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Research Article

An inactivated bivalent vaccine effectively protects Asian sea bass (*Lates calcarifer*) against *Streptococcus iniae* and *Vibrio harveyi* infections

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Abstract

Co-infection with *Streptococcus iniae* and *Vibrio harveyi* is the major health challenge in Asian sea bass (*Lates calcarifer*) farms. Vaccination is the most effective means of preventing these diseases, therefore, this study presents an efficacious bivalent vaccine against *S. iniae* and *Vibrio harveyi* infections in Asian sea bass. 420 juvenile sea bass (weighing 20.35 ± 0.37 g) were separated into seven equal groups in triplicates: group A: received Phosphate-buffered saline as a control group, groups B and E: immunized with *S. iniae* vaccine via injection (*S.i*-Inj) and immersion (*S.i*-Im) routes, groups C and D: immunized with *V. harveyi* vaccine via immersion (*V.h*-Im) and injection (*V.h*-Inj) routes, and groups F and G: immunized with bivalent *S. iniae*+*V. harveyi* vaccine via immersion (Bi-im) and injection (Bi-inj) routes. The blood, serum, and gene samples were taken on 0, 30, and 60 days of the experiment and to measure specific and non-specific immune parameters, hematological and immune-related gene expression. The bacterial challenge test was used as a protective immunity measure at the end of the experiment. The results showed that the injection bivalent vaccine (*S. iniae*+*V. harveyi*) route was more effective than monovalent vaccines in enhancing the specific (antibody titer) and non-specific (lysozyme and complement activity, Nitro Blue Tetrazolium (NBT) reduction, protein, and globulin level) immune responses. Meanwhile, the vaccine efficacy and expression of *IL-10* and *GM-CFC* genes in the bivalent vaccine were significantly higher ($P<0.05$) than monovalent vaccines. These results indicate that the high immunogenicity and protective immunity provided by the bivalent vaccine (*S. iniae*+*V. harveyi*) strongly support its use as a general preventive measure against streptococosis and vibriosis in Asian sea bass farms.

Introduction

Given the scarcity of freshwater resources, particularly in drought-affected countries, the cultivation of marine fish in brackish water has emerged as a critical aquaculture practice. The farming of saltwater fish species has seen a significant uptick worldwide in the last decade (Tičina *et al*., 2020). The Asian sea bass, being a euryhaline fish, stands out as a prime candidate for aquaculture due to several reasons, including its ability to thrive across a wide range of salinities, reproduce in captivity, rapid growth, and its status as a high-value iconic species (Mozanzadeh *et al*., 2021). Despite its favorable biological traits for cultivation, the species' susceptibility to various infectious diseases, such as Vibriosis and Streptococosis, has led to substantial financial losses. (Nor NM *et al.,* 2019; Van Khang *et al.,* 2019; Thu Lan *et al.,* 2021). Recently Asian Sea bass farming in marine cages in the Persian Gulf is increasingly developing. Vibriosis is a disease caused by pathogenic *Vibrio* spp., which negatively affects marine aquaculture through high mortality, other than being a potential zoonotic disease vector to humans (Helmi *et al.,* 2020). This disease is one of the most common diseases in Asian sea bass in Australia, Malaysia, and Indonesia (Charoenwai et al., 2019). The most serious bacterial fish disease that cause Vibriosis in aquatic animals include *Vibrio anguillarum*, *V. harveyi*, *V. ordalli*, and *V. parahaemolyticus*. Compared to other bacterial species, *V. harveyi* is considered the most numerous bacterium in farmed marine fishes and is implicated in many problems that threaten economic stability, causing a reduction in income (Mohamad *et al.,* 2019). Because antibiotics are no longer recommended for administration in aquaculture due to the emergence of antibiotic-resistant bacteria (Vincent *et al*., 2019), an alternative control measure through vaccination is recommended (Sarker *et al.,* 2020).

The bacterium *Streptococcus iniae* stands as one of the most common etiological agents of streptococcosis. In Asian sea bass, this disease is attributed to *S. iniae*, characterized by symptoms such as hemorrhage in the skin and fins, as well as protruding eyes. The first epidemic of streptococcosis was reported in Australia in 1999 (Bromage *et al*., 1999). Numerous investigations have revealed high mortality rates associated with *S. iniae* infections in Asian sea bass across various countries (Piamsomboon *et al*., 2020). Meanwhile, the disease has continued to afflict sea bass farms in Australia, with reported mortality rates of up to 70% in the early life stages in 2006 (Creeper and Buller., 2006). This disease has been reported in cultured Asian sea bass in Thailand and Vietnam (Lan *et al*., 2021).

Moreover, the control of *V. harveyi* and *S. iniae* in aquaculture predominantly relies on the use of a wide range of antibiotics. However, due to the associated risks and side effects of antibiotic compounds, including the selection of antibiotic-resistant mutant bacterial variants, contamination of water ecosystems, and potential food safety hazards, alternative means of disease control, such as vaccines, have been under study in recent decades. Fish vaccinology has shown remarkable improvement in recent years and has been applied to provide and assist in eliciting a

long-lasting immune response to microsporidian infection in cultured fish (Adams and Subasinghe, 2019; Halimi et al., 2019).

Evaluation of the expression of immune-relevant genes reveals the status of immune responses at the cellular and molecular levels. GM-CSF cytokine was initially identified as a colony-stimulating factor due to its ability to induce granulocyte and macrophage populations from precursor cells. It is a kind of cytokine with hemato-immunological functions that is mostly produced by monocytes, fibroblasts, and endothelial cells, and it is responsible for the proliferation in general, survival, and differentiation of neutrophils, monocytes macrophages, and their respective progenitors (Fleetwood *et al*., 2005). Meanwhile, the *IL-10* cytokine is widely expressed in various cells, including macrophages, monocytes, dendritic cells, mast cells, eosinophils, neutrophils, natural killer cells, and lymphocytes (Ouyang *et al*., 2011).

The development of vaccine strategies against infectious pathogens in Asian sea bass is still in its nascent stages. Several studies have identified promising monovalent vaccine candidates against *V. harveyi* and *S. iniae*, demonstrating reliable efficacy. However, to the best of our knowledge, no bivalent vaccines with high efficacy via different routes against both *V. harveyi* and *S. iniae* have been documented in sea bass. Therefore, this study aims to evaluate the efficacy of monovalent and bivalent Streptococcus and Vibrio vaccines administered through different routes in Asian sea bass (*Lates calcalifer*). Additionally, this study aims to assess the effects of these vaccines on hematological parameters, as well as specific and non-specific immune responses in *Lates calcalifer*.

Materials and methods

Fish husbandry and experimental design

In total, 420 healthy sea bass (20.35±0.37 g) with no clinical signs of disease and normal appearance were obtained from Ramooz Marine Fish Farming Co, Bushehr, Iran and moved to the Aquatic Animal Health research laboratory, faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran. After two weeks of acclimatization, the fish were randomly divided into seven equal groups, each with three replicates. Each replicate contained 20 fish, allocated as follows: Group A served as the control group and received PBS. Group B was immunized with the *S. iniae* vaccine via the injection route (Si-Inj). Group C was immunized with the *V. harveyi* vaccine via the immersion route (Vh-Im). Group D was immunized with the *V. harveyi* vaccine via the injection route (Vh-Inj). Group E was immunized with the *S. iniae* vaccine via the immersion route (Si-Im). Group F was immunized with the bivalent *S. iniae* + *V. harveyi* vaccine via the immersion route (Bi-Im). Group G was immunized with the bivalent *S. iniae*+*V. harveyi* vaccine via the injection route (Bi-Inj).

Each tank received equal water flow and continuous central aeration and was equipped with thermostatic hitters (Autumn season), Fish were fed 3% of body weight, twice a day for two months

with a commercial pellet during this trial (Beiza Co, Iran). Throughout the duration of the experiment the natural photoperiod was maintained. The items which were checked daily were temperature (28.5±0.5°C) and dissolved oxygen (7.1±0.3 mg) and those which were measured weekly were salinity (10.61±1.43), NO^2-N (<0.05 mgL^{-1}); NH^3-N (<0.05 mgL^{-1}) , and pH (7.4±0.4) of water.

Bacterial strains

Streptococcus iniae and *Vibrio harveyi* were isolated from diseased fish in Asian sea bass cage farms of the southern provinces of Iran as the experimental vaccine seeds. Both bacteria were identified based on biochemical and molecular methods using 16s RNA genes. PCR products were directly used for Sanger sequencing to confirm the amplified fragments.

Vaccine preparation

Formalin-killed vaccines of *Streptococcus iniae* and *Vibrio harveyi* were prepared according to (Tulaby Dezfuly *et al*., 2020). Briefly, the bacteria were first cultured in a Trypticase Soy Broth (1.5% NaCl) for 48 h in 30 ºC and then bacteria were washed twice with PBS and inactivated using 1% formalin for 12 h. Then, suspension was centrifuged and formalin removed from the vaccine by washing with PBS 3 times (pH: 7.4) and then resuspended in PBS to prepare a density of 10^{10} CFU/mL. For administration of each vaccine by injection route, the fish were starved for 24 h prior to each vaccination. Later, Fish were anesthetized with a concentration of 400 ppm 2-phenoxy ethanol, and 100 µl of the vaccine was injected intraperitoneally. In the immersion route, the 24 h starved fish were immersed for 2 minutes at a concentration of 10^9 bacteria per mL. Immunization was performed on days zero and 14 of the experiment with the same route and the same amount of vaccine. The bivalent vaccine was prepared by mixing two vaccines in equal proportions.

Sampling procedure

On days 0 (pre-vaccination), 30, and 60 of the experiment, nine fish were chosen from each group (three fish from each replicate). Following 24 h of starvation and anesthesia using 300 mgL^{-1} of 2-phenoxyethanol, heparinized blood samples were obtained from the fish for hematological and immunological assays, respectively. Following blood sampling and euthanasia, the fish were dissected, and gene expression samples were extracted from the anterior kidneys and hindgut. These samples were preserved in a freezer at -70°C until further use.

The *in vivo* phase of this experiment has been conducted according to the guidelines of the Institutional Animal Ethics Committee, Faculty of Veterinary, Shahid Chamran University, Iran (Cod: EE/1401.3.24.78971 /scu.ac.ir).

Antibody titer against S. iniae and V. harveyi via ELISA method

*S. iniae and V.harveyi*specific antibody titers in serum, were measured by Enzyme-linked immunosorbent assay (ELISA) as recommended by Halimi *et al.* (2020), with slight modifications. Briefly, 50 µL of

soluble whole-cell antigens of Bacteria (*S. iniae or V. harveyi*) (100 µg mL^{-1}) in carbonate/bicarbonate buffer (pH=7.6) were added to each well of ELISA plates (SPL, South Korea) and incubated overnight at 4°C. Plates were washed with PBS containing 0.05% of Tween-20 (PBS-T) and blocked with 2.5% skim milk in PBST for 1 hour at room temperature (RT). Following three times washing, 100 µL of serum was then poured at a 1:20 dilution respectively. The plate was incubated for 90 minutes at RT and after washing, 100 µL of rabbit anti-sea bass polyclonal antibody at a 1:2000 dilution were added to wells and the plate was incubated at RT for an hour. Following washing, 50 µL of goat anti-rabbit IgG HRP conjugate (Sigma-Aldrich) at a 1:2500 dilution was added to each well. After incubation for 60 minutes at RT the plate was washed and 50 µL of standard chromogen-substrate solution was added to wells and incubated for 10 minutes at RT. Finally, 50 µL of stop solution (2% of sulfuric acid) were added to the wells and the optical density (OD) of individual wells was read at 450 nm by a plate reader (Accu Reader, Taiwan). Sera of hyperimmune sea bass (against *S. iniae* or *V. harveyi*) was used as the positive control. Antibody titer showed an S/P ratio as follows (Halimi *et al.,* 2020):

S/P ratio=(OD of Test Sample - Mean OD of negative control)/ (Mean OD of Positive control – Mean OD of negative control) ×100

Non-specific immune parameters

Serum alternative complement activity was assayed according to (Barta and Brata, 1993) using Rabbit red blood cells (RaRBC) as the target cell. RaRBC was provided in 1.5% agarose (pH=7.2), containing 0.5 mM MgCl2 and 1.5 mM CaCl2. RaRBC in agarose was washed with PBS (0.1 M pH=7.0) by centrifugation at 750 g for five min and the cell concentration was adjusted to 1×108 cell mL^{-1}. Agarose containing RaRBC was dispensed into a plate, incubated at 4°C, and punched (3 mm in diameter). Subsequently, each hole was filled with 15 µL of serum and incubated at room temperature. After 24 h of incubation, the zone of lysis was measured and expressed in Arbitrary Unit mL^{-1}.

Serum lysozyme activity

Serum lysozyme activity was determined using the *Micrococcus lysoplate* assay according to Osserman and Lawlor (1966). The serum bactericidal activity was carried out based on Budino *et al*. (2006) method with some modifications. Briefly, *A. hydrophila* was adjusted to 10^7 CFU mL^{-1}, next 33 µL of fresh serum and 133 µL of bacterial dilution were added to each well and mixed. PBS and inactive serum (at 50˚C for 30 min) were used as control. All samples were incubated at the same time at 20°C. Then 86 µl of MTT (2mg mL^{-1}) was added, mixed, and incubated for 15 min at 20˚C. At last MTT reduction (by live bacteria) was measured at 630 nm and the bactericidal index was determined as the mean absorbance of 4 wells versus the absorbance of the control.

NBT reduction assay

Phagocytic power of leukocytes was assayed according to the protocol of (Geng *et al.,* 2012) on blood cells using the nitroblue tetrazolium (NBT, Merck) and N-dimethyl formamide (Merck). The optical density (OD) of the supernatant was measured at 540 nm in the microplate reader (Model: Eurolyser).

Total serum protein, albumin, and globulin

The total serum protein level was estimated by the method of Bradford (1976) using the standard protein estimation kit (Zist shimi co, Iran). For globulin estimation 50 µL saturated ammonium sulfate solution was added dropwise to 50 µL serum followed by vortexing.

Centrifugation was done at 10000 was done at 10,000 gr for min. Then 20 µL of this sample was dissolved with 80 µL carbonate-bicarbonate buffer (pH 9.3) and the protein content was estimated by the method of Bradford using the standard protein estimation kit (Zist shimi co, Iran). Albumin content was measured using a standard albumin estimation kit (Zistchem Diagnostics, Iran)

Hematological parameters

Heparinized blood samples were immediately analyzed for the estimation of numbers of erythrocytes (RBC), White blood cells (WBC), hemoglobin (Hb), and hematocrit (Hct). RBC and WBC were determined using the Neubauer Haemocytometry method, and hemoglobin measurement was determined by the cianometa-hemoglobin method and hematocrit (Hct) measured by microhematocrit centrifuge (10 g for 15 minutes) (Schaperclaus *et al*., 1991).

Expression assay of IL-10 and GMCFC genes

RNA extraction and synthesis of cDNA

RNA extraction of samples from the head kidney and hint intestine were evaluated via the SinaPure RNA, Animal tissues, isolation kit (Sina Clone Co, Iran) based on the instructions of the company. Quantification and amount of purified RNA were detected spectrophotometrically (Eppendorf, Germany). Isolated and purified RNA evaluated via 260/280 nm absorption in spectrophotometers with a ratio > 1.8 were subjected to cDNA synthesis. RT-PCR was carried out with the reverse transcriptase Sina Clone cDNA synthesis kit according to the protocol of the manufactory (Sina Clone Co, Iran).

Design of Primers for quantitative PCR

For designing quantitative PCR primers of *IL10* and *GMCFC* gene sequences, the status of these genes in the GenBank of NCBI database was assayed, using specific software (Gene Runner, version 6). The sequences of designed primers were brought in Table 1. For standardization of expression levels, the *EF1α* gene was used as the housekeeping gene.

Gene expression via real-time PCR (qPCR)

In this study for evaluation of the alterations of *IL10* and Granulocyte-Macrophage Colony-Forming Cells (*GMCFC*) gene expression, quantitative real-time PCR was used by a Step One Real-Time PCR ABI (ABI, USA). The housekeeping or reference gene was Elongation factor 1α (EF1 α). Sequences of gene primers used in this study are shown in Table 1.

Table 1: Sequences of oligonucleotide primers used for real-time PCR.

Gene	Accession number	qPCR primers Forward/Reverse	Amplicon (bp)
EF1α	XM_018679392.1	TCAGAGACAGGTGACAACCA GGAAAGTGGTGTTGGCTTGA	180
IL 10	XM_018687637.1	CCAATGTGCAACCACCAGTG **TTCGACGGTCTGATCTAGCA**	149
GMCFC	XM_018705122.1	GCTTGTCATCCCCTTTGACT GACCATCCTGAGTTTCTAGCTC	206

Determination of LD50 of S. iniae and V. harveyi in Asian sea bass

To estimate the Lethal dose (LD_{50}) of *S. iniae* and *V. harveyi*, the method recommended by Halimi *et al*. (2019) was used. Median lethal dose (LD_{50}) was estimated as follows: LD_{50} of *S. iniae*=4×10^8 CFU mL^{-1} and LD_{50} of *V. harveyi*=2.7×10^8 CFU mL^{-1}

Bacterial Challenge

At the end of the experiment on day 60, fish in each replicate were separately intraperitoneally injected with live *S. iniae* (LD_{50} concentration=4×10^8 CFU mL^{-1}) and *V. harveyi* (at a concentration of 2.7×10^8 CFU mL^{-1}). Challenged fish were monitored for 14 days and the mortality was recorded daily then cumulative mortality percentage was calculated. One group was kept non-infected but under similar conditions as a negative control group (Laith *et al*., 2019). Bacteria were re-isolated from the kidney of dead fish using tryptone soy agar containing 1.5% NaCl and incubated at 22'C for 24h. For confirmation of the cause of death, standard PCR using *S. iniae and V. harvei*-specific 16S ribosomal RNA gene primers were used for confirmation of *S. iniae and V. harveyi* (Donati *et al.,* 2021).

Statistical analysis

Data was analyzed by using the SPSS software Version 23. One-way analysis of variance (ANOVA) compares the means of two or more independent groups in order to determine whether there is statistical evidence that the associated population means are significantly different. The Tukey's test compares the differences between means of values rather than comparing pairs of values with a 95% confidence level. Comparison of cumulative mortality percentage among the groups was analyzed using the Kaplan–Meier survival (Log-rank value) analysis test. Results of Gene Expression level were evaluated using ΔΔCt comparative method (LightCycler SW1 /1 software) and resulted in a fold change compared to the control group reported based on the $2^{-\Delta\Delta Ct}$ general formula.

Results

Antibody titer

The highest serum anti *S. iniae* antibody titer (using the ELISA method) was observed in Bi-inj group and monovalent strep vaccine respectively with significantly increased compared to other groups in both sampling points (p<0.05). Meanwhile, a significant increase in Bi-im and *S. iniae* monovalent immersion groups was recorded in both sampling points too.

Similarly, serum anti *V. harveyi* antibody titer in Bi-inj and V.h-inj groups were significantly higher than other groups on days 30 and 60 of the experiment ($p<0.05$). A slighter increase of anti *V.harveyi* antibody titer was observed in bivalent and *V. harve*yi vaccinated through immersion route ($p<0.05$) (Figs. 1 and 2).

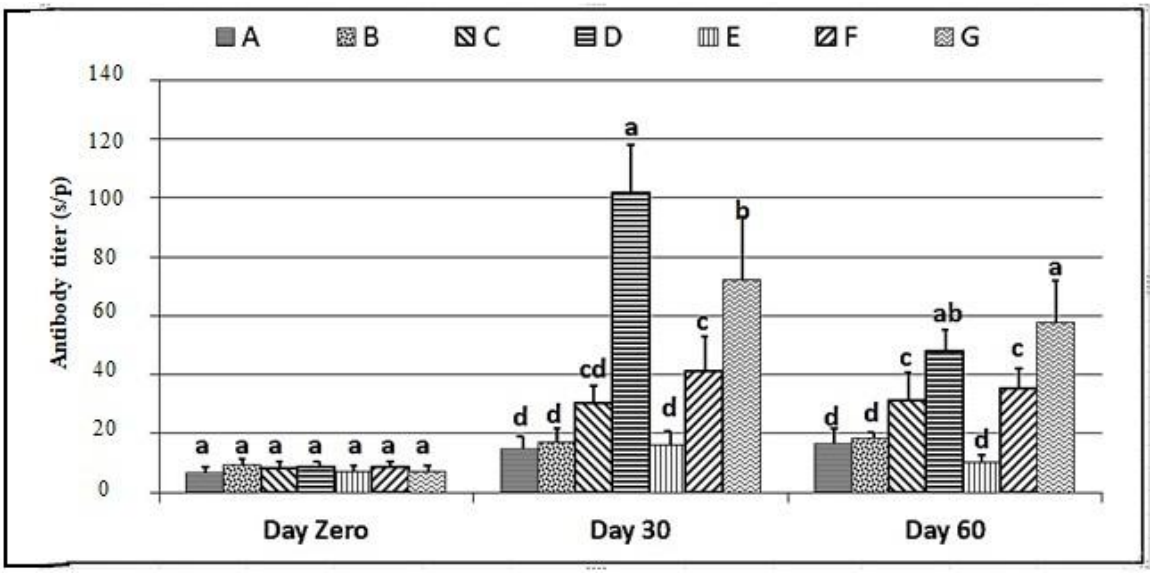

Figure 1: Serum Antibody titer against *V. harveyi* in different experimental groups (n=9): "Significant differences among groups at each sampling point are indicated by means with different lowercase letters." (*P*<0.05). (A: Control group, B: Fish vaccinated with *S. iniae* via injection route, C: Fish vaccinated with *V. harveyi* via immersion route, D: Fish vaccinated with *V. harveyi* via injection route, E: Fish vaccinated with *S. iniae* via immersion route, F: Fish vaccinated with *bivalent* vaccine via immersion route, G: Fish vaccinated with *bivalent* vaccine via injection route.

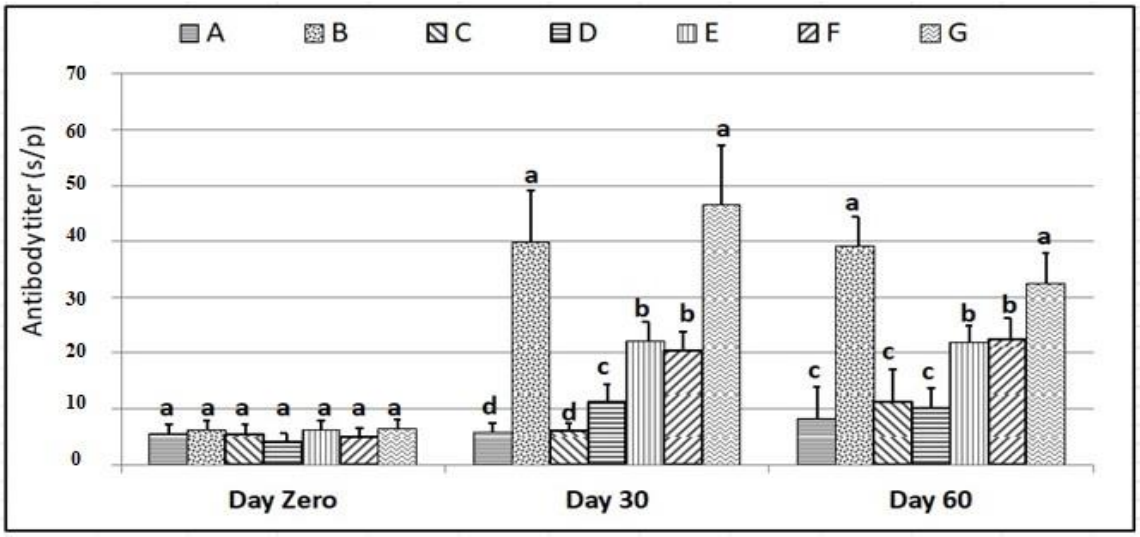

Figure 2: Serum Antibody titer against *S.iniae* in different experimental groups (n=9): "Significant differences among groups at each sampling point are indicated by means with different lowercase letters" (*p*<0.05). (A: Control group, B: Fish vaccinated with *S. iniae* via injection route, C: Fish vaccinated with *V. harveyi* via immersion route, D: Fish vaccinated with *V. harveyi* via injection route, E: Fish vaccinated with *S. iniae* via immersion route, F: Fish vaccinated with *bivalent* vaccine via immersion route, G: Fish vaccinated with *bivalent* vaccine via injection route.

Innate immune components

Most of the evaluated non-specific immune parameters were significantly enhanced in intraperitoneally injected groups, particularly in the bivalent vaccinated group.

Serum lysozyme activity, total protein, and IgM level of sampled fish showed a significant (p<0.05) increase in Bi-inj group followed by Si-Inj and Vh-inj on days 30 and 60 of the experiment compared to the control group. Although serum lysozyme activity increased significantly (p<0.05) in immersion groups compared to the control group in both sampling times, protein and globulin levels as well as complement activity showed no differences in all immersion vaccinated groups on days 30 and 60 of the experiment (Table 2 and Fig. 3).

Table 2: Immunological parameters of Asian sea bass in 7 experimental groups Different lowercase superscripts in each column indicate statistically significant differences between the values . (n=9; p<0.05). (A: Control group, B: Fish vaccinated with *S .iniae* via injection route, C: Fish vaccinated with *V.harveyi* via immersion route, D: Fish vaccinated with *V.harveyi* via injection route, E: Fish vaccinated with *S .iniae* via immersion route, F: Fish vaccinated with *bivalent* vaccine via immersion route, G: Fish vaccinated with *bivalent* vaccine via injection route.

Ave	Groups	NBT OD in 640nm	Bacteicidal activity OD	Complement activity(OD)	Total protein(g/dl)	Albumin (g/dl)	Globulin (g/dl)
Day zero	A	0.28±0.08 ^{a}	32.16±6.17 ^{a}	13.12±3.31 ^{a}	4.52±0.37 ^{a}	1.11±0.22 ^{a}	3.16±0.47 ^{a}
	B	0.29±0.06 ^{a}	31.23±8.57 ^{a}	13.48±2.32 ^{a}	4.48±0.33 ^{a}	1.22±0.21 ^{a}	3.22±0.48 ^{a}
	C	0.29±0.11 ^{a}	33.13±9.17 ^{a}	14.14±2.76 ^{a}	4.42±0.43 ^{a}	1.29±0.20 ^{a}	3.16±0.42 ^{a}
	D	0.28±0.07 ^{a}	32.34±6.79 ^{a}	12.40±2.08 ^{a}	4.29±0.89 ^{a}	1.17±0.26 ^{a}	3.12±0.69 ^{a}
	E	0.29±0.09 ^{a}	34.86±3.11 ^{a}	11.67±1.28 ^{a}	4.40±0.33 ^{a}	1.24±0.18 ^{a}	3.19±0.31 ^{a}
	F	0.30±0.06 ^{a}	33.36±6 9 ^{a}	11.79±1.90 ^{a}	4.32±0.33 ^{a}	1.19±0.20 ^{a}	3.12±0.42 ^{a}
	G	0.28±0.07 ^{a}	31.34±8.79 ^{a}	12.40±2.88 ^{a}	4.29±0.89 ^{a}	1.17±0.26 ^{a}	3.12±0.69 ^{a}
Day 30	A	0.28±0.07 ^{b}	36.54±16.00 ^{a}	13.65±4.24 ^{c}	4.40±0.33	1.24±0.18	3.19±0.31 ^{b}
	B	0.34±0.05 ^{a}	39.66±9.24 ^{a}	18.15±3.77 ^{a}	4.95±0.72 ^{a}	1.14±0.16 ^{a}	3.67±0.49 ^{b}
	C	0.29± 0.04 ^{b}	37.71±5.21 ^{a}	11.96±0.96 ^{c}	4.34±0.52 ^{b}	1.14±0.08 ^{a}	3.41±0.54 ^{b}
	D	0.32± 0.07 ^{a}	36.77±10.23 ^{a}	16.67±3.87^{b}	5.39±0.63 ^{a}	1.28±0.27 ^{a}	4.02±0.35 ^{a}
	E	0.30± 0.04 ^{ab}	34.68±7.39 ^{a}	11.07±0.68 ^{c}	4.67±0.65 ^{b}	1.14±0.23 ^{a}	3.46±0.67 ^{b}
	F	0.31±0.07 ^{ab}	37.91±8.70 ^{a}	11.70±1.60 ^{c}	4.77±0.88 ^{b}	1.13±0.36 ^{a}	3.51±0.69 ^{b}
	G	0.36±0.05 ^{a}	35.86±10.59 ^{a}	22.15±4.39 ^{a}	5.84±0.57 ^{a}	1.23±0.47 ^{a}	4.40±0.82 ^{a}
Day 60	A	0.29±0.06 ^{b}	30.13±9.17 ^{a}	12.00±2.31 ^{b}	4.32±0.33 ^{b}	1.19±0.20 ^{a}	3.12±0.42 ^{b}
	B	0.44±0.17 ^{a}	32.34±6.79 ^{a}	15.02±4.31 ^{a}	4.96±0.57 ^{a}	1.27±0.17 ^{a}	3.90±0.55 ^{ab}
	C	0.41±0.12 ^{a}	32.86±3.11 ^{a}	13.48±2.02 ^{b}	4.55±0.90 ^{b}	1.17±0.2 ^{a}	3.41±0.81 ^{b}
	D	0.46±0.03 ^{a}	31.73±11.77 ^{a}	14.84±3.3 ^{ab}	5.16±0.52 ^{a}	1.2±0.43 ^{a}	3.98±0.60 ^{a}
	E	0.40±0.12 ^{a}	32.45±15.13 ^{a}	13.26±3.58 ^{b}	4.55±0.84 ^{b}	1.17±0.35 ^{a}	3.48±0.95 ^{b}
	F	0.47±0.02 ^{a}	34.15±9.11 ^{a}	14.26±4.3 ^{ab}	4.54±0.54 ^{b}	1.20±0.08 ^{a}	3.47±0.59 ^{b}
	G	0.48±0.08 ^{a}	32.26±7.82 ^{a}	17.26±2.17 ^{a}	5.55±0.82 ^{a}	1.26±0.25 ^{a}	4.43±0.90 ^{a}

Different lowercase superscripts in each column indicate statistically significant differences between the values (p<0.05).

The highest blood respiratory burst activity (showed as NBT reduction level) and alternative complement activity, were detected in Bi-inj, Si-Inj, and Vh-inj groups, respectively. However, serum NBT reduction and complement activity were significantly higher in intraperitoneal vaccine injected groups compared to the

control group on day 30 and day 60 of the experiment (p<0.05), and no significant change was found in monovalent and bivalent immersion vaccinated groups (Bi-Im, Si-Im, and Vh-Im) compared to the control group (p>0.05). The serum bactericidal power and serum albumin level were not affected by different vaccination regimes and routes of vaccination which was explained in this experiment (p>0.05).

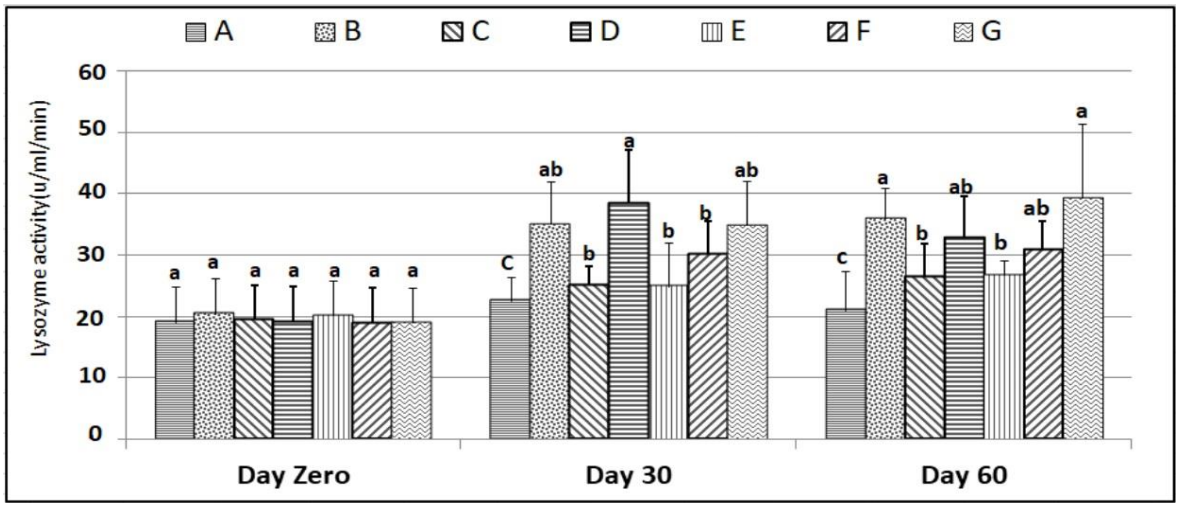

Figure 3: Serum Lysozyme activity in different experimental groups. Significant differences (p<0.05) among groups at each sampling point are indicated by means with different lowercase letters. (n=9 ; p<0.05). (A: Control group, B: Fish vaccinated with *S .iniae* via injection route, C: Fish vaccinated with *V. harveyi* via immersion route, D: Fish vaccinated with *V. harveyi* via injection route, E: Fish vaccinated with *S .iniae* via immersion route, F: Fish vaccinated with bivalent vaccine via immersion route, G: Fish vaccinated with bivalent vaccine via injection route.

Hematological parameters

No statistically significant differences (p>0.05) were detected among all experimental groups in terms of the globular index of hematological parameters (RBC, PCV, and Hb) in both sampling times. Inspire of mentioned results in globular-related hematological parameters, a significant increase in WBC value was found in Bi-inj, Si-Inj, and Vh-inj groups on days 30 and 60 of the experiment (Table 3). Changes in monovalent and bivalent immersion vaccinated groups (Bi-Im, Si-Im, and Vh-Im) were insignificant compared to the control group (p>0.05).

Expression of IL-10 and GMCFC genes in kidney

The relative expression of *IL-10* was significantly lower (Fig. 4, p<0.05) at days 30 and 60 of the experiment in the Si-Inj group and Bi-Inj group compared to the control group. In other vaccinated groups, relatively lower expressions of *IL-10* were seen but not to a significant extent.

The highest relative gene expression of GMCFC was detected on days 30 and 60 of the experiment in the Si-Inj group and Bi-Inj group (Fig. 5, p<0.05) which was significantly higher than that control group and all zero-day samples. In *V. harveyi* vaccinated groups, only the intraperitoneally injected group (Vh-Inj) on day 60 demonstrated a particular increase in expression of the GMCFC gene.

Table 3: Hematological parameters. Immunological parameters of Asian sea bass in 7 experimental groups Different lowercase superscripts in each column indicate statistically significant differences between the values (n=9; p<0.05). (A: Control group, B: Fish vaccinated with *S. iniae* via injection route, C: Fish vaccinated with *V. harveyi* via immersion route, D: Fish vaccinated with *V. harveyi* via injection route, E: Fish vaccinated with *S .iniae* via immersion route, F: Fish vaccinated with *bivalent* vaccine via immersion route, G: Fish vaccinated with *bivalent* vaccine via injection route.

Ave	Groups	Hemoglobin	PCV(%)	WBC(×10^4 /µ«)	RBC(×10^6 /µL)
Day zero	A	6.17±1.15^a	24.60±3.29 ^{a}	17.40±2.70 ^{a}	1.40±0.28 ^{a}
	B	6.16±0.69 ^{a}	26.23±7.8 ^{a}	16.47±1.67 ^{a}	1.44±0.26 ^{a}
	C	6.09±1.09 ^{a}	25.20±5.41 ^{a}	16.17±3.43 ^{a}	1.39±0.24 ^{a}
	D	6.21±1.13 ^{a}	24.12±7.53 ^{a}	16.21±3.43 ^{a}	1.38±0.24 ^{a}
	E	6.16±0.69 ^{a}	26.00±6.58 ^{a}	16.40±1.67 ^{a}	1.41±0.24 ^{a}
	F	6.07±1.15^a	24.67±3.26 ^{a}	16.43±2.73 ^{a}	1.42±0.28 ^{a}
	G	6.11±1.19 ^{a}	25.12±7.48 ^{a}	16.21±3.43 ^{b}	1.37±0.24 ^{a}
Day 30	A	6.09±1.09 ^{a}	25.00±7.56 ^{a}	16.17±3.43 ^{b}	1.39±0.24 ^{a}
	B	6.78±1.03 ^{a}	24.60±5.22 ^{a}	18.80±2.07 ^{a}	1.50±0.19 ^{a}
	C	6.44±1.20 ^{a}	26.00±3.94 ^{a}	16.33±1.53 ^{b}	1.35±0.13 ^{a}
	D	6.59±2.30 ^{a}	24.60±5.81 ^{a}	17.90±3.56 ^{ab}	1.38±0.16 ^{a}
	E	6.54±1.89 ^{a}	25.60±5.37 ^{a}	16.60±2.61 ^{b}	1.40±0.30 ^{a}
	F	6.16±2.09 ^{a}	28.60±4.34 ^{a}	17.25±3.86 ^{b}	1.33±0.21 ^{a}
	G	6.86±1.89 ^{a}	26.80±2.28 ^{a}	19.83±3.66 ^{a}	1.49±0.29 ^{a}
Day 60	A	6.16±0.69 ^{a}	26.00±7.58 ^{a}	16.40±1.67 ^{b}	1.44±0.13 ^{a}
	B	6.43±1.22 ^{a}	26.00±4.64 ^{a}	18.87±3.11 ^{a}	1.33±0.13 ^{a}
	C	6.52±0.85 ^{a}	24.40±6.02 ^{a}	17.75±3.10 ^{b}	1.45±0.34 ^{a}
	D	6.25±1.10 ^{a}	23.00±2.35 ^{a}	18.90±2.77 ^{a}	1.36±0.17 ^{a}
	E	6.87±1.34 ^{a}	27.20±4.32 ^{a}	17.00±4.10 ^{b}	1.42±0.31 ^{a}
	F	6.76±1.04 ^{a}	25.40±4.22 ^{a}	17.80±2.10 ^{b}	1.36±0.24 ^{a}
	G	6.63±1.37 ^{a}	24.80±3.11 ^{a}	18.89±2.39 ^{a}	1.50±0.26 ^{a}

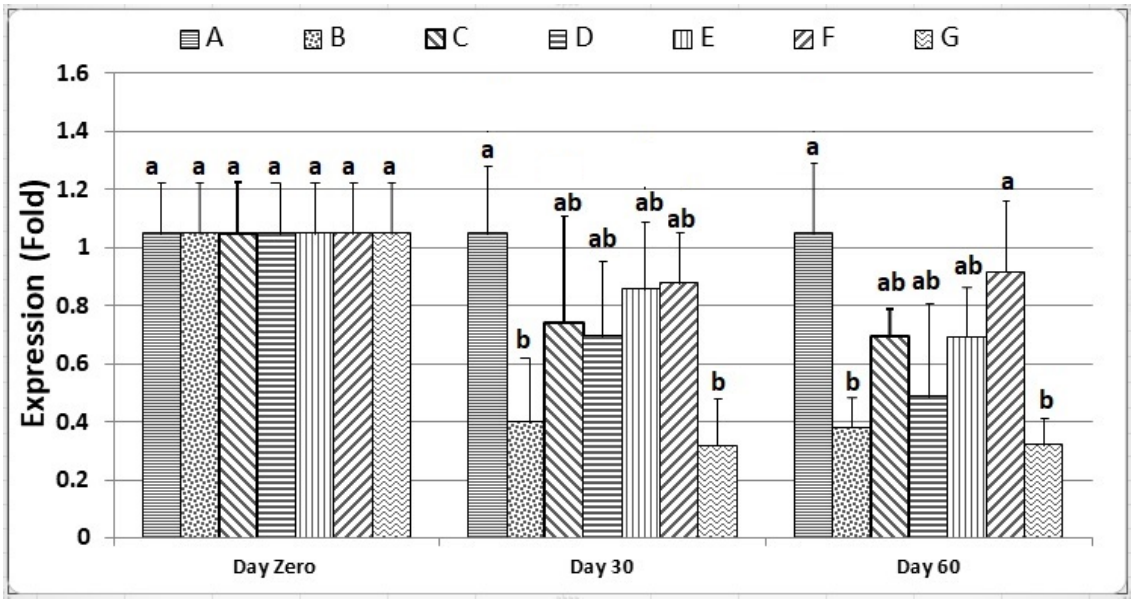

Figure 4: *IL-10* gene expression rate of Asian sea bass in 7 experimental groups. Different lowercase superscripts in each column indicate statistically significant differences between the values (n=9; p<0.05).

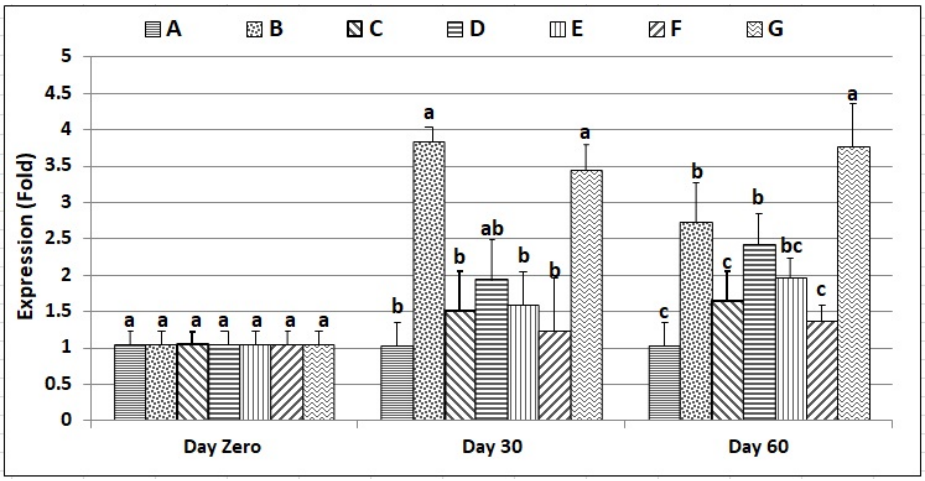

Figure 5: GMCFC gene expression rate of Asian sea bass in 7 experimental groups. Different lowercase superscripts in each column indicate statistically significant differences between the values (n=9; p<0.05).

The immersion vaccinated groups demonstrated a relative enhancement in GMCFC gene expression, but not to a statistically significant extent.

Challenge with S. iniae and V. harveyi

"The results of the challenge with *S. iniae* demonstrated that the lowest cumulative mortality occurred in the bivalent and *S. iniae* intraperitoneal vaccinated groups (10% and 16.67±5.77%, respectively), revealing a significant difference compared to all other groups." (p<0.05). Additionally, mortality in bivalent and *S. iniae* bath vaccinated groups was 46.7±5.77% and 53.34±10% which was significantly lesser than control groups (100%) (p<0.05). Results of *V. harveyi* challenge showed that i.p vaccinated bivalent group (16.67±5.77) and i.p vaccinated *V. harveyi* groups (13.34±5.77) showed significantly lowest mortality rate (p<0.05). Mortality in immersion vaccinated with bivalent and *V. harveyi* groups were 36.67±5.77 and 33.34±1, respectively which was significantly lower than the control and S. inia vaccinated groups (Figs. 6 and 7).

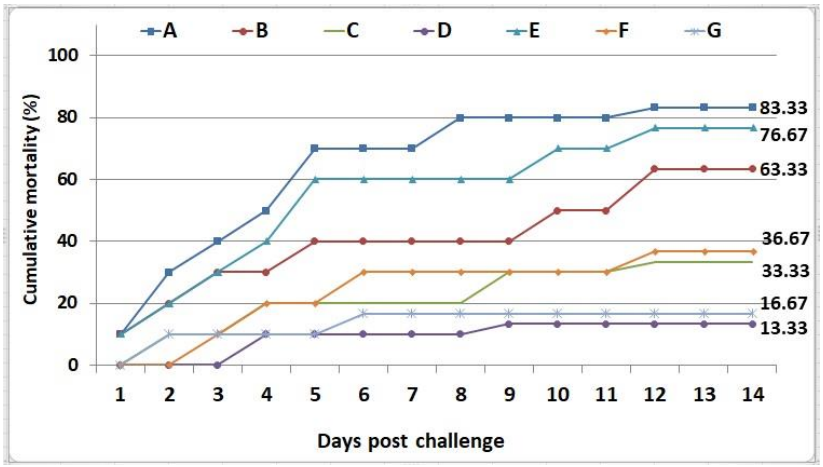

Figure 6: Cumulative mortality of Asian sea bass after challenge with *Vibrio harveyi* in 7 experimental groups. Different lowercase superscripts in each column indicate statistically significant differences between the values (n=9; p<0.05).

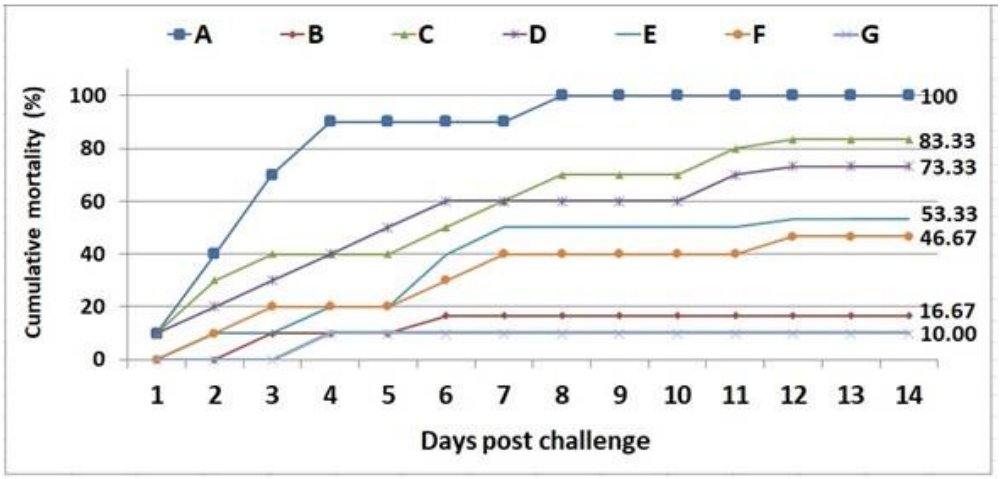

Figure 7: Cumulative mortality of Asian sea bass after challenge with *S iniae* of in 7 experimental groups. Different lowercase superscripts in each column indicate statistically significant differences between the values. (n=9; $p<0.05$).

Discussion

The results of this study demonstrate that the protective efficacy of the bivalent vaccine (*S. iniae* and *V. harveyi*) and its immunogenicity, whether administered through injection or immersion routes, were more effective compared to each monovalent vaccine. While various immunological processes such as antigenic cross-reaction, antigenic competition, and affinity maturation may potentially have negative impacts on the efficacy and immunogenicity of bivalent and polyvalent vaccines, the bivalent vaccine (*S. iniae*+*V. harveyi*) developed in this study had a positive effect on vaccine properties.The most intriguing aspect of the results lay in the comparison of the protective efficacy between the bivalent vaccine (*S. iniae*+*V. harveyi*) and the monovalent *S. iniae* and *V. harveyi* vaccines. Cumulative mortality following challenge with *S. iniae* in the Bi-inj group (10%) was lower than that in the Si-Inj group (16.7%). Conversely, mortality in the Bi-Im group (46.67%) was lower than in the Si-Im group (53.33%). We hypothesize that the presence of the *V. harveyi* vaccine in the bivalent vaccine likely plays an adjuvant-like role in enhancing the effectiveness of the *S. iniae* vaccine in both injection and immersion routes. Additionally, mortality following challenge with *S. iniae* in the Vh-Inj group (73.33%) and Vh-Im group (83.33%) was remarkably lower compared to the control group (100%). This intriguing finding can be attributed to cross-protection between the *V. harveyi* vaccine and *S. iniae* infection. The most intriguing aspect of the results pertained to the comparison of the protective efficacy of the bivalent vaccine *(S. iniae*+*V. harveyi*) and the monovalent *S. iniae* and *V. harveyi* vaccines. Cumulative mortality following the challenge with *S. iniae* in the Bi-inj group (10%) was lower than that in the Si-Inj group (16.7%). Conversely, mortality in the Bi-Im group (46.67%) was lower than in the Si-Im group (53.33%). We hypothesize that the inclusion of the *V. harveyi* vaccine in the bivalent vaccine likely plays an adjuvant-like role in enhancing the effectiveness of

the *S. iniae* vaccine in both the injection and immersion routes. Furthermore, mortality subsequent to challenge with *S. iniae* in the Vh-Inj group (73.33%) and Vh-Im group (83.33%) was significantly lower compared to the control group (100%). This intriguing discovery could be attributed to cross-protection between the *V. harveyi* vaccine and *S. iniae* infection (Nguyen *et al*., 2018).

Results of the challenge with *V. harveyi* showed that the least cumulative mortality was recorded in Vh-Inj group (13.37%) and Bi-Inj vaccine (16.67%) respectively, which was significantly lower than all other groups. An acceptable protective immunity with lower post-challenge mortality was seen in Vh-Im group (33.33%) and Bi-Im group (36.67%) which was significantly lower than the control group (83.33%). Due to the various advantages of polyvalent vaccines over monovalent vaccines in aquaculture, numerous researches designed to confirm these effects. Sun *et al.* (2011) showed that protective immunity of injectable multivalent vaccine containing *E. tarda*, *V. anguillarum*, *S. iniae*, *V. harveyi* was more efficient than monovalent vaccine of each bacteria in Japanese flounder. They report a mutual and adjuvant-like effect between *E. tarda* and *V. anguillarum*, which enables the bivalent vaccine (*E. tarda*+*V. anguillarum*) to induce more effective protective immunity against these bacteria to compared to monovalent vaccines. In line with previous studies (Nguyen *et al*. 2018) declared that a bivalent inactivated vaccine increased definite and indefinite immune responses in cobia (*Rachycentron canadum*), and gave 100% protection upon challenge with a highly virulent *Streptococcus dysgalactiae* strain. Shoemaker *et al.* (2012) announced effective laboratory indication/evidence of bivalent inactivated vaccine with high RPS of 89% and 100% against *Vibrio vulnificus* and *S. iniae* in hybrid tilapia (*O. niloticus*×*O. aureus*) has been reported. In addition, a trivalent inactivated vaccine formulated from *V. alginolyticus, V. parahaemolyticus,* and *Photobacterium damselae* stimulated both specific antibodies and protection (84.7-93.8% survival) in cobia against corresponding bacterial pathogen infections (Lin *et al.,* 2006). The application of bivalent or multivalent vaccines gives some useful advantages over monovalent vaccines for commercial scale since they decrease the number of injections, stress from handling the fish, time and cost of immunization (Wang *et al.,* 2020).

Some multivalent vaccines against 3 to 7 pathogens have been commercially available in the salmon industry (Lan *et al.,* 2021).. This experiment's findings show that Bi-Inj and Si-Inj groups demonstrate the highest anti *S. iniae* antibody titer, while Si-Im and Bi-Im groups, Relatively lesser increase of antibody titer. These elevated antibody titers were statistically significant compared to the control group. In the same way, anti *V. harveyi* antibody titer was significantly higher in both bivalent Bi-Inj and monovalent Vh-Inj groups compared to the control group and a lesser elevated antibody titer was detected in immersion vaccine treated groups (Vh-Im and Bi-Im). These results are consistent with previous studies that showed elevated serum antibody titer after vaccination with a polyvalent vaccine. For instance, Sun *et al.*

(2012) reported comparable even higher antibody titers in bivalent (*S. iniae and V.anguillarum*) vaccinated Japanese flounder with monovalent vaccinated fish. Likewise Wang *et al*. (2020) demonstrated the cross-protection and synergetic effect of a component of the bivalent vaccine (*S. agalactiae* and *S. iniae*) in their immunogenicity and antibody production of tilapia. The current study demonstrated a continuous long-term response-kinetics (60 days) of the immunized fish with a single antigen and a combination of two antigens in one vaccine. Contrary to the outcomes of the current study which demonstrated the synergetic impact of two vaccine seeds of bivalent vaccine in enhancing the antibody titer. Lan *et al.* (2021) reported suppression of antibody titer in sea bass vaccinated with bivalent *S. agalactiae*/ *S. iniae* vaccine compared to monovalent vaccine. They guessed that such inhibition is caused by possible antigen competition of two similar closely related Gram-positive bacteria (Nikoskelainen *et al.,* 2007). The lack of close antigen similarity of our bacteria (gram-negative *V. harveyi* and gram-positive *S. iniae*) may cause a kind of mutual synergetic effects in antibody responses. According to our knowledge, this is the first study which is on the specific and non-specific immune response of Asian sea bass following immunization with *S. iniae* and *V.* harveyi vaccine in the form of monovalent and bivalent vaccines. It is worth noting that a positive correlation has occurred between antibody titer (specific immune response) and protective immunity in both bivalent and monovalent vaccinated groups. The low mortality percentage in vaccination treatment is mainly caused because of the formation of the particular immune response that is followed an increase in the nonspecific immune system. These results further confirmed the role of specific humeral immune responses in the protective immunity of fish. There are numerous reports of correlation between antibody titer (specific immune response) and protective immunity against infectious disease in different fish species (Nikoskelainen *et al.,* 2007; Thu Lan *et al.,* 2021).

Our research demonstrates that immunization with the inactivated *S. iniae* and *V. harveyi* in the form of monovalent and bivalent vaccines in the intraperitoneal injection route affected some of the non-specific immune responses of Asian sea bass. Serum lysozyme activity, total protein, and IgM level as well as complement and NBT activity notably enhanced in i.p vaccinated groups, particularly in Bi- Inj group. Conversely mentioned parameters showed no significant increase in all immersion vaccinated groups on days 30 and 60 of the experiment. Despite the elevation in activity of the mentioned immune parameters, no significant differences were observed in the serum albumin and bactericidal activity in experimental groups.

Although there is a general consensus that the stimulating of the immune response in vaccinated fish correlates with an increase in lysozyme activity and serum protein level, there are some reports of reliable protective immunity in vaccinated fish despite the lack of change in lysozyme activity (Monir *et al.,* 2021). The highest lysozyme activity, total protein and IgM

level, complement and NBT reduction in Bi-inj and Si-Inj groups in this study showed efficient immune responses which cooperated in protective immunity against experimental bacterial infection. This elevation of the mentioned immune parameters along with an increase in the WBC level can be interpreted as a general stimulation in the immune responses of vaccinated fish. In the current study, the highest blood respiratory burst activity (NBT reduction test) and alternative complement activity were detected in Bi-inj group, Si-Inj, and Vh-inj groups respectively. Although serum NBT and complement activity were significantly higher in i.p vaccine injected groups compared to the control group, no significant change was found in immersion vaccinated groups (Bi-Im, Si-Im, and Vh-Im) compared to the control group. A primary and important role in the innate immunity of fish is related to the complement system because of its role in leukocytes activation, phagocytosis, chemotaxis, inflammatory reaction, and opsonizes foreign organisms and pathogens studied by Boshra and Sunyer (2006). Elevated complement and Phagocytosis activity in Bi-inj group which vaccinated with bivalent injectable vaccine, deeply confirmed the role of non-specific immunity in protective immunity against *S. iniae* and *V. harveyi* infection. Moreover, respiratory burst activity (showed by NBT activity) following phagocytosis procedure of invasive pathogens by leukocytes is a widely used indicator of the nonspecific immune response evaluation (Abarike *et al.,* 2019).

The elevated complement and NBT activity in injectable vaccine groups, particularly bivalent vaccines may be due to the higher efficacy of stimulating complement receptor expression as well as increasing the serum antimicrobial properties like protective proteins and various antimicrobial peptides to inhibit the pathogenicity of infectious microorganisms (Esteban, *et al.,* 2015). In agreement with mentioned results (Nikoskelainen *et al.,* 2007; Karami *et al.,* 2019) reported the elevated serum complement activity and phagocytic power of leukocytes following bivalent and polyvalent vaccination in rainbow trout respectively. Although there is some evidence that showed many immunological processes including antigenic cross-reaction, antigenic competition, and immune-suppressing competent, may affect negatively the efficacy and immunogenicity of bivalent and polyvalent vaccines, our result confirmed the positive effect of a bivalent vaccine containing a gram-negative (*V. harveyi*) and gram-positive (*S. iniae*) killed bacteria. Our bivalent vaccines induced high antibody titer, as well as elevated non-specific immune responses along with higher protective efficacy against both virulent bacteria of vaccine seed.

In this study, although red cell dependent haematological parameters (RBC, Hb, and PCV) didn't affect by different bivalent and monovalent groups, WBC count demonstrated a particular increase in Bi_Inj and Si-Inj groups in 30 and 60 days of the experiment compared with the control group. Haematological parameters have been usually used for observing the health status and

immunological responses of fish and aquatic animals. The findings of hematological parameters following vaccine administration in fish are often contradictory. An increase in the total leukocyte count in vaccinated rainbow trout with the polyvalent vaccine was reported which showed a correlation with other non-sp-ecific immune parameters (Nikoskelainen *et al.,* 2007). Similarly, higher WBC was reported in rainbow trout (Karami *et al*., 2019), sturgeon (Khoshbavar-Rostami *et al.,* 2007) and red tilapia (Monir *et al.,* 2021) in i.p vaccinated with polyvalent bacterial vaccines. Among the different haematological parameters, the leukocyte count is very important for functioning in the non-specific and specific immune systems of fish (Fazio, 2019). As most cytokines and immune-modulatory substances generate from leukocytes (WBC), the increase in WBC positively affects antibody production, nonspecific humeral and cellular immune parameters, which leads to higher protective immunity (Silva *et al.,* 2009; Sukenda *et al.,* 2017). It is interesting that the lack of negative effects of different developed vaccines in this study (mono and bivalent) in intraperitoneally and immersion routes on hematological parameters of tilapia can be interpreted as a positive safety test of vaccines. The relative expression of *IL-10* gene in the head kidney of sea bass was significantly decreased (Fig 4, $p<0.05$) on days 30 and 60 of the experiment in Si-Inj group and Bi-Inj group compared to the control group. Although the reduction of *IL-10* expression in injected groups, in immersion vaccinated groups, relatively lower expression of *IL-10* was seen but not to a significant extent. IL-10 is one of the most important cytokines with anti-inflammatory properties and plays a key role as a negative regulator of immune responses to pathogens Rutz and Ouyang (2016). IL-10 is widely expressed in various cells including macrophages, monocytes, dendritic cells, mast cells, eosinophils, neutrophils, natural killer cells, and lymphocytes. The biological activities of IL-10 include the inhibition of various immune-related genes expression including major histocompatibility complex (MHC) class II antigens in monocytes and dendritic cells (Ouyang *et al.,* 2011). In accordance with the outcomes of the current study, an anti-inflammatory role for *IL10* has been previously shown in carp (Piazzon *et al.,* 2015) and in zebrafish (Coronado *et al.,* 2019). Moreover, (Harjula *et al.,* 2018) used the mutant IL-10 gene of zebrafish and confirmed enhanced interferon-gamma response and improved survival against a *Mycobacterium marinum* infection for confirming the negative role of IL-10 on fish immune responses. To the best of our knowledge, our study is the first report of *IL-10* expression in the head kidney of Asian sea bass after vaccination.

In this study groups Si-Inj and Bi-Inj showed the highest relative gene expression of Granulocyte colony-stimulating factor (GMCSF) on days 30 and 60 of the experiment which was significantly higher than that control group and all groups of zero-day. In *V. harveyi* vaccinated groups, only in the intraperitoneally injected group (Vh-Inj) on day 60 showed a significant increase in the expression of GMCFC gene. The immersion-vaccinated groups exhibited a relative increase in **GMCFC** gene expression;

however, the observed increase was not statistically significant.

The GMCFC is a kind of cytokine with hemato-immunological functions which is mostly produced by monocytes, fibroblasts, and endothelial cells, and it is responsible for the proliferation in general, survival, and differentiation of neutrophils, monocytes macrophages, and their respective progenitors. (Fleetwood *et al.,* 2005). Our outcomes agreed with others, which describe the higher expression of the GMCSF gene in immune-activated fish such as fugu (Stachura *et al.,* 2013), rock bream (*Oplegnathus fasciatus*) (Jeswin *et al.,* 2017), Japonese flounder (Jeswin *et al.,* 2017). The GM-CSF gene expression assay of the fish mostly concentrated on its transcription levels in vital immune organs under the immune stimulation condition or infections, showing its crucial functions in immune responses by defending against pathogen invasion (Guo *and Li,* 2021). It has also been reported that recombinant GM-CSF as an adjuvant can promote, anti-*E. tarda* antibody titer in flounder (Guo *et al.,* 2018; Guo *and Li,* 2021). In this study, the Increase in GM-CSF genes as a pro-inflammatory cytokine, along with decreased expression of *IL-10* which plays a central role by suppressing the inflammatory process can be a rational and convincing cause for protective immunity and native immune stimulation that occurred in the bivalent vaccinated group. The adjuvant-like effects of each bacterium in bivalent bacterial vaccine for its counterpart can justify the higher expression of GMCFC (an immunostimulating gene) and lower expression of *IL-10* (An immunosuppressive gene).

Conclusions

Based on the findings of this study, the bivalent vaccine (*S. iniae+V. harveyi*) demonstrated its ability to significantly enhance both specific and nonspecific immune responses, as well as vaccine efficacy and immune-modulatory gene expression in Asian sea bass. This effectiveness surpasses that of monovalent vaccines. The adoption of this bivalent vaccine could offer cost-effective strategies for large-scale immunization against *S. iniae* and *V. harveyi*, thereby reducing losses in Asian sea bass co-infected with these bacteria. Further investigation into the vaccine's efficacy in real-world settings is warranted. As far as our knowledge extends, this bivalent vaccine may represent the first of its kind, providing a combination of two bacterial antigens to safeguard Asian sea bass against streptococcosis and vibriosis. The demonstrated efficacy of this bivalent vaccine strongly advocates for its implementation as a standard preventive measure against mortalities induced by both pathogens in Asian sea bass cage culture.

Conflicts of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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