Hindawi
Aquaculture Research
Volume 2023, Article ID 7063186, 20 pages
https://doi.org/10.1155/2023/7063186

Research Article

Maternal Immunity Transfer of Zebrafish (*Danio rerio*) following Vaccination with a Live-Attenuated *Vibrio harveyi* Vaccine

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Received 15 March 2023; Revised 2 May 2023; Accepted 10 May 2023; Published 24 May 2023

Academic Editor: Mohamed Abdelsalam

Vibriosis causes a major setback in the marine aquaculture industry. In controlling vibriosis, vaccination of the broodstock could help in transferring the initial immunity to the fry and perhaps giving immunity to them during the culture period. This study determines the efficacy of three live-attenuated *Vibrio harvey*i vaccine (LAVh) derivatives on zebrafish broodstock in providing immunological protection to their offspring against pathogenic *Vibrio* spp. Zebrafish were vaccinated intraperitoneally with full effective dose (ED_{80}) and monitored for 14 weeks. On week 6, the specimens were intraperitoneally (i.p.) challenged with pathogenic 10^7 CFU/ml of *Vibrio* spp., and subsequently another batch was left to spawn. Thereafter, the antibody level of larvae was monitored for 4 weeks post-hatching. Groups of juvenile zebrafish were given booster vaccination via immersion (1×10^7 CFU/mL) with vaccines similar to their predecessor. The fish were sampled weekly for four weeks to determine their antibody profile and pro-inflammatory gene expression. Finally, at week 3, the juvenile fish were challenged with pathogenic strains of *Vibrio* spp. Results of antibody profiling in the adult zebrafish model indicated that freeze-dried and stale LAVh delivers a longer immunological protective duration, while the FKVh vaccination group failed to provide immunological protection against *V. alginolyticus*. However, the gene expression of pro-inflammatory interleukin 1β (IL-1β) in the FKVh vaccinated group was elevated longer than that in the freeze-dried LAVh vaccinated group. Nevertheless, the relative percent survival (RPS) of both vaccination groups against pathogenic *Vibrio* spp. displayed 100% immunity. Overall, the freeze-dried LAVh vaccine was found to confer maternal immune protection for its offspring, provide a long duration of immunological protection and cross-protection coverage against pathogenic *Vibrio* spp., and have a longer shelf life. It is proposed to be commercially available for farmers to protect against *Vibrio* spp. infection.

1. Introduction

Vibriosis causes deadly hemorrhagic septicaemia towards its infecting host. It is identified as one of the main aquaculture problems in marine aquaculture production [1] in many countries like Malaysia, the Philippines, Vietnam, and China [2–5]. It is a rod-shaped, Gram-negative bacterium and tends to propagate significantly in marine and estuaries of warm climate environments with a high dependency on salinity for its survival [6]. In Malaysia and neighboring

tropical countries, cases of outbreaks are frequent in a variety of caged cultured groupers (*Epinephelus* sp.), Asian seabass (*Lates calcarifer*), and snappers (*Lutjanus* sp.) [7].

Clinical signs featured in teleost have been described as skin darkening, lethargy, anorexia [8], loss of scale [5], lesions of necrotic muscle fibers, multifocal necrosis in the renal tubular, and hepatocytes of kidney and liver, respectively [2]. From the year 2000 until 2003, China saw a massive loss of cage-cultured yellow croaker (*Pseudosciaena crocea*) with mortalities leading between 30% and 40%, and the highest was recorded at 80% mortality [9]. Vibriosis also affects the crustacean farming industry where de Souza Valente and Wan [10] had reported that the production loss due to acute hepatopancreatic necrosis disease (AHPND) caused by *Vibrio* infection costed around USD 44 billion between 2010 and 2016 in Mexico, China, Thailand, Vietnam, and Malaysia combined.

Disease outbreaks in fish larvae could be due to undeveloped immunity in its gastrointestinal tract while having a pH that is not low enough to inhibit pathogenic *Vibrio* spp. [11]. A study showed the tendency of pathogenic *Vibrio* spp. to infiltrate and infect young fishes (<200 g) stocked in open-net cages, with mortality up to 40% [5]. A common practice for fish farmers to counter the outbreak of vibriosis is by using antibiotics [12]. However, the utilization of antibiotics has also encouraged the development of resistance in pathogens by selectively pressuring them into a horizontal transfer of resistance genes to the different organisms [3, 12].

Vaccination is convenient for use in open and closed culture water as they target a specific antigen inside the host without causing harm to other beneficial microorganisms [13]. Vaccination intends to imitate a natural cascade of effects caused by foreign material entering a host, where the vaccines activate both the innate and adaptive immune system by producing antibodies from memory cells and establishing lasting protection [14]. A unique way of developing protection against disease is via the transfer of maternal immunity towards the larvae [15]. The quality and quantity of immune factors transferred to the offspring are significantly affected during vitellogenesis. Female fish not exposed to antigens cannot produce specific antibodies against those pathogens, thus limiting the transfer of immunity [16].

Studies showed that a live-attenuated vaccine could increase antibody production and reduce the time required for revaccination as the protective period is prolonged compared with the conventional killed vaccine [17]. In addition, equipping the vaccine with a freeze-drying technique helps preserve its shelf life and ease access to remote and isolated hatcheries [18]. The capability of the live-attenuated *Vibrio harveyi* (LAVh) vaccine to promote relevant immunity response favors not only the adult fish and its overall health but also the health conditions of its offspring. A healthy maternal transferred immunity from the broodstock to fish embryos is essential for the survival of its offspring against microbial infections [19]. With a history of repeated vaccination, broodstock would reinforce the maternal immunity delivered towards the offspring to have a protective immune response for their defense [20].

This study aimed to determine the antibody profiles of zebrafish and their offspring following vaccination with LAVh and FKVh vaccines. Besides, this study also observed the protective efficacy of the offspring and vaccinated juvenile zebrafish post-challenge with different *Vibrio* spp. as well as the innate immune-related gene profiling of juvenile zebrafish at post-vaccination.

2. Materials and Methods

2.1. Experimental Fish. Adult zebrafish (*Danio rerio*) (length of 2.9 ± 0.1 cm; weight of 0.20 ± 0.5 g) were brought from Sanwa Aquatics Sdn. Bhd., Kuala Lumpur, Malaysia. However, the zebrafish larvae and juvenile fish were acquired from vaccinated adults in this study. All fish were maintained and cultured at Aquatic Animal Health and Therapeutics Laboratory, Institute of Bioscience, Universiti Putra Malaysia, Malaysia. Approval for bacterial use and the fish host was recognized in accordance with the guideline stipulated by the Department of Biosafety, the Ministry of Natural Resource and Environment, Malaysia, with approval no. JBK(S) 602-1/2/136(6). All approaches performed in studies involving animals were in accordance with the ethical standards of the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia (UPM), under approval no. UPM/IACUC/AUPR059/2016.

2.1.1. Adult Fish. Adult zebrafish ($n = 525$) were acclimatized for 7 days and screened for any bacterial and parasite infections before being used for vaccination trial and challenge study. Fish were placed in 70 L tanks of unchlorinated water with 24 h of aeration and salinity of less than 0.5 ppt. Feed was given two times a day at 0800 h and 1700 h. The water exchange was conducted once a week at 25%. The fish were stationed inside a closed environment with an average water temperature of 23°C and 12 : 12 h of light : dark using an automated fluorescence lamp system. Adult fish were fed with commercial micro-pellets (Sanyu Ichiban, Malaysia; crude protein 35%, crude fat 4%, crude fiber 3%, and moisture 10%) *ad libitum* once a day. During the vaccination trial and challenge test, fish were placed in 25 L tanks with 24 h aeration.

2.1.2. Fish Larvae. Spawning fish eggs was done artificially using a mesh-covered container to collect the eggs [21]. The eggs in the container were sifted out and maintained in the same condition as the adult in a 1 L plastic tank with 24 h of aeration, and the larvae were maintained in this condition after hatching. Larvae ($n = 225$) were fed with fine-crushed, commercial feed (Sanyu Ichiban, Malaysia; crude protein 35%, crude fat 4%, crude fiber 3%, and moisture 10%) *ad libitum* once a day.

2.1.3. Juvenile Fish. Juvenile fish ($n = 135$) were raised from eggs spawned by vaccinated adult zebrafish. The fish fry age was 28 days post-hatching (dph) and was maintained in groups according to the vaccination group of their

predecessor. They were maintained in the same condition as the adult zebrafish in 1 L plastic tanks with 24 h aeration and fed with commercial feed twice a day at 0800 h and 1700 h. The water exchange was conducted once a week at 25%. Juvenile fish were fed with commercial micro-pellets (Sanyu Ichiban, Malaysia; crude protein 35%, crude fat 4%, crude fiber 3%, and moisture 10%) *ad libitum* once a day.

2.2. Bacterial Strains and Culturing Conditions. Pathogenic strains of *V. alginolyticus* (Strain VA2; GenBank KJ930426.1), *V. parahaemolyticus* (Strain FORC_008; GenBank CP009983.1), and *V. harveyi* (Strain VH1; GenBank KT266880.1) were isolated and identified from diseased groupers [22] (Supplementary Table 1). All bacterial strains, including live-attenuated *V. harveyi* (LAVh) (Strain MVH-vhs; GenBank EU344975.1) vaccine [23], were maintained at the Aquatic Animal Health and Therapeutics Laboratory, Institute of Bioscience, Universiti Putra Malaysia. Bacterial identification was carried out using PCR (Supplementary Table 2) and sent for DNA sequencing at First BASE Laboratories Sdn. Bhd., Selangor, Malaysia.

The bacteria were cultured on thiosulfate-citrate-bile salt-sucrose agar (TCBS; Oxoid, Hampshire, UK) and incubated at 30°C for 12 h. Five colonies were selected and transferred into 10 mL tryptic soy broth (TSB; Oxoid) with 1.5% NaCl (w/v). They were then incubated at 30°C, 150 rpm for 12 h. Bacterial cultures were spun down at 8000 g for 10 min at 4°C, and the supernatant was discarded. The pellets were washed three times with 10 mL sterile 0.01 M phosphate buffer saline (PBS) before resuspending with 10 mL sterile 0.01 M PBS.

To determine the bacterial concentration, 100 μL of diluted working culture from each dilution factor of 10^0 to 10^{-7} was transferred onto TCBS agar with 1.5% NaCl (w/v) and spread using a glass spreader [24] before being incubated. The bacterial colonies formed on the plates were then counted using the plate count technique following the equation for the colony-forming unit (CFU mL^{-1}). Once the total bacterial concentration had been established, the working culture was diluted to the desired concentration needed throughout the study [25].

2.3. Vaccine Preparation. Live-attenuated *V. harveyi* vaccine was prepared based on previous study [22]. In brief, by utilizing the combined broth-membrane filtration mating method and mixing both the pathogenic *V. harveyi* strain Vh1 with a recombinant *E. coli* SM10λpir that harbors the mutagenesis plasmid, positive colonies with presence of chloramphenicol resistant gene (CmR) and vhs gene (vhs) with deleted catalytic triad were screened, sub-cultured, and later designated as live-attenuated *Vibrio harveyi* strain MVH-vhs (LAVh) vaccine. The live-attenuated *V. harveyi* vaccine was further divided into three groups; fresh, stale, and freeze-dried live-attenuated vaccine (Table 1).

2.3.1. Fresh and Stale Live-Attenuated V. harveyi Vaccine. Preparation of fresh and stale live-attenuated LAVh vaccine follows the previously described methods in Section 2.2. In short, prior to use, the LAVh vaccine was incubated in tryptic soy broth (TSB) with 1.5% NaCl (w/v) at 30°C, 150 rpm for 12 h. The vaccines are designated as fresh LAVh due to them being inoculated within 24 h post-incubation. Vaccines that were prepared after 24 h and stored at 4°C before use were designated as stale LAVh vaccines.

2.3.2. Freeze-Dried V. harveyi Vaccine. The freeze-dried LAVh vaccine has undergone a freeze-drying process and represents the option for a vaccine with longer shelf life. However, in the final phase of the vaccine preparation, the bacterial suspension was spun down at $8000 \times g$ for 10 minutes at 4°C (Eppendorf, Hamburg, Germany), and the supernatant was discarded. The bacterial pellets were mixed in PBS with 15% sucrose and 15% skimmed milk. The opened end of the centrifuge tube was covered with parafilm, and small holes were poked with a sterile wooden toothpick. This is to ensure the contents of the centrifuge tube are thoroughly dried and to avoid spillage. The centrifuge cap is gently placed without damaging the parafilm and stored in a 4°C fridge for 24 h [26]. The vaccines were later transferred to a −30°C biomedical freezer (Sanyo, Osaka, Japan) for 24 h before finally being stored in a −80°C ultra-low temperature (ULT) freezer until further processing. Before the samples were placed in the freeze-drying glass jars, the caps were taken off, leaving only the parafilm coat as a barrier to stop any samples from coming out of the centrifuge tubes. The freeze-drying process was conducted for 12 h following the protocol established using the Freeze Dry System Freezone 12 (Labconco, Kansas, USA).

2.3.3. Formalin-Killed Vibrio harveyi Vaccine. Whole-cell formalin-killed *V. harveyi* vaccine (FKVh) represents positive control in this study. The bacterial broth culture was spun down, and the pellet was washed three times with 0.01 M of PBS. The final pellet was mixed and suspended in 0.5% formalin (v/v) for 24 h at 4°C. The following day, the formalin suspension was centrifuged at $3000 \times g$ and discarded, and the bacterial pellet was then washed with 0.01 M PBS and spun down to eliminate any remaining residue of formalin in the vaccine. Total mortality was determined by inoculating 100 μL from the cell suspension onto a TCBS agar and culturing for 16 h at 30°C. The method was adapted from Mohamad et al. [27] with minor modification.

2.4. Vaccination Regime. This experimental design for the vaccination trial consists of three phases: the adult, larvae, and juvenile phases. The vaccination regime is displayed in Figure 1.

2.4.1. Adult Phase. Adult zebrafish were separated into five groups with triplicates ($n = 35$) (Table 2), and each group was vaccinated with their vaccine's corresponding effective dosage ($ED_{80-144\,h}$) at 10^4 CFU/m, except for control group. The vaccination regime starts at week 0, with a booster vaccination on week 2 and week 5. Each treatment group was let to spawn on week 7.

Table 1: Vaccination and treatment groups for vaccine efficacy test.

Group	Designation	Description	Application method
1	Fresh live-attenuated *V. harveyi* vaccine	A freshly prepared live-attenuated *V. harveyi* (LAVh) vaccine to be used 24 h post-production. Represents the industry's capability to manufacture on-site.	Can be applied via injection or immersion
2	Stale live-attenuated *V. harveyi* vaccine	LAVh that has been prepared 24 h and stored at 4°C before use. Represents outsourcing supplies of vaccines and transportation.	
3	Freeze-dried live-attenuated *V. harveyi* vaccine	LAVh that has been freeze-dried and stored at 4°C for 1 month before use. Represents long storage and transportation in industries.	
4	Whole-cell formalin-killed *V. harveyi* vaccine	A pathogenic strain of *V. harvey*i that has been formalin killed and maintained in 0.01 M PBS.	
5	0.01 M PBS, 25°C	PBS only. Represents the negative control group.	

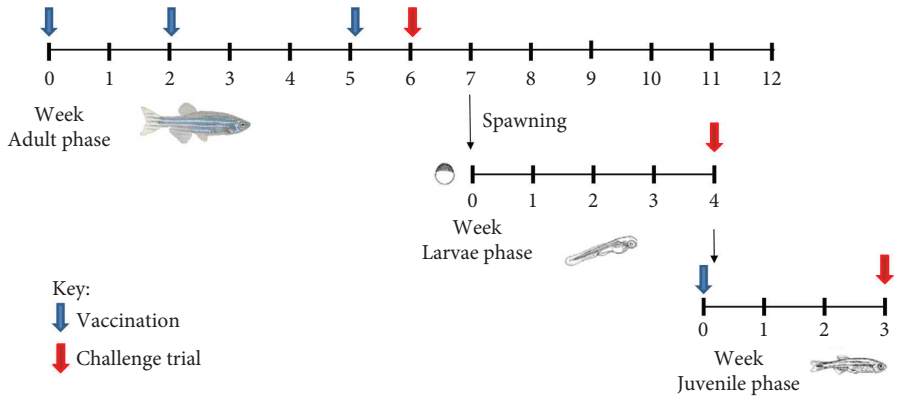

Figure 1: Vaccination and challenge trial.

2.4.2. Larvae Phase. After spawning, the zebrafish eggs and larvae were maintained as previously described. They were maintained until 28 days post-hatching (dph) until they are considered juvenile zebrafish.

2.4.3. Juvenile Phase. The zebrafish juveniles whose broodstocks were fed with freeze-dried LAVh vaccines, FKVh vaccines, and PBS-inoculated groups were selected for further experiments. They were vaccinated by immersion with 10^7 CFU/mL at week 0. This dose was selected as the exact dosage needed to induce 50% mortality in the $LD_{50-144\,h}$ of *Vibrio* spp. infection in 21 dph zebrafish fry.

The freeze-dried LAVh vaccine was selected due to its advantage over its other derivatives of LAVh vaccines in terms of antibody production, longer shelf life, and ease of use. FKVh acts as a positive control, while the PBS-inoculated group is the negative control.

2.5. Blood Sampling. Ten adult fish from each group were anesthetized by four-second immersion in crushed ice water with a temperature ranging between 1 and 3°C to prepare for vaccination administration [28]. The blood samples were collected from each group biweekly for ELISA. Blood was collected from a cut on the caudal fin using a capillary tube and transferred into a gel and blood clot vacutainer [29]. The vacutainer (Sterilizasyon Medikal, Istanbul, Turkey) was then spun down at 750 g for 10 min, and the separated homogenate was collected and transferred before being stored at −30°C until further use.

2.6. Egg and Larvae Sample Preparation. Eggs and larvae were maintained as previously described. A total of 20 larvae from the broodstock of each vaccination group were collected weekly (0 dph, 7 dph, 14 dph, 21 dph, and 28 dph) and crushed using a hand-held pestle (Duran Wheaton Kimble Life Science, Mainz, Germany) for ELISA. Homogenized material was collected in a gel and blood clot vacutainer and spun down at 750 g for 10 minutes. Separated serum was collected and transferred into a 1.2 mL mini-centrifuge tube (Biologix, Kansas, USA) before being stored at −30°C until further use.

2.7. Enzyme-Linked Immunosorbent Assay Analysis. The indirect ELISA method was used to determine the antibody level after vaccination, following Mohamad et al. [7]. Coating antigens of *V. alginolyticus*, *V. parahaemolyticus*, and *V. harveyi* were prepared initially. Flat-bottomed microtitre plates (6 wells, Sigma-Aldrich) were coated per well with 100 μL of the prepared coating antigen and kept at 4°C overnight before being washed with sterile washing buffer (PBST consisting of PBS + 0.05% Tween 20) two times. After washing, each well was blocked by adding 200 μL of 1% bovine serum albumin (PBS + 0.05% Tween-20 + BSA, Sigma-Aldrich) to prevent non-specific binding and kept for 1 h at 25°C, and each well of the plates was again washed thrice with PBST. Serum samples (1 : 300 serum in blocking buffer) were added in the well at 100 μL each well and incubated for 1 h at 25°C. After the incubation period has been met, the wells were washed thrice with PBST and 100 μL of primary antibody consisting of 1 : 1000 mouse anti-zebrafish IgM monoclonal antibody (Aquatic Diagnostics Ltd., Scotland, United Kingdom) was added to the wells and incubated for 2 h at 25°C. The plates were again washed thrice with PBST, and 100 μL of secondary antibody of 1 : 1000 anti-mouse-HRP (Aquatic Diagnostics Ltd., Scotland, United Kingdom) was incubated for 1.5 h at 25°C. After washing with PBST, 100 μL of TMB substrate solution (Sigma-Aldrich, Inc., Missouri, USA) was added to the reaction well to detect the bound conjugate for 30 minutes at 25°C in dark before the reaction was stopped with 100 μL of 0.16 M sulphuric acid. Values were obtained by measuring the absorbance using a Multiskan spectrum microplate reader (Thermo-Fisher Scientific, Massachusetts, USA) with the OD at 450 nm.

2.8. Challenge Test. A total of 30 adult zebrafish in triplicates from each group were intraperitoneally challenged at week 6 with $LD_{50-144\,h}$ pathogenic *Vibrio* spp. (*V. alginolyticus*, 1×10^5 CFU/mL; *V. parahaemolyticus*, 1×10^6 CFU/mL; and *V. harveyi* 1×10^6 CFU/mL), while the remaining batches were left to spawn ((A) in Table 2). Thereafter, at the offspring level, the larvae were maintained until 28 dph, and 90 larvae from each group were challenged by immersion with 10^7 CFU/mL of pathogenic *V. alginolyticus*,

Table 2: Treatment groups for challenge trial in adult, larvae, and juvenile zebrafish.

Group	Designation	Vaccine dosage	Challenged pathogen	Number of fish (triplicate)
(A) Challenge trial in adult zebrafish				
1	Fresh live-attenuated *V. harveyi* vaccine	10^4 CFU/mL	*V. alginolyticus* (10^5 CFU/mL), *V. parahaemolyticus* (10^6 CFU/mL), *V. harveyi* (10^6 CFU/mL)	$3 \times 35 = 105$
2	Stale live-attenuated *V. harveyi* vaccine	10^4 CFU/mL		$3 \times 35 = 105$
3	Freeze-dried live-attenuated *V. harveyi* vaccine	10^4 CFU/mL		$3 \times 35 = 105$
4	Whole-cell formalin-killed *V. harveyi* vaccine	10^4 CFU/mL		$3 \times 35 = 105$
5	Control	0.01 M PBS		$3 \times 35 = 105$
				Total = 525
(B) Challenge trial in zebrafish larvae				
1	Fresh live-attenuated *V. harveyi* vaccine	No vaccine was given during the larval stage	*V. alginolyticus* (10^7 CFU/mL), *V. parahaemolyticus* (10^7 CFU/mL), *V. harveyi* (10^7 CFU/mL)	$3 \times 15 = 45$
2	Stale live-attenuated *V. harveyi* vaccine			$3 \times 15 = 45$
3	Freeze-dried live-attenuated *V. harveyi* vaccine			$3 \times 15 = 45$
4	Whole-cell formalin-killed *V. harveyi* vaccine			$3 \times 15 = 45$
5	Control			$3 \times 15 = 45$
				Total = 225
(C) Challenge trial in juvenile zebrafish				
1	Freeze-dried live-attenuated *V. harveyi* vaccine	10^7 CFU/mL	*V. alginolyticus* (10^7 CFU/mL), *V. parahaemolyticus* (10^7 CFU/mL), *V. harveyi* (10^7 CFU/mL)	$3 \times 15 = 45$
2	Whole-cell formalin-killed *V. harveyi* vaccine	10^7 CFU/mL		$3 \times 15 = 45$
3	Control	0.01 M PBS		$3 \times 15 = 45$
				Total = 135

V. parahaemolyticus, and *V. harveyi* ((B) in Table 2). At the same time, the remaining larvae were left to grow until the juvenile stage. At week 3, after entering the juvenile stage (28 dph), the fish was challenged by immersion with 10^7 CFU/mL of pathogenic *V. alginolyticus*, *V. parahaemolyticus*, and *V. harveyi* ((C) in Table 2). In every challenged trial, the fish were immersed in 11 ppt salinity for 10 minutes before being placed back in their previous container (<0.5 ppt), and this method was adapted from Zhang et al. [30] with major modifications. They were not fed for 144 h (6 days) throughout the challenge test.

2.9. Relative Percent Survival. The relative percent survival is to determine the protective efficacy of a vaccine against pathogenic bacterial species. The calculation method and formula were adapted from Reed and Muench [31].

$$\text{Cumulative percent survival} = (\text{total number of fish mor tality/total number of fish}) \times 100$$

$$\text{Relative percent survival} = 1 - (\text{average CPM in the treatment group/average CPM in the control group}) \times 100$$

2.10. Quantitative Polymerase Chain Reaction. The expression of the immune-related genes was only analyzed in vaccinated juvenile zebrafish groups (5 homogenized whole fish samples/group) using SYBR-based quantitative real-time PCR (qPCR) assay [32]. In brief, the reaction was performed in a 20 μL reaction containing 1000 ng of cDNA, 10 μL of 2 × SYBR Green PCR master mix (Qiagen, Gmbh, Hilder, Germany), 1.4 μL of each forward and reverse primers of immune-related genes (TNF-α, IL-1β, IL-8, and β-actin; Table 3), and nuclease-free water adjusted to the final volume of 20 μL. The reactions were performed in a real-time PCR Rotor-Gene Q machine (Qiagen) in triplicates. At the end of each cycle, the melting curve analysis of the PCR products was performed at 65°C to 95°C with 0.5°C per 5 s increment to determine that only specific PCR products were acquired and there were no primer dimers [33]. The relative expression of each immune-relative gene was determined by comparing it with the expression level of the β-actin gene as the housekeeping gene using the Livak method, $2^{-\Delta\Delta^{CT}}$ method.

2.11. Data Analysis. The antibody level during the vaccination regime for adults, offspring, and vaccinated juvenile zebrafish was analyzed using ANOVA following Tukey's HSD for multiple comparisons [34]. All statistical analyses were performed via IBM SPSS Statistics ® version 25.0 (SPSS Inc., Chicago, IL, USA). The graph of antibody level was plotted using GraphPad Prism 7 (Graphpad Software Inc., San Diego, CA, USA). Values were considered significantly different at $p < 0.05$.

3. Results

3.1. Antibody Profiling of Vaccinated Adult Zebrafish. The antibody profiling of adult zebrafish was categorized into antibody levels against *V. alginolyticus*, *V. parahaemolyticus*, and *V. harveyi*.

3.1.1. Antibody Level of Vaccinated Adult Zebrafish against V. alginolyticus. Initial vaccination of zebrafish at week 0 induced the production of antibodies against *V. alginolyticus* with a significant difference ($p < 0.05$) compared with the control group. By week 2, after the second booster vaccination, all vaccination groups had their antibodies elevated before slightly decreasing as they reached week 4. After the third booster vaccination on week 5, the expression of antibody levels within the vaccinated groups was steadily increased but gradually decreased after their peak until week 12 (Figure 2(a)).

The initial progression of antibody levels in the freeze-dried LAVh vaccinated group was significantly different ($p < 0.05$) against antibody levels of the control and stale LAVh vaccinated group. However, the antibody level that had initially gained momentum after the second booster vaccination in week 2 had dropped in week 4. After the third booster vaccination at week 5, the antibody gradually increased until week 8, when it peaked and was significantly different from that of fresh LAVh and formalin-killed *V. harveyi* vaccine (FKVh (whole cell)) antibody level. After week 8, the antibody level gradually decreased but remained significantly different than that of the control group's antibody level.

Upon the initial vaccination of stale LAVh in zebrafish, the antibody was observed to have remained sustained until after the second booster vaccination on week 2. From here on, the antibody level had increased before slightly decreasing to week 5. Upon the third booster vaccination on week 5, the antibody level had boosted significantly ($p < 0.05$) until it reached its peak point at week 10 and gradually decreased by week 12. The antibody of zebrafish from the control group against *V. alginolyticus* was constant from week 0 until week 12, with a slight decrease on week 8.

Zebrafish vaccinated with fresh LAVh displayed an increase in antibody levels after the initial and second booster vaccinations. Their antibody level had slightly dropped before the third booster vaccination at week 5 before the antibody peaked and was significantly ($p < 0.05$) elevated on week 6. By week 8, the antibody level had dropped significantly ($p < 0.05$) compared with other vaccination groups and expressed the same antibody level against *V. alginolyticus* as that of the control group from week 10 to week 12.

Formalin-killed *V. harveyi* vaccine (FKVh (whole cell)) expressed antibody levels after the initial vaccination on week 0 and continued to express antibody levels on week 2 and week 5 before their booster vaccination administration. However, the expressed antibody level had increased slightly and peaked at around week 6 until week 8 before decreasing again to the same antibody level as that of the control group at week 12 in a continuous manner.

3.1.2. Antibody Level of Vaccinated Adult Zebrafish against V. parahaemolyticus. Zebrafish vaccinated with freeze-dried LAVh had a significant ($p < 0.05$) antibody expression against *V. parahaemolyticus* at week 0 before the initial vaccination of the zebrafish group. The antibody level had

Table 3: Primers designed to detect targeted innate immune-related genes.

Primers	Functions	Primer sequence (5′-3′)	Tm (°C)	Accession number
TNF-α	Regulation of the recruitment of immune cells	GCGCTTTTCTGAATCCTACG AAGTGCTGTGGTCGTGTCTG	60	AY427649
IL-1β	Mediator of key inflammatory response	CGCTCCACATCTCGTACTCA ATACGCGGTGCTGATAAACC	60	BC098597
IL-8	Attracts and activates neutrophils in the inflammation area	CTCGGACTGAAGGTGACTCC AAGCTGTTTACTGCACATGTT GT	60	HF674400.1
β-Actin	A housekeeping gene	ACCACGGCCGAAAGAGAAAT ATGTCCACGTCGCACTTCAT	60	NM_131031.2

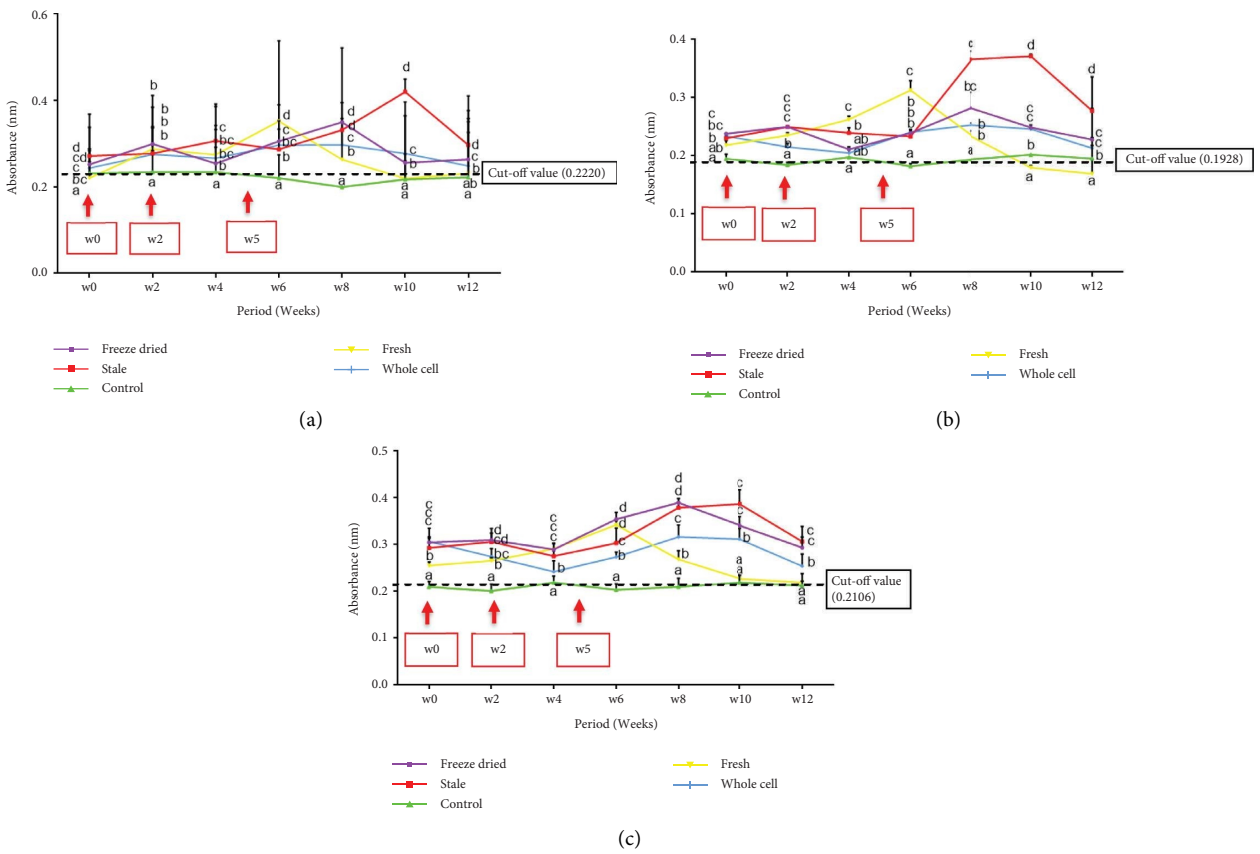

Figure 2: Antibody level of adult zebrafish from vaccination group following challenge with pathogenic *V. alginolyticus* (a), *V. parahaemolyticus* (b), and *V. harveyi* (c). Different letters represent statistically significant differences between treatments ($p < 0.05$).

increased until the second booster vaccination on week 2 before decreasing sharply on week 4. By week 5, the third booster vaccination was administered, and the antibody level was seen to have increased until it peaked in week 8 before gradually decreasing until week 12 (Figure 2(b)).

Expression of antibody level in stale LAVh vaccinated zebrafish group after the initial vaccination on week 0 increased until week 2. From there, the antibody level had slightly decreased before the third booster vaccination on week 5. After week 6, the antibody level jumped significantly ($p < 0.05$) at week 8 and peaked at week 10. The antibody level was still significantly higher ($p < 0.05$) compared with the other treatment groups when the antibody level started to decrease until week 12. The antibody level of the control group against *V. parahaemolyticus* remained constant throughout the experiment, with a slight elevation of antibodies around week 10 until week 12.

Zebrafish vaccinated with fresh LAVh display a constant increase in antibody levels against *V. parahaemolyticus* from the initial vaccination on week 0, continuously throughout the second and third booster vaccination on week 2 and week 5. The antibody level was significantly higher ($p < 0.05$) than other treatment groups on week 4 and peaked at week 6 before the antibody level dropped significantly ($p < 0.05$) on week 8. Antibody level was significantly lower ($p < 0.05$) than that of the control group between week 10 and week 12.

Vaccination of zebrafish with the FKVh vaccine expressed a continuous smooth expression of antibody levels as compared with treatment groups from the derivatives of LAVh vaccines. A slight drop in antibody level was detected from week 2 until week 5 before increasing again before administering booster vaccination. The antibody level peaked on week 8 before decreasing gradually until week 12.

3.1.3. Antibody Level of Vaccinated Adult Zebrafish against V. harveyi. Expression of antibody levels against *V. harveyi* in freeze-dried vaccinated zebrafish group indicated a smooth increase of antibody level from the initial vaccination until the second booster vaccination on week 2. There was a slight decrease in antibodies by week 4, before the antibody level then increased after the third booster vaccination on week 5 and peaked on week 6. There was a significant difference ($p < 0.05$) in antibody level on week 6 compared to the other treatment groups except against stale LAVh. The antibody level of freeze-dried vaccinated zebrafish then gradually decreased until week 12 while being significantly higher ($p < 0.05$) than fresh LAVh and FKVh vaccinated groups (Figure 2(c)).

Stale LAVh vaccinated zebrafish had shown antibody levels against *V. harveyi* to be on par with that of freeze-dried vaccine expression of antibody. This vaccine, however, peaked on week 10, a week later than the freeze-dried vaccine, and gradually decreased until week 12. The expression of antibodies in the control group remained constant until week 12.

The antibody level of the fresh LAVh vaccinated zebrafish group showed a continual increase in antibodies from the initial vaccination on week 0 to the second and third booster vaccination on weeks 2 and week 5. Fresh LAVh group peaked on week 6 before gradually decreasing its antibody expression at the same level as the control group from week 10 until week 12.

FKVh antibody level expression decreased after the initial vaccination on week 0. This trend continued after the second booster vaccination before increasing again prior to the third booster vaccination on week 5. The antibody level peaked in-between week 8 and week 10 before decreasing at week 12.

3.2. Antibody Profiling of Offspring from Vaccinated Zebrafish. The antibody profiling of zebrafish offspring from vaccinated adult zebrafish was categorized into antibody levels against *V. alginolyticus*, *V. parahaemolyticus*, and *V. harveyi*.

3.2.1. Antibody Level of Zebrafish Fry from Vaccinated Adult against V. alginolyticus. The antibody level of freeze-dried LAVh vaccinated offspring against *V. alginolyticus* decreased after the egg hatched and continued throughout the larval stage and the early juvenile I stage until week 2 post-fertilized. The antibody level then significantly increased ($p < 0.05$) and peaked at week 3 before decreasing at week 4 (Figure 3(a)).

In offspring of stale LAVh vaccinated zebrafish, the antibody level began to increase as early as week 1 in the larvae stage. The trend continued until it peaked in week 3 and decreased again by week 4. The control group's offspring showed no elevation of antibody levels throughout the 4-week study period. The offspring from fresh LAVh treatment groups display the same antibody level as that of offspring of the freeze-dried LAVh treatment group. The antibody level started to increase only after week 3; however, the antibody level peaked in week 4.

The same can be said for observing offspring of the FKVh treatment group. The antibody started to rise only after week 3 when the offspring were in the juvenile I stage. The antibody level of this treatment group also peaked on week 3 and decreased slightly by week 4.

3.2.2. Antibody Level of Zebrafish Fry from Vaccinated Adult against V. parahaemolyticus. The antibody level of offspring of freeze-dried LAVh vaccinated zebrafish displayed an increase of antibody expression as early as week 1 against *V. parahaemolyticus*. The antibody level slightly dropped by week 2 before increasing and peaked at week 3. There was a significant difference ($p < 0.05$) in the antibody expressed by freeze-dried treatment groups as compared with fresh LAVh and FKVh treatment groups; however, there was no significant difference ($p < 0.05$) against the treatment group of stale LAVh group (Figure 3(b)).

The antibody level of offspring from stale LAVh-vaccinated zebrafish displayed the same trend as that of the freeze-dried LAVh offspring. The offspring from the control group displayed a slight increase in antibody levels against *V. parahaemolyticus* on weeks 1 and 3; however, the antibody level decreased throughout the study period. Antibody level from fresh LAVh-vaccinated zebrafish offspring followed the trend of its other LAVh derivative groups; however, the antibody level continues to increase after week 3, coming into week 4.

Expression of antibody levels by offspring from the FKVh treatment group peaked at week 3; however, it did not show a significant difference against that of the fresh and control groups within the same period. The low expression indicates that the antibody level in the offspring of the FKVh group against *V. parahaemolyticus* was negligible.

3.2.3. Antibody Level of Zebrafish Fry from Vaccinated Adult against V. harveyi. The antibody level against *V. harveyi* in freeze-dried LAVh offspring of vaccinated zebrafish displayed a continuous increase in antibody level from week 0 and peaked at week 3. The antibody level then dropped at week 4 (Figure 3(c)). This is also true for the expression of antibodies of offspring from the stale LAVh treatment.

In the antibody level of offspring from the fresh LAVh treatment group, the antibody level against *V. harveyi* increased gradually until week 4 and displayed a significant difference ($p < 0.05$) against the other treatment groups within that period (week 4). However, there was a significant increase ($p < 0.05$) of antibody levels in the control group against *V. harveyi* in the initial period of week 1, and the antibody level then continued to drop from week 2 until week 4. The antibody level of offspring from the FKVh treatment group gradually increased from week 0 until it peaked at week 3 and decreased by week 4.

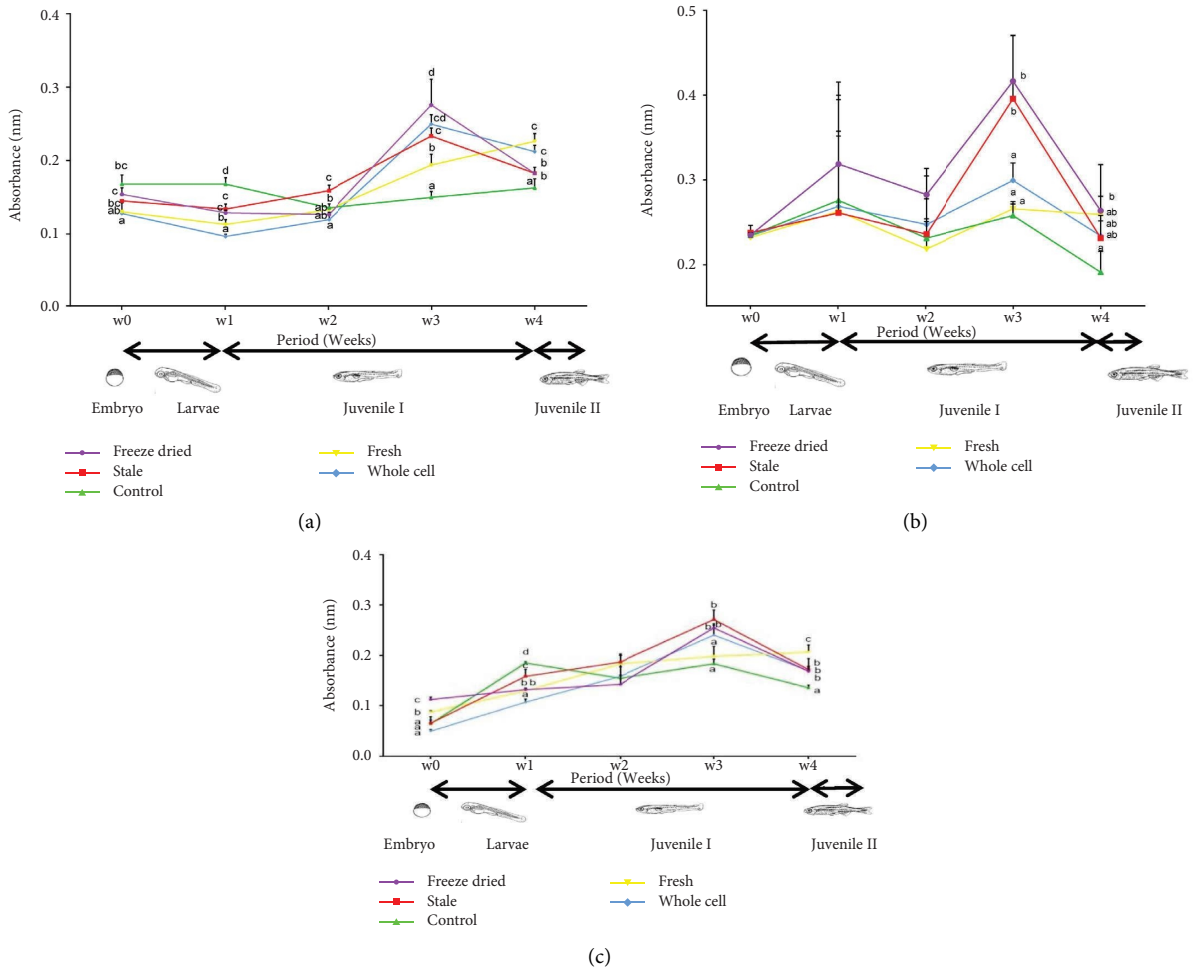

FIGURE 3: Antibody level of zebrafish fry from vaccination group following challenge with pathogenic *V. alginolyticus* (a), *V. parahaemolyticus* (b), and *V. harveyi* (c). Different letters represent statistically significant differences between treatments ($p < 0.05$).

3.3. Antibody Profiling of Vaccinated Juvenile Zebrafish. The antibody profiling of immerse vaccinated zebrafish fry from maternal vaccinated adult zebrafish was categorized into antibody levels against *V. alginolyticus*, *V. parahaemolyticus*, and *V. harveyi*.

3.3.1. Antibody Level of Vaccinated Zebrafish Fry from Vaccinated Adults against V. alginolyticus. Initial immerse vaccination of 28 days post-hatching (dph) freeze-dried LAVh offspring from freeze-dried vaccinated adults revealed an increase in antibody expression after 2 weeks against *V. alginolyticus* antigen. At week 2, the antibody level of freeze-dried vaccinated fry had peaked and was significantly higher ($p < 0.05$) than that of the control group (Figure 4(a)).

The antibody expressed by 28 dph fry from the formalin-killed *V. harveyi* vaccination group displayed the same antibody expression as the freeze-dried vaccination group. Its peak was seen on week 2 and showed no significant difference ($p < 0.05$) with antibodies from freeze-dried vaccinated fry throughout this study.

3.3.2. Antibody Level of Vaccinated Zebrafish Fry from Vaccinated Adults against V. parahaemolyticus. Vaccination of freeze-dried LAVh vaccine by immersion in the maternal offspring of the freeze-dried LAVh vaccinated group displayed significantly higher ($p < 0.05$) antibody expression against *V. parahaemolyticus* (Figure 4(b)). The vaccination of offspring from the maternal FKVh vaccinated group did not display a significant difference ($p < 0.05$) in antibody levels against other treatment groups and the control group from week 0 until week 1 and week 3. At week 2, this group showed no significant difference ($p < 0.05$) against the antibody level of the freeze-dried LAVh group vaccinated offspring.

3.3.3. Antibody Level of Vaccinated Zebrafish Fry from Vaccinated Adults against V. harveyi. Interestingly, the antibody level of both freeze-dried vaccinated fry and that of FKVh vaccinated fry had higher antibody levels in the initial stage of vaccination at week 0 as compared with the control group (Figure 4(c)). However, the antibody level had then dropped to the same level as the control group and showed

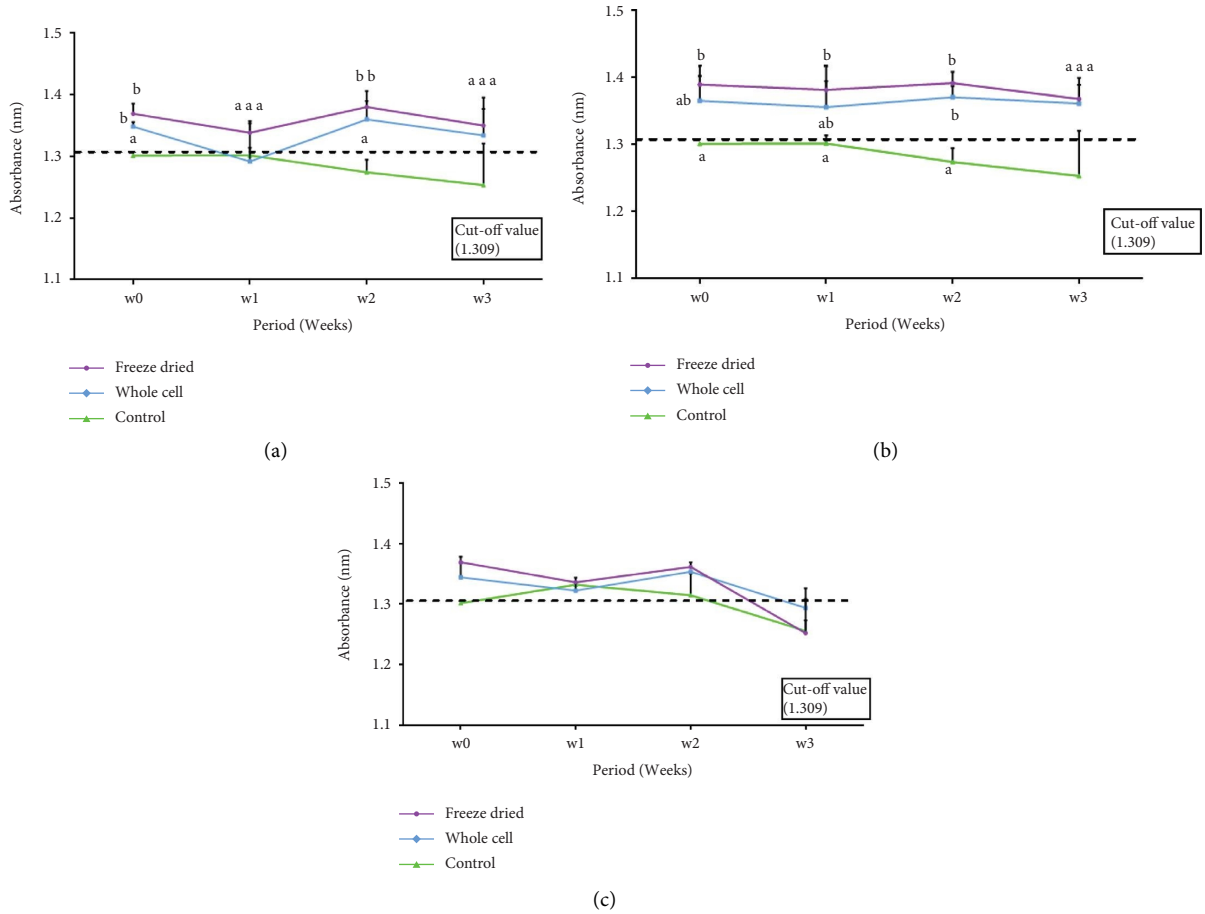

Figure 4: Antibody profiling of vaccinated zebrafish fry from vaccinated broodstock against antigens from *V. alginolyticus* (a), *V. parahaemolyticus* (b), and *V. harveyi* (c). Different letters represent statistically significant differences between treatments ($p < 0.05$).

no significant difference between all treatment groups from week 1 until week 3.

3.4. Challenge Test. The results of the challenge test are categorized into three compartments: the challenge test of adult zebrafish, the challenge test of offspring from vaccinated zebrafish, and the challenge test of vaccinated zebrafish juveniles.

3.4.1. Challenge Test for Offspring from Vaccinated Zebrafish. Upon challenge test using the doses for larvae LD_{50-144} of a pathogenic strain of *Vibrio* spp. against adult zebrafish and offspring from treatment groups of vaccinated adults, the relative percent survival of these offspring was measured.

Adult zebrafish vaccinated with derivatives of LAVh vaccine managed to present highest RPS values when challenged against $LD_{50-144\,h}$ of *V. harveyi* (Table 4). Stale LAVh, however, displayed lowest RPS value at 29.8% when challenged against *V. alginolyticus*. FKVh vaccine displayed a low RPS value in adult zebrafish challenge.

On the other hand, the challenge test against the pathogenic strain of *V. parahaemolyticus* of all treatment groups showed that offspring from the FKVh treatment group had the lowest RPS value at 68.10%. When challenged against the pathogenic strain of *V. harveyi*, the offspring from the fresh LAVh treatment group displayed the lowest relative percent survival (57.45%) among the other treatment groups (Table 5).

3.4.2. Challenge Test for Vaccinated Juvenile Zebrafish. All zebrafish fry vaccinated with freeze-dried LAVh and FKVh survived the study and showed no clinical signs of infection against all three pathogenic strains of *Vibro* spp. (Table 6). There was no significant difference between treatment groups ($p < 0.05$).

3.5. Expression of Innate Immunity Post-Vaccination. The expression of innate immune genes post-vaccination of immersed vaccinated zebrafish fry from maternal vaccinated adult zebrafish were categorized into their respective genes of interleukin-1 beta (IL-1β), interleukin-8 (IL-8), and tumor necrotic factor-alpha (TNF-α). Overall results indicated that fry of freeze-dried LAVh and FKVh vaccinated group showed high expression of innate immune-related gene during the initial post-vaccination period which subsequently decreased afterward.

Table 4: Relative percent survival of vaccinated adult zebrafish.

Group	*Vibrio* spp.	Average mortality at a specific hour post-challenge of adult zebrafish							Total fish sample	Cumulative mortality (%)	RPS (%)
		0 h	24 h	48 h	72 h	96 h	120 h	144 h			
Fresh LAVh	*V. alginolyticus* (10^5 CFU/mL)	0	1	1	1	0	0	0	30	3 (10.0)	78.70
Stale LAVh		0	2	5	2	1	0	0	30	10 (33.3)	29.80
Freeze-dried LAVh		0	1	1	1	0	0	0	30	3 (10.0)	78.70
Whole-cell FKVh		0	6	2	2	1	1	0	30	12 (40.0)	28.48
Control		0	4	2	5	2	1	0	30	14 (46.7)	0.00
Fresh LAVh	*V. parahaemolyticus* (10^6 CFU/mL)	0	1	0	1	0	1	0	30	3 (10.0)	78.70
Stale LAVh		0	0	0	0	0	0	0	30	0 (0.0)	100.0
Freeze-dried LAVh		0	1	2	0	0	0	0	30	3 (10.0)	78.70
Whole-cell FKVh		0	4	4	2	1	1	0	30	12 (40.0)	28.48
Control		0	1	4	7	1	1	0	30	14 (46.7)	0.00
Fresh LAVh	*V. harveyi* (10^6 CFU/mL)	0	0	0	0	0	0	0	30	0 (0.0)	100.00
Stale LAVh		0	0	0	0	0	0	0	30	0 (0.0)	100.00
Freeze-dried LAVh		0	0	0	0	0	0	0	30	0 (0.0)	100.00
Whole-cell FKVh		0	6	1	1	1	1	0	30	10 (33.3)	33.40
Control		0	2	2	5	4	2	0	30	15 (50.0)	0.00

LAVh, live-attenuated *Vibrio harveyi* vaccine; FKVh, whole-cell formalin-killed *V. harveyi* vaccine; RPS, relative percent survival.

Table 5: Relative percent survival of 28 dph larvae from vaccinated zebrafish.

Group	*Vibrio* spp.	Average mortality at a specific hour post-challenge of 28 dph larvae							Total fish sample	Cumulative mortality (%)	RPS (%)
	$LD_{50-144\,h}$ (10^7 CFU/mL)	0 h	24 h	48 h	72 h	96 h	120 h	144 h			
Fresh LAVh	*V alginolyticus*	0	2	0	1	0	0	0	45	3 (6.7)	78.72
Stale LAVh		0	2	2	1	0	1	0	45	6 (13.3)	68.10
Freeze-dried LAVh		0	1	1	1	0	0	0	45	3 (6.7)	78.72
Whole-cell FKVh		0	2	2	2	0	0	0	45	6 (13.3)	68.10
Control		0	7	5	6	7	2	0	45	27 (60.0)	0.00
Fresh LAVh	*V. parahaemolyticus*	0	1	1	1	0	0	0	45	3 (6.7)	78.72
Stale LAVh		0	1	0	1	1	0	0	45	3 (6.7)	78.72
Freeze-dried LAVh		0	1	1	1	0	0	0	45	3 (6.7)	78.72
Whole-cell FKVh		0	3	1	2	0	0	0	45	6 (13.3)	68.10
Control		0	6	7	5	4	2	0	45	24 (53.3)	0.00
Fresh LAVh	*V. harveyi*	0	3	2	2	2	0	0	45	9 (20.0)	57.45
Stale LAVh		0	1	2	0	0	0	0	45	3 (6.7)	78.72
Freeze-dried LAVh		0	3	2	1	0	0	0	45	6 (13.3)	68.10
Whole-cell FKVh		0	1	1	1	0	0	0	45	3 (6.7)	78.72
Control		1	7	5	5	3	0	0	45	21 (46.7)	0.00

LAVh, live-attenuated *Vibrio harveyi* vaccine; FKVh, whole-cell formalin-killed *V. harveyi* vaccine; RPS, relative percent survival.

Table 6: The mortality of vaccinated juvenile zebrafish post-challenge test and the relative percent survival.

Group	*Vibrio* spp. $LD_{50-144h}$ (10^7 CFU/mL)	Vaccine dose	Average mortality at a specific hour post-vaccination							Total fish sample	Cumulative mortality (%)	RPS (%)
			0 h	24 h	48 h	72 h	96 h	120 h	144 h			
Freeze-dried LAVh	*V. alginolyticus*	1×10^7 CFU/mL	0	0	0	0	0	0	0	45	0 (0%)	100
	V. parahaemolyticus		0	0	0	0	0	0	0	45	0 (0%)	100
	V. harveyi		0	0	0	0	0	0	0	45	0 (0%)	100
Formalin-killed *V. harveyi*	*V. alginolyticus*	1×10^7 CFU/mL	0	0	0	0	0	0	0	45	0 (0%)	100
	V. parahaemolyticus		0	0	0	0	0	0	0	45	0 (0%)	100
	V. harveyi		0	0	0	0	0	0	0	45	0 (0%)	100
Control	*V. alginolyticus*	0.01 M PBS	0	16	4	2	0	0	0	45	22 (48.9%)	0
	V. parahaemolyticus		0	9	0	0	1	9	0	45	19 (42.2%)	0
	V. harveyi		0	11	6	5	2	0	0	45	24 (53.3%)	0

LAVh, live-attenuated *Vibrio harveyi* vaccine; FKVh, whole-cell formalin-killed *V. harveyi* vaccine; RPS, relative percent survival.

3.5.1. Expression of Interleukin-1 Beta (IL-1β). The expression of IL-1β from immerse-vaccinated freeze-dried LAVh zebrafish fry showed high expression at week 0 before vaccination. During week 1 and week 2, the expression of IL-1β had decreased to a level lower than the expression of the control group. However, in week 3, the expression of the IL-1β gene increased slightly (Figure 5(a)). Zebrafish fry vaccinated by immersion with FKVh vaccine displayed high expression of IL-1β at week 0 and continued until week 1. However, this gene's expression decreased to a lower level than that of the control group on week 2 and week 3. No significant differences were detected between treatment groups within each sampling week throughout the study period.

3.5.2. Expression of Interleukin-8 (IL-8). Zebrafish fry vaccinated with freeze-dried LAVh vaccine displayed high IL-8 gene expression at week 0 following immerse vaccination. The gene expression had dropped sharply by week 1, below the expression levels of the control group. However, it increased slightly until week 3 (Figure 5(b)). FKVh displayed a higher expression of IL-8; however, there was no significant difference ($p < 0.05$) between the treatment groups in each sampling week throughout the study period; the expression, later on, decreased as the study progressed.

3.5.3. Expression of Tumor Necrotic Factor-Alpha (TNF-α). Before the vaccination of freeze-dried vaccines of zebrafish fry via immersion, high expression of TNF-α was detected on week 0. On week 1, the expression dropped significantly ($p > 0.05$) compared with FKVh-vaccinated zebrafish fry. However, in week 2, the expression of TNF-α increased slightly before decreasing again in week 3 (Figure 5(c)). TNF-α expression from zebrafish fry of the FKVh vaccinated group showed a high expression value in the early period of the study, week 0. The expression decreased by week 1 and continued until week 3 in a gradual state.

4. Discussion

Live-attenuated bacterial vaccines can provide a higher immunological protective barrier against intracellular pathogens while inducing better immuno-responses towards the host than conventional killed vaccines [35]. Additionally, live-attenuated bacterial vaccines can provide a higher function by providing both homologous and heterologous protection across multiple species of bacteria by displaying similar antigen-recognizing outer membrane protein (OMP) of other species expressed on its surface [36].

This study conducted LAVh derivative vaccination of adult zebrafish according to the effective dose of 80% survival ($ED_{80-144\,h}$) of the vaccine when challenged against the median lethal dose ($LD_{50-144\,h}$) of pathogenic *Vibrio* spp. as a baseline. The vaccination of adult zebrafish using the $ED_{80-144\,h}$ of LAVh derivatives was established as a reference to determine the transfer of maternal immunity in zebrafish fry by using a predetermined and justified vaccination dose.

The vaccination points for adult zebrafish were established to desire the phase at which the gonad was developing. Nisaa et al. [37] stated that gonad development during vaccination impacts antibody accumulation in fish eggs and seeds. Vaccination in the second stage of gonad development would yield higher antibodies compared to later stages in tilapia (*Oreochromis niloticus*).

Maternal immunity is the transfer of antibodies and immune-related material from the progenitor parent to their offspring. Fish larvae depend highly on their maternal-derived immune materials and nutrient composition for their development. Akbary et al. [38] highlighted the high mortality in fish fry recorded globally due to outbreaks of microbial pathogens, hence the importance of maternally derived immunity for the survival of offspring in the early stage before hatching [19].

Multiple studies were conducted on different fish species, both commercially and model species, to highlight the protective efficiency of maternal immune transfer. Sukenda et al. [39] recorded that vaccination of broodstock increased the survival of offspring against 10^7 CFU/mL *Streptococcus agalactiae* by 95% on 7 days post-hatching (dph) as compared with 56% survival after 28 dph with the same bacterial dose. Another study on larvae of sea bream from vaccinated broodstock concluded that there was a significant increase in anti-protease activity and high total antibody level after spawning compared with larvae from unvaccinated broodfish [40]. These studies indicated the effectiveness of vaccinating broodfish to confer higher survival for their offspring by increasing the transfer of maternally derived antibodies to counter microbial threats in the early stage of fish fry. As a side note, Ye et al. [41] conducted a study in vaccinating adult zebrafish with live-attenuated *V. anguillarum* MVM425, which resulted in the enhancement of myelopoiesis development in their offspring.

A successful upbringing of fish fry largely coincides with the broodstock's overall health and an environment that is supportive of the survival of the fish fry [11]. The vaccination of broodstock to confer the transfer of maternal immunity helps increase the survival rate of fish fry against disease infection. This measure ensures reliable and sustainable fish fry production for the aquaculture industry.

In adult zebrafish, stale antibody level was seen to have peaked at the later stage of the vaccination profile as compared with fresh and freeze-dried LAVh. Fresh LAVh was the earliest vaccine to peak and drop significantly, continuing onwards. The increase in antibody buildup could be linked to the age of the inoculated bacterium. Older bacterium expresses a higher array of outer membrane protein (OMP) than younger ones. This increases the binding competence of the produced antibodies in the host against invading antigens. The peaking of antibody levels in adult zebrafish determined that the freeze-dried and stale LAVh vaccine delivers a longer immunological protective duration than fresh LAVh.

It had also been noted that the expression of antibody levels against the antigen of *V. harvey*i in adult zebrafish showed a smoother expression of antibody level than the

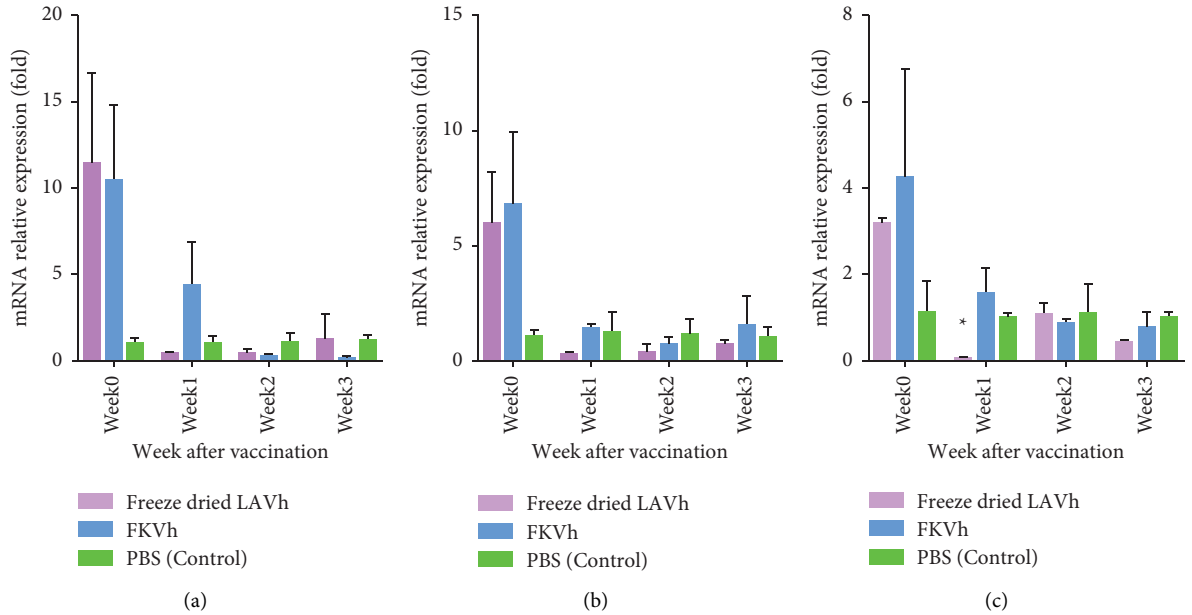

FIGURE 5: Gene expression of innate immune-related genes of (a) IL-1β, (b) IL-8, and (c) TNF-α from whole fish fry for 28 days post-vaccination. Asterisks (*) indicate the significance of differences ($p < 0.05$) between the vaccination groups of the targeted gene during the same period.

sudden spikes of that from antibody level expression against *V. alginolyticus* and *V. parahaemolyticus*. There was no explanation for this condition that the authors were aware of at the time of this writing. Despite that, it is suggested that due to the use of the same species of the bacterium as a vaccine candidate (*V. harveyi*), the antibodies produced were more compatible to attach with the pathogenic strain of *V. harveyi*, hence the smoother expression of antibody level. However, in comparison with other strains of pathogenic *Vibrio* spp., it is suggested that the antibodies bind more coarsely to the displayed antigens of *V. alginolyticus* and *V. parahaemolyticus*. This could be because of the difference in compatibility between the antibodies and the invading pathogen.

As stated earlier, the lower antibody production of fresh live-attenuated *V. harveyi* (LAVh) vaccine as compared to stale and freeze-dried LAVh. Therefore, a more extended incubation period is suggested to promote additional multiplication and higher bacterial maturity. Winslow and Walker [42] described that the early phase of bacterial culture consisted of rapid multiplication of cell with high metabolic activity but low resistance to environmental conditions. In the later phase, the culture contained more developed bacteria. This resulted in more matured pathogen recognition proteins, which in turn increased the marker expressed on macrophages as well as affinity of antibodies. Vaccination with the live-attenuated vaccine is presumed to have promoted myelopoiesis development while regulating cells to mature into macrophages [41].

During the initial stage of zebrafish fry, the antibody level started to increase gradually, suggesting the accomplishment of maternal immunity transfer to the larvae from their immunized parents. Offspring of the fresh LAVh vaccinated group had a more extended peaked antibody level than the other vaccination groups.

Other studies, such as Hanif et al. [40], recorded that antibodies in the larvae of sea bream (*Sparus aurata*) were reported to decrease gradually until the eighth day post-hatching (dph) before increasing again. It was thought to be because of the absorption of egg yolks and natural protein catabolism in fish larvae during this period. Zebrafish egg yolk had been completely absorbed by the fourth dph; moreover, during this period, the thymus of zebrafish fry was recorded to have been developed as an organ; it is then followed by rapid growth and replication until the 3rd week post-hatching (wph) and organized into a thymic cortex [43]; this can be seen with the striking increase of antibody on the third wph. Zapata et al. [43] then highlighted that despite having distinguished the lymphoid organs, other factors would determine the immunocompetence of the fish; it is noted that full maturity of immunological competence can only be observed later, despite having noticeable T and B lymphocytes much earlier. Adams [44] later wrote that in regard to B cell responses, the number of antibodies produced does not correlate with the level of protection that can be conferred to the host.

The relative percent survival in offspring of the FKVh vaccinated adult zebrafish had failed to increase the antibody of offspring against *V. alginolyticus*. It was suggested that the maternal transfer of immune-related material was limited in the FKVh vaccination group as compared to that of LAVh derivatives.

Freeze-dried LAVh vaccine demonstrated reliable antibody expression in adult zebrafish with an $ED_{80\text{–}144\,h}$ of 1×10^4 CFU/mL. Freeze drying of live-attenuated (LAVh) vaccine increased the vaccine's shelf life while retaining its

ability to mimic natural pathogens. By deploying the exact vaccine derivative for immerse vaccination dose at 1×10^7 CFU/mL, immersion vaccination is a more suitable method to be utilized as the fish fry is small in size and comes in a large group. The immersion would be hard to tackle compared to intraperitoneal vaccination, which is laborious and time intensive.

Guo et al. [45] previously stated that bacterial dose of 107 CFU/mL is usually required to induce innate immunity in zebrafish larvae via immersion. Their suggestion was based on a compilation of studies conducted on the immune responses of zebrafish larvae from bacterial infection [46–49].

Initially, the antibody level of fry from vaccinated broodstock from freeze-dried LAVh and FKVh vaccines was elevated compared to the control. The capability of maternally transferred immunity to confer protection is essential to help their offspring during their initial days in their hostile environment.

In weeks following the initial vaccination, the antibody level then leveled out. This can be seen at week 3 post-vaccination; the antibody level of both freeze-dried LAVh and FKVh vaccination group and their control counterpart showed no significant difference in terms of antibody detection via absorbance analysis.

It is proposed that a more extended study duration on the antibody profiling of vaccinated fry of two (2) months would indicate the peaking point of antibody levels and the downtrend of the antibody. From here, providing a booster vaccination for the fry can be suggested to increase the antibody level and prolong immunological protection against *Vibrio* spp. infection.

Theoretically, when a foreign material enters the external barrier of the host, it will cause inflammation of the entry site due to the recruitment and responses of non-specific inflammatory responses such as the macrophage, neutrophil, and dendritic cells. The robust and immediate action of the immune cells is mediated by pro-inflammatory cytokines such as pro-inflammatory interleukin (-1α, -1β, -2,-6, -7, -12, and -15), the tumor necrosis factor-α (TNF-α), and the interferon-γ (IFNγ) [50, 51].

Vaccination of freeze-dried LAVh and FKVh vaccine manages to trigger the expression of pro-inflammatory genes during the initial week; the expression of genes then drops in the weeks following as the immune system has triggered the adaptive immunity and advances to deploy their humoral stage with the inclusion of developing antibodies. Interestingly, genes related to innate immunity recruitment were highly expressed during the initial vaccination week, as can be seen for cytokine IL-1β compared with TNF-α, which was moderately expressed.

It is worth mentioning that a study using *Edwardsiella tarda* exposed to zebrafish larvae also stimulated the same amount of IL-1β and TNF-α [46] as this study. These results, however, differ from another study by Oehlers et al. [52] by using zebrafish larvae exposed *to Listonella anguillarum,* which resulted in a strong expression of IL-1β and TNF-α. Both these cytokines were described as working to coincide with each other to mediate the pro-inflammatory responses [51]. However, Hasegawa et al. [53] stated that prolonging the expression of pro-inflammatory cytokines has detrimental effects on the host due to the ongoing recruitment of macrophages and neutrophils.

High expression of IL-1β in freeze-dried LAVh vaccinated fry on week 0 indicated that the vaccine had promoted the recruitment of innate phagocytes to the site and carried on with the immunity-cascading effects. Compared with FKVh, the expression of IL-1β continued until week 1, suggesting that the FKVh vaccine still requires the recruitment of innate cells to the site of infection. It is suggested that high expression but the short duration of pro-inflammatory genes is required in the early phase of infection to promote more immune cells to the infected site vigorously. A long duration of pro-inflammatory expression suggests that the vaccine is weak at promoting the incursion of immune cells to the site of interest.

In the case of cytokine IL-1β, prolonged exposure has been cited for causing epidermal damage to surrounding cells cascading into apoptosis. Although unlikely to happen in healthy fish as the macrophages can efficiently attenuate the cytokines, fish that are immuno-suppressed or immuno-compromised have a high chance of this occurring. TNF-α was suggested to function as an inhibitor to activate the endothelium in response to the agitation of foreign material rather than direct action on the leukocytes [52]; in other terms, they attract neutrophils [54]. However, Pan et al. [55] reported that upon infection with Vibrio *vulnificus, both grouper* (*Epinephelus coioides*) and zebrafish exhibited an over-expression of immune genes (TNF-α, il-6), which may contribute to endotoxemic shock and increases the mortality rate of the fishes. Hence, a high expression of TNF-α does not indicate a reliable and competent immune response.

Secretion of the IL-8 cytokine by macrophages is vital to the progression and reaction of innate immunity [56]. They are hardly detectable when healthy fish have no stimulant compared to other mediated stimulants such as TNF-α, IL-1β, and other cellular stress [57], although their primary function is to recruit and guide neutrophils to the targeted site of infection [58]. The freeze-dried LAVh and FKVh vaccines are assumed to have been cleared by week 2 due to the significant drop in IL-8 expression (Figure 5(a)). The drop of IL-8 can also signify that the foreign material has been cleared from the host, and the neutrophils have dispersed to other lymphoid organs.

The relative survival rate of both vaccination groups was on par with each other. They could equally confer complete protection against multiple strains of *Vibrio* spp. Nevertheless, a deep understanding of the innate immune response has to be taken into account, as this could have dire consequences due to the overexpression of pro-inflammatory cytokines of the host during inoculation.

5. Conclusion

The application of LAVh to delivering maternal immunity from broodstock to fish fry for their survival during the hatchery and nursery phases has been established. The application, in turn, would help increase the output of the

relevant parties in tackling the supply shortage. The use of freeze-dried LAVh vaccine to confer maternal immune protection for its offspring is desired because it has better cross-protection coverage against *V. alginolyticus*, *V. parahaemolyticus*, and *V. harveyi* as compared with the FKVh vaccine. Although zebrafish were not the intended species for the vaccine, they play a major role in determining the early response of antibodies from the maternal transfer. Immersion with 1×10^7 CFU/mL of freeze-dried LAVh was able to induce good immunity response and full protection towards their host when challenged with multiple strains of pathogenic *Vibrio* spp. Vaccination of fish fry is important to maintain the immune protection brought down by their parent. Although the maternal transfer of immunity manages to confer some protection for the offspring, it is limited and only provides protection for a limited period, hence the need for a booster vaccination for the fish fry.

Data Availability

The source of *Vibrio* strains (Supplementary Table 1) and primers for *Vibrio* detection (Supplementary Table 2) used in this study is available in the supplementary information files accompanying this article. All other data that support the findings of this study are available from the corresponding author upon reasonable request.

Ethical Approval

Approval for bacterial use and the fish host was recognized following the guideline stipulated by the Department of Biosafety, Ministry of Natural Resource and Environment, Malaysia (approval number: JBK(S) 602-1/2/136(6)). All approaches performed in studies involving animals were in accordance with the ethical standards of the Institutional Animal Care and Use Committee, Universiti Putra Malaysia (approval number: UPM/IACUC/AUPR059/2016).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

This research was supported by using the grants of Long Term Research Grant Scheme (LRGS), code: LRGS/1/2019/UPM/01/1/2; Higher Institution Centre of Excellence (HiCoE), vot no.: 6369100 provided by the Ministry of Higher Education Malaysia.

Supplementary Materials

Supplementary Table 1: the *Vibrio* spp. strains used in experiments. Supplementary Table 2: primer for detection of *V. alginolyticus* and *V. parahaemolyticus* (gyrB) and *V. harveyi* (serine protease). (*Supplementary Materials*)

References

[1] T. Chu, L. Guan, P. Shang et al., "A controllable bacterial lysis system to enhance biological safety of live attenuated *Vibrio anguillarum* vaccine," *Fish & Shellfish Immunology*, vol. 45, no. 2, pp. 742–749, 2015.

[2] E. A. Tendencia, "*Vibrio harveyi* isolated from cage-cultured seabass *Lates calcarifer* Bloch in the Philippines," *Aquaculture Research*, vol. 33, no. 6, pp. 455–458, 2002.

[3] K. Zhu, Z. Chi, J. Li et al., "The surface display of haemolysin from *Vibrio harveyi* on yeast cells and their potential applications as live vaccine in marine fish," *Vaccine*, vol. 24, no. 35-36, pp. 6046–6052, 2006.

[4] 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*, vol. 8, no. 2, pp. 104–115, 2012.

[5] H. T. Dong, S. Taengphu, P. Sangsuriya et al., "Recovery of *Vibrio harveyi* from scale drop and muscle necrosis disease in farmed barramundi, *Lates calcarifer* in Vietnam," *Aquaculture*, vol. 473, pp. 89–96, 2017.

[6] C. A. Broberg, T. J. Calder, and K. Orth, "*Vibrio parahaemolyticus* cell biology and pathogenicity determinants," *Microbes and Infection*, vol. 13, no. 12-13, pp. 992–1001, 2011.

[7] A. Mohamad, F. 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*, vol. 12, no. 2, p. 133, 2022.

[8] J. Ransangan and S. Mustafa, "Identification of *Vibrio harveyi* isolated from diseased Asian seabass *Lates calcarifer* by use of 16S ribosomal DNA sequencing," *Journal of Aquatic Animal Health*, vol. 21, no. 3, pp. 150–155, 2009.

[9] Y. Deng, L. Xu, H. Chen et al., "Prevalence, virulence genes, and antimicrobial resistance of *Vibrio* species isolated from diseased marine fish in South China," *Scientific Reports*, vol. 10, no. 1, Article ID 14329, 2020.

[10] C. de Souza Valente and A. H. L. Wan, "Vibrio and major commercially important vibriosis diseases in decapod crustaceans," *Journal of Invertebrate Pathology*, vol. 181, Article ID 107527, 2021.

[11] I. Frans, C. W. Michiels, P. Bossier, K. A. Willems, B. Lievens, and H. Rediers, "*Vibrio anguillarum* as a fish pathogen: virulence factors, diagnosis and prevention," *Journal of Fish Diseases*, vol. 34, no. 9, pp. 643–661, 2011.

[12] A. M. Aris, I. Yasin, M. Zamri-saad, H. Mohd, and N. M. Alipiah, "Molecular characterization of Vibrio harveyi virulence- associated serine protease and outer membrane protein genes for vaccine development," *Biosci International Journal*, vol. 6655, pp. 10–28, 2016.

[13] S. Nikoskelainen, S. Verho, S. Järvinen, J. Madetoja, T. Wiklund, and E. Lilius, "Multiple whole bacterial antigens in polyvalent vaccine may result in inhibition of specific responses in rainbow trout (*Oncorhynchus mykiss*)," *Fish & Shellfish Immunology*, vol. 22, no. 3, pp. 206–217, 2007.

[14] B. Magnadottir, "Immunological control of fish diseases," *Marine Biotechnology*, vol. 12, no. 4, pp. 361–379, 2010.

[15] W. Pasaribu, S. Sukenda, and S. Nuryati, "The efficacy of Nile tilapia (*Oreochromis niloticus*) broodstock and larval immunization against *Streptococcus agalactiae* and *Aeromonas hydrophila*," *Fishes*, vol. 3, no. 1, p. 16, 2018.

[16] S. Zhang, Z. Wang, and H. Wang, "Maternal immunity in fish," *Developmental & Comparative Immunology*, vol. 39, no. 1-2, pp. 72–78, 2013a.

[17] A. A. Laith, M. A. Abdullah, W. W. I. Nurhafizah et al., "Efficacy of live attenuated vaccine derived from the *Streptococcus agalactiae* on the immune responses of *Oreochromis niloticus*," *Fish & Shellfish Immunology*, vol. 90, pp. 235–243, 2019.

[18] L. Yang, Y. Ma, and Y. Zhang, "Freeze-drying of live attenuated *Vibrio anguillarum* mutant for vaccine preparation," *Biologicals*, vol. 35, no. 4, pp. 265–269, 2007.

[19] P. Swain and S. K. Nayak, "Role of maternally derived immunity in fish," *Fish & Shellfish Immunology*, vol. 27, no. 2, pp. 89–99, 2009.

[20] Z. Wang, S. Zhang, Z. Tong, L. Li, and G. Wang, "Maternal transfer and protective role of the alternative complement components in zebrafish *Danio rerio*," *PLoS One*, vol. 4, no. 2, Article ID e4498, 2009.

[21] J. P. Van Eenennaam, J. Linares-Casenave, J.-B. Muguet, and S. I. Doroshov, "Induced spawning, artificial fertilization, and egg incubation techniques for green sturgeon," *North American Journal of Aquaculture*, vol. 70, no. 4, pp. 434–445, 2008.

[22] 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*, vol. 27, no. 1, pp. 125–140, 2018.

[23] A. Mohd-Aris, M. H. N. Muhamad-sofie, M. Zamri-saad, H. M. Daud, and M. Y. Ina-Salwany, "Live vaccines against bacterial fish diseases: a review," *Vet World*, vol. 12, no. 11, pp. 1806–1815, 2019.

[24] D. J. Reasoner, "Heterotrophic plate count methodology in the United States," *International Journal of Food Microbiology*, vol. 92, no. 3, pp. 307–315, 2004.

[25] M. N. Neely, J. D. Pfeifer, and M. Caparon, "*Streptococcus*-zebrafish model of bacterial pathogenesis," *Infection and Immunity*, vol. 70, no. 7, pp. 3904–3914, 2002.

[26] M. F. Nazarudin, M. N. Shamsudin, and H. S. Universiti, "Scanning and transmission electron microscopy evaluation of the effects and efficiency of formulated *L. Lactis* cell suspended in skim milk in the presence of starch and gellan gum as excipients," *Australian Journal of Basic and Applied Sciences*, vol. 7, pp. 343–349, 2013.

[27] 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*, vol. 9, no. 4, p. 368, 2021.

[28] G. K. Iwama and P. A. Ackerman, *Anesthetics in Biochemistry and Molecular Biology of Fishes*, P. W. Hochachka and T. P. Mommsen, Eds., pp. 1–15, Elsevier, Amsterdam, Netherlands, 1994.

[29] Z. Zhang, H. Wu, J. Xiao, Q. Wang, Q. Liu, and Y. Zhang, "Immune responses of zebrafish (*Danio rerio*) induced by bath-vaccination with a live attenuated *Vibrio anguillarum* vaccine candidate," *Fish And Shellfish Immunology*, vol. 33, no. 1, pp. 36–41, 2012.

[30] Z. Zhang, H. Wu, J. Xiao, Q. Wang, Q. Liu, and Y. Zhang, "Immune responses evoked by infection with *Vibrio anguillarum* in zebrafish bath-vaccinated with a live attenuated strain," *Veterinary Immunology and Immunopathology*, vol. 154, no. 3–4, pp. 138–144, 2013.

[31] L. J. Reed and H. Muench, "A simple method of estimating fifty percent endpoints," *American Journal of Epidemiology*, vol. 27, no. 3, pp. 493–497, 1938.

[32] 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*, vol. 137, no. 3, pp. 167–173, 2020.

[33] 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*, vol. 34, no. 3, pp. 762–769, 2013.

[34] A. Akçay, "The calculation of LD_{50} using probit analysis," *The FASEB Journal*, vol. 27, no. 1, pp. 1217–1228, 2013.

[35] Y. Gao, H. Wu, Q. Wang et al., "A live attenuated combination vaccine evokes effective immune-mediated protection against *Edwardsiella tarda* and *Vibrio anguillarum*," *Vaccine*, vol. 32, no. 45, pp. 5937–5944, 2014.

[36] K. L. Roland, S. A. Tinge, K. P. Killeen, and S. K. Kochi, "Recent advances in the development of live, attenuated bacterial vectors," *Current Opinion in Molecular Therapeutics*, vol. 7, no. 1, pp. 62–72, 2005.

[37] K. Nisaa, U. C. Makassar, and S. Sukenda, "Fry tilapia (*Oreochromis niloticus*) antibody improvement against *Streptococcus agalactiae* through broodstock vaccination," *Pakistan Journal of Biotechnology*, vol. 14, pp. 9–16, 2017.

[38] P. Akbary, A. R. Mirvaghefi, M. Akhlaghi, and M. S. Fereidouni, "Influence of maternal and larval immunisation against *Lactococcus garviae* infection in rainbow trout *Oncorhynchus mykiss* (Walaum) lysozyme activity and IgM level," *Open Journal of Animal Sciences*, vol. 05, no. 03, pp. 258–269, 2015.

[39] S. Sukenda, R. Rahman, K. Nisaa, D. Hidayatullah, and A. Vinasyiam, "The efficacy of *Streptococcus agalactiae* vaccine preparations, administered to tilapia broodstock, in preventing streptococcosis in their offspring, via transfer of maternal immunity," *Aquaculture International*, vol. 26, no. 3, pp. 785–798, 2018.

[40] A. Hanif, V. Bakopoulos, and G. J. Dimitriadis, "Maternal transfer of humoral specific and non-specific immune parameters to sea bream (*Sparus aurata*) larvae," *Fish And Shellfish Immunology*, vol. 17, no. 5, pp. 411–435, 2004.

[41] N. Ye, H. Wu, and Y. Zhang, "Maternal transfer and protection role in zebrafish (*Danio rerio*) offspring following vaccination of the brood stock with a live attenuated *Vibrio anguillarum* vaccine," *Aquaculture Research*, vol. 47, no. 11, pp. 3667–3678, 2016.

[42] C.-E. A. Winslow and H. H. Walker, "The earlier phases of the bacterial culture cycle," *Bacteriological Reviews*, vol. 3, no. 2, pp. 147–186, 1939.

[43] A. Zapata, B. Diez, T. Cejalvo, C. Gutiérrez-De Frías, and A. Cortés, "Ontogeny of the immune system of fish," *Fish And Shellfish Immunology*, vol. 20, no. 2, pp. 126–136, 2006.

[44] A. Adams, "Progress, challenges and opportunities in fish vaccine development," *Fish And Shellfish Immunology*, vol. 90, pp. 210–214, 2019.

[45] X. Guo, C. Ji, X. Du et al., "Comparison of gene expression responses of zebrafish larvae to *Vibrio parahaemolyticus* infection by static immersion and caudal vein microinjection," *Aquaculture and Fisheries*, vol. 6, no. 3, pp. 267–276, 2021.

[46] M. E. Pressley, P. E. Phelan, P. Eckhard Witten, M. T. Mellon, and C. H. Kim, "Pathogenesis and inflammatory response to *Edwardsiella tarda* infection in the zebrafish," *Developmental And Comparative Immunology*, vol. 29, no. 6, pp. 501–513, 2005.

[47] R. O'Toole, J. von Hofsten, R. Rosqvist, P. Olsson, and H. Wolf-watz, "Visualisation of zebrafish infection by GFP-

labelled *Vibrio anguillarum*," *Microbial Pathogenesis*, vol. 37, no. 1, pp. 41–46, 2004.

[48] J. J. Van Soest, O. W. Stockhammer, A. Ordas, G. V. Bloemberg, H. P. Spaink, and A. H. Meijer, "Comparison of static immersion and intravenous injection systems for exposure of zebrafish embryos to the natural pathogen *Edwardsiella tarda*," *BMC Immunology*, vol. 12, no. 1, p. 58, 2011.

[49] Y. Shan, C. Fang, C. Cheng, Y. Wang, J. Peng, and W. Fang, "Immersion infection of germ-free zebrafish with Listeria monocytogenes induces transient expression of innate immune response genes," *Frontiers in Microbiology*, vol. 6, Article ID 373, 2015.

[50] K. R. Maddipati, "Non-inflammatory physiology of "inflammatory" mediators–unalamation, a new paradigm," *Frontiers in Immunology*, vol. 11, Article ID 580117, 2020.

[51] J. Watzke, K. Schirmer, and S. Scholz, "Bacterial lipopolysaccharides induce genes involved in the innate immune response in embryos of the zebrafish (*Danio rerio*)," *Fish And Shellfish Immunology*, vol. 23, no. 4, pp. 901–905, 2007.

[52] S. H. B. Oehlers, M. V. Flores, C. J. Hall et al., "Expression of zebrafish cxcl8 (interleukin-8) and its receptors during development and in response to immune stimulation," *Developmental A Comparative Immunology*, vol. 34, no. 3, pp. 352–359, 2010.

[53] T. Hasegawa, C. J. Hall, P. S. Crosier et al., "Transient inflammatory response mediated by interleukin-1β is required for proper regeneration in zebrafish fin fold," *Elife*, vol. 6, pp. e22716–e22722, 2017.

[54] L. Y. Du, H. Darroch, P. Keerthisinghe et al., "The innate immune cell response to bacterial infection in larval zebrafish is light-regulated," *Scientific Reports*, vol. 7, Article ID 12657, 2017.

[55] C. Y. Pan, T. C. Huang, Y. Wang et al., "Oral administration of recombinant epinecidin-1 protected grouper (*Epinephelus coioides*) and zebrafish (*Danio rerio*) from *Vibrio vulnificus* infection and enhanced immune-related gene expressions," *Fish & Shellfish Immunology*, vol. 32, no. 6, pp. 947–957, 2012.

[56] L. Jiang, M. Yin, J. Xu et al., "The transcription factor Bach1 suppresses the developmental angiogenesis of zebrafish," *Oxidative Medicine and Cellular Longevity*, vol. 2017, Article ID 2143875, 10 pages, 2017.

[57] N. K. Abdelkhalek, A. Komiya, Y. Kato-Unoki, T. Somamoto, and M. Nakao, "Molecular evidence for the existence of two distinct IL-8 lineages of teleost CXC-chemokines," *Fish And Shellfish Immunology*, vol. 27, pp. 763–767, 2009.

[58] S. de Oliveira, C. C. Reyes-Aldasoro, S. Candel, S. A. Renshaw, V. Mulero, and A. Calado, "Cxcl8 (IL-8) mediates neutrophil recruitment and behavior in the zebrafish inflammatory response," *The Journal of Immunology*, vol. 190, no. 8, pp. 4349–4359, 2013.