by Cryptocaryon Irritans," *Fish & Shellfish Immunology* 34, no. 3 (2013): 762–769.

25. Y. K. Chin, N. Al-saari, Z. Zulperi, et al., "Efficacy of Bath Vaccination with a Live Attenuated *Vibrio harveyi* Against Vibriosis in Asian Seabass Fingerling, *Lates calcarifer*," *Aquaculture Research* 51, no. 1 (2020): 389–399.

26. S. H. Hoseinifar, S. Yousefi, G. Capillo, et al., "Mucosal Immune Parameters, Immune and Antioxidant Defence Related Genes Expression and Growth Performance of Zebrafish (*Danio rerio*) Fed on *Gracilaria gracilis* Powder," *Fish & Shellfish Immunology* 83 (2018): 232–237.

27. M. C. Piazzon, J. Galindo-Villegas, P. Pereiro, et al., "Differential Modulation of IgT and IgM upon Parasitic, Bacterial, Viral, and Dietary Challenges in a Perciform Fish," *Frontiers in Immunology* 7 (2016): 1–16.

28. A. Mohamad, M. Zamri-Saad, M. N. A. Amal, et al., "Vaccine Efficacy of a Newly Developed Feed-Based Whole-Cell Polyvalent Vaccine against Vibriosis, Streptococcosis and Motile Aeromonad Septicemia in Asian Seabass, *Lates calcarifer*," *Vaccines (Basel)* 9, no. 4 (2021): 368.

29. M. S. Monir, M. S. M. Yusoff, M. Zamri-Saad, et al., "Effect of an Oral Bivalent Vaccine on Immune Response and Immune Gene Profiling in Vaccinated Red Tilapia (Oreochromis spp.) During Infections With *Streptococcus iniae* and Aeromonas hydrophila," *Biology* 11, no. 9 (2022): 1268.

30. A. Mohd-Aris, M.-Z. Saad, H. M. Daud, M. T. Yusof, and M. Y. Ina-Salwany, "*Vibrio harveyi* Protease Deletion Mutant as a Live Attenuated Vaccine Candidate against Vibriosis and Transcriptome Profiling Following Vaccination for *Epinephelus fuscoguttatus*," *Aquaculture International* 27, no. 1 (2019): 125–140.

31. C.-F. Lin, H.-K. Jiang, N.-C. Chen, T.-Y. Wang, and T.-Y. Chen, "Novel Subunit Vaccine With Linear Array Epitope Protect Giant Grouper Against Nervous Necrosis Virus Infection," *Fish & Shellfish Immunology* 74 (2018): 551–558.

32. B. Yin, H. Liu, B. Tan, et al., "Preliminary Study of Mechanisms of Intestinal Inflammation Induced by Plant Proteins in Juvenile Hybrid Groupers (♀*Epinephelus fuscoguttatus*×♂*E. lanceolatus*)," *Fish & Shellfish Immunology* 106 (2020): 341–356.

33. S. Kumar, G. Stecher, M. Suleski, M. Sanderford, S. Sharma, and K. Tamura, "MEGA12: Molecular Evolutionary Genetic Analysis Version 12 for Adaptive and Green Computing," *Molecular Biology and Evolution* 41, no. 12 (2024): msae263.

34. S. Bravo and P. J. Midtlyng, "The use of Fish Vaccines in the Chilean Salmon Industry 1999-2003," *Aquaculture* 270, no. 1–4 (2007): 36–42.

35. J. Ransangan, T. M. Lal, and A. H. Al-Harbi, "Characterization and Experimental Infection of *Vibrio harveyi* Isolated from Diseased Asian Seabass (*Lates calcarifer*)," *Malaysian Journal of Microbiology* 8 (2012): 104–115.

36. J. K. Chettri, R. M. Jaafar, J. Skov, P. W. Kania, I. Dalsgaard, and K. Buchmann, "Booster Immersion Vaccination Using Diluted *Yersinia ruckeri* Bacterin Confers Protection against ERM in Rainbow Trout," *Aquaculture* 440 (2015): 1–5.

37. M. S. Ismail, A. Siti-Zahrah, M. R. M. Syafiq, M. N. A. Amal, M. Firdaus-Nawi, and M. Zamri-Saad, "Feed-Based Vaccination Regime Against Streptococcosis in Red Tilapia, *Oreochromis niloticus* x *Oreochromis mossambicus*," *BMC Veterinary Research* 12, no. 1 (2016): 1–6.

38. A. Mohamad, M. Zamri-Saad, M. N. A. Amal, et al., "Vaccine Efficacy of a Newly Developed Feed-Based Whole-Cell Polyvalent Vaccine Against Vibriosis, Streptococcosis and Motile Aeromonad Septicemia in Asian Seabass, *lates calcarifer*," *Vaccines* 9, no. 4 (2021): 368.

39. H. T. Nguyen, T. T. T. Nguyen, Y. T. Wang, P. C. Wang, and S. C. Chen, "Effectiveness of Formalin-Killed Vaccines Containing CpG Oligodeoxynucleotide 1668 Adjuvants Against *Vibrio harveyi* in Orange-Spotted Grouper," *Fish & Shellfish Immunology* 68 (2017): 124–131.

40. S.-P. Liu, S.-C. Chuang, and C.-D. Yang, "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," 8, no. 1 (2020).

41. S. Chakraborty, T. Cao, A. Hossain, et al., "Vibrogen-2 Vaccine Trial in Lumpfish (*Cyclopterus lumpus*) Against Vibrio anguillarum," *Journal of Fish Diseases* 42, no. 7 (2019): 1057–1064.

42. F. Buonocore, N. Nuñez-Ortiz, S. Picchietti, et al., "Vaccination and Immune Responses of European Sea Bass (*Dicentrarchus labrax* L.) Against Betanodavirus," *Fish Shellfish Immunol* 85 (2019): 78–84.

43. K. Sun, W.wei Zhang, J.hui Hou, and L. Sun, "Immunoprotective Analysis of VhhP2, a *Vibrio harveyi* Vaccine Candidate," *Vaccine* 27, no. 21 (2009): 2733–2740.

44. J. Wang, T. Wang, O. Benedicenti, et al., "Characterisation of ZBTB46 and DC-SCRIPT/ZNF366 in Rainbow Trout, Transcription Factors Potentially Involved in Dendritic Cell Maturation and Activation in Fish," *Developmental & Comparative Immunology* 80 (2018): 2–14.

45. K. Syahputra, P. W. Kania, A. Al-Jubury, R. M. Jafaar, R. P. Dirks, and K. Buchmann, "Transcriptomic Analysis of Immunity in Rainbow Trout (*Oncorhynchus mykiss*) Gills Infected by *Ichthyophthirius multifiliis*," *Fish & Shellfish Immunology* 86 (2019): 486–496.

46. P. Bao, X. Sun, Q. Liu, Y. Zhang, and X. Liu, "Synergistic Effect of a Combined Live *Vibrio anguillarum* and *Edwardsiella piscicida* Vaccine in Turbot," *Fish & Shellfish Immunology* 88 (2019): 84–90.

47. M. Chen, C. Wang, Z. Li, and J. Chen, "Involvement of JNK Signaling Pathway in Lipopolysaccharide-Induced Complement C3 Transcriptional Activation From Amphioxus *Branchiostoma belcheri*," *Fish & Shellfish Immunology* 86 (2019): 196–203.

48. D.-H. H. Xu, G. S. A. Moreira, C. A. Shoemaker, D. Zhang, and B. H. Beck, "Expression of Immune Genes in Systemic and Mucosal Immune Tissues of Channel Catfish Vaccinated With Live Theronts of *Ichthyophthirius multifiliis*," *Fish & Shellfish Immunology* 66 (2017): 540–547.

49. P. Kayansamruaj, H. T. Dong, N. Pirarat, D. Nilubol, and C. Rodkhum, "Efficacy of α-Enolase-Based DNA Vaccine Against Pathogenic *Streptococcus iniae* in Nile Tilapia (*Oreochromis niloticus*)," *Aquaculture* 468 (2017): 102–106.

50. Z. Zhang, A. Yu, J. Lan, et al., "GapA, a Potential Vaccine Candidate Antigen Against *Streptococcus agalactiae* in Nile Tilapia (*Oreochromis niloticus*)," *Fish & Shellfish Immunology* 63 (2017): 255–260.

51. Y. Y. Yao, D. D. Chen, Z. W. Cui, et al., "Oral Vaccination of Tilapia Against *Streptococcus agalactiae* Using *Bacillus subtilis* Spores Expressing Sip," *Fish & Shellfish Immunology* 86 (2019): 999–1008.

52. F. Buonocore, G. Scapigliati, A. Pallavicini, and M. Gerdol, "Identification of an IgD/IgT Chimera in the European Sea Bass (*Dicentrarchus labrax* L.)," *Fish & Shellfish Immunology* 105 (2020): 224–232.

53. N. G. Thu Lan, H. T. Dong, N. T. Vinh, et al., "A Novel Vaccination Strategy against *Vibrio harveyi* Infection in Asian Seabass (*Lates calcarifer*) With the Aid of Oxygen Nanobubbles and Chitosan," *Fish & Shellfish Immunology* 149 (2024): 109557.

54. T. T. Mai, P. Kayansamruaj, S. Taengphu, et al., "Efficacy of Heat-Killed and Formalin-Killed Vaccines Against Tilapia Tilapinevirus in Juvenile Nile Tilapia (*Oreochromis niloticus*)," *Journal of Fish Diseases* 44, no. 12 (2021): 2097–2109.

55. N. G. T. Lan, H. T. Dong, N. T. Vinh, et al., "Immersion Prime and Oral Boost Vaccination With an Inactivated *Vibrio harveyi* Vaccine Confers a Specific Immune Response and Protection in Asian Seabass (*Lates calcarifer*)," *Fish & Shellfish Immunology* 144 (2024): 109293.