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Research Article

Stability and Efficacy of Live-Attenuated *Vibrio harveyi* Vaccines Under Different Storage Conditions in Zebrafish (*Danio rerio*) Models

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Abstract

Vibriosis poses a significant threat to marine teleosts, causing substantial losses in the global aquaculture industry. Previous work in our lab led to the development of a live-attenuated *V. harveyi* vaccine (LAVh) candidate that targets the serine endoprotease gene with a three-point knockout and has shown promise in protecting against vibriosis. However, further investigation is necessary to evaluate the stability and efficacy of its various storage conditions for broader applications. This study aims to determine how well the three different LAVh vaccine storage (fresh, stale, and freeze-dried LAVh) worked against vibriosis. A total of 1000 adult zebrafish (*Danio rerio*) (mean weight: 0.20±0.5 g) were divided into four groups. Groups 1, 2, and 3 were intraperitoneally injected with different LAVh vaccine storage (fresh, stale, and freeze-dried, respectively), while Group 4 received 0.01 M phosphate-buffered saline (PBS) and served as the unvaccinated control. Fish were monitored for 21 days post-vaccination for safety, stability, efficacy, and antibody analysis. The results showed that a modest dosage of 1 x 10^4 CFU/mL of LAVh vaccine from all storage conditions provided 80% survival upon intraperitoneal challenge with pathogenic strains of pathogenic *V. harveyi, V. alginolyticus,* and *V. parahaemolyticus*. This dosage induced significant antibody production and conferred cross-protection against different *Vibrio* spp., indicating the LAVh vaccine's potential for commercial application. The LAVh vaccine demonstrated high effectiveness and suitability for storage as a freeze-dried powder. This study might offer significant insights into practical strategies for reducing vibriosis, especially in aquaculture settings with limited infrastructure.

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1. Introduction

By the end of this century, the human global population is projected to exceed 10 billion. Simultaneously, addressing the protein needs of such a substantial population has emerged as a significant concern. Aquaculture is considered a crucial solution for addressing future human protein needs because of its rapid growth and significant production and trade volume (Yang *et al*., 2024) Notably, in 2022, the worldwide production of aquaculture reached 130.9 million tonnes, with a value of USD 312.8 billion. This value accounted for 59 percent of the total fisheries and aquaculture production globally, with aquaculture surpassing capture fisheries for the first time in aquatic animal production (94.4 million tonnes). This value represented 51 percent of the total global production and a record-breaking 57 percent of the production intended for human consumption (FAO, 2024).

Although aquaculture production is increasing, diseases in aquaculture pose a possible limitation on productivity. Pathogenic bacteria and other diseases have hindered the effectiveness of fish and other aquatic animal cultures. For example, the occurrence of vibriosis, a bacterial disease caused by *Vibrio* species infection, resulted in a substantial increase in fish and shrimp death rates and negatively impacted productivity in aquaculture. *Vibrio* spp. are a group of bacteria frequently present in water environments and can flourish in various saltiness and temperatures. Their capacity to adapt has enabled them to thrive in several ecological niches, including artificial environments like wastewater and aquaculture systems (Triga *et al*., 2024). *Vibrios* are considered opportunistic pathogens since the bacteria mainly cause severe infections in immunosuppressed hosts or under environmental stress. Additionally, their position as primary pathogens has received increased attention in recent years, partly due to climate change's effects (Zupičić *et al*., 2024)

Notably, one *Vibrio* species, *Vibrio harveyi*, has become a prevalent and significant disease for cultured marine species (Zhou *et al*., 2024). *V. harveyi* is a member of the Vibrionaceae family and is commonly found in natural water environments. The harveyi clade consists of *V. harveyi* and 11 additional species with a close evolutionary relationship. The other species include *Vibrio rotiferianus, V. parahaemolyticus*, *V. azureus*, *V. sagamiensis*, *V. campbellii*, *V. owensii*, *V. jasicida*, *V. alginolyticus*, *V. natriegens*, and *V. mytili* (Zupičić *et al*., 2024). *V. harveyi* infection can cause significant mortality in many fish species, frequently accompanied by surface sores and tail erosion. Thus far, many types of teleosts have been documented as being impacted by *V. harveyi* (Zhou *et al*., 2024).

The use of antibiotics in aquaculture for disease treatment has resulted in a rise in bacterial resistance to drugs and the presence of drug residues, which may have implications for the industry, environment, and human health. The fundamental approach to enhancing production efficiency is the development of efficient disease prevention and control technologies. Gaining a comprehensive understanding of the response mechanisms that lead to disease resistance is crucial for advancing and implementing novel disease management strategies, such as developing vaccines that exhibit protection against disease infection (Wang *et al*., 2024).

Vaccination is a non-antibiotic method used to reduce the spread of infectious diseases in aquaculture. Various types of *Vibrio* vaccines for Asian seabass have been created, ranging from inactivated whole-cell vaccines to subunit and DNA vaccines (Lan *et al*., 2024). Formalin-inactivated vaccines, DNA vaccines, subunit vaccines, and live attenuated vaccines (LAV) were commonly examined in research related to fish farming. The live attenuated vaccine (LAV) has several advantages over other vaccine types. Firstly, it confers robust and durable protection without requiring adjuvants. Secondly, it efficiently stimulates both non-specific and specific immune responses. Lastly, it is easily administered in fish farming operations (Wan *et al*., 2024).

Previously, a live attenuated vaccine candidate against vibriosis has been developed by Tan *et al*. (2022). They successfully developed a live attenuated vaccine candidate against vibriosis by creating a ΔVAGM003125 mutant strain. This vaccine elicited a robust immune response, as evidenced by significantly elevated antibody titers and an effective protection rate, with a relative survival rate of 81% in vaccinated subjects. Importantly, this protective effect was achieved without any observable histopathological abnormalities, underscoring the vaccine's safety and efficacy. In a separate study, Pang *et al.* (2022) reported on the development of a HY9901ΔvscB mutant strain vaccine, which conferred a relative percent survival (RPS) of 77.6% following challenge with the wild-type *Vibrio alginolyticus* strain HY9901. Real-time quantitative PCR (qPCR) assays revealed that vaccination with HY9901ΔvscB significantly upregulated the expression of key immune-related genes, including MHC-I, IgM, and CD8α, in both the liver and spleen. These findings suggest that the HY9901ΔvscB vaccine induces a strong humoral immune response and enhances cell-mediated immunity in pearl gentian grouper.

Nevertheless, the challenges are in ensuring fish immunity and preventing the reversion of bacterial pathogens when employing the live-attenuated vaccine. Besides, when managing live attenuated vaccines, storing them at low temperatures is necessary to maintain their viability. Therefore, it is crucial to carefully consider sufficient storage while shipping and delivering vaccinations over extended distances (Ben Hamed *et al*., 2021). Even so, the inherent volatility of these vaccines can be reduced by utilizing freeze-drying procedures during their production.

This study utilized zebrafish (*Danio rerio*) as an experimental model to investigate the immunological responses, including those related to vibriosis, following the administration of a live-attenuated *Vibrio harveyi* (LAVh) vaccine (Ye *et al*., 2016). The study further evaluated the efficacy of LAVh vaccines stored under different conditions (fresh, stale, and freeze-dried) against various *Vibrio* strains in zebrafish. The findings may provide valuable insights into practical strategies for mitigating vibriosis, particularly in aquaculture systems with limited infrastructure.

2. Materials and Methods

2.1 Materials

For bacterial culture, an orbital shaker incubator (Thermo-Fisher Scientific, Massachusetts, USA) was used to agitate cultures at 150 rpm for 12 hours at 30°C in tryptic soy broth (TSB; Oxoid, Hampshire, UK) with the addition of 1.5% NaCl (Merck, Darmstadt, Germany). After incubation, bacterial cells were pelleted using a refrigerated centrifuge (Eppendorf 5810R, Hamburg, Germany) at 8000 × g for 10 minutes at 4°C. Meanwhile, for vaccine storage, the vaccine was kept at 4°C for 24 hours using an incubator (Major Science, CA, USA), while freeze-drying of the vaccine was conducted using a Freeze Dry System FreeZone 12 (Labconco, Japan) for optimal preservation. In addition, for antibody analysis, samples were measured with a microplate reader (Thermo-Fisher Scientific, Massachusetts, USA) at a wavelength of 450 nm to quantify antibody binding and concentration levels accurately.

2.1.1 Ethical approval

The use of bacteria and fish hosts was authorized by the regulations established by the Department of Biosafety, Ministry of Natural Resources and Environment, Malaysia, under the approval number JBK(S)602-1/2/136(6). The Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (UPM) has approved all animal-related operations (with approval number UPM/IACUC/AUPR059/2016).

2.2 Fish

A total of 1000 adult zebrafish (*D. rerio*) were acquired from Sanwa Aquatics Sdn. Bhd., Kuala Lumpur, Malaysia. The zebrafish had an average body length of 2.9 ± 0.1 cm and a 0.20 ± 0.05 g weight. Subsequently, the fish were relocated to the Fish Hatchery Unit, Laboratory of Aquatic Animal Health and Therapeutics (AquaHealth), Institute of Bioscience (IBS) at Universiti Putra Malaysia in Selangor, Malaysia. The fish were maintained for seven days of acclimation in controlled laboratories. Afterward, 10 fish were chosen randomly and examined for bacterial infections using tryptic soy agar (TSA, Oxoid, Hampshire, England), following the procedure outlined by Mohamad *et al.* (2021). Throughout the experiment, the fish were kept in a freshwater supply system with a salinity of 0 ppt, both throughout the acclimatization phase and after that. The temperature was controlled within a narrow range of 27 to 28°C, dissolved oxygen (DO) levels were maintained between 5.82 and 6.67 mg/L, pH was checked and held between 7.21 and 7.74, and ammonia-nitrogen levels were kept below 0.01 mg/L. The fish were given sufficient commercial feed (Sanyu Ichiban, C.S.L. Thean Yeang Aquarium (M) Sdn. Bhd., Malaysia) twice daily, from 8:00 a.m. to 5:00 p.m., and fed until satiation.

2.3 Bacteria

Vibrio harveyi, *V. alginolyticus*, and *V. parahaemolyticus,* which are disease-causing strains, were obtained from the laboratory stock and were cultured accordingly following the methodology described by Mohd-Aris *et al.* (2019) (Table 1). The bacterial strains were initially cultivated on thiosulfate-citrate-bile salts-sucrose agar (TCBS; Oxoid, Hampshire, UK) and placed in an incubator (Major Science, CA, USA) at 30°C for 12 hours. Afterward, 10 colonies were chosen and sub-cultured into 10 mL of tryptic soy broth (TSB; Oxoid, Hampshire, UK) with the addition of 1.5% NaCl (w/v). The culture was subsequently cultivated at a temperature of 30°C with agitation (Thermo-Fisher Scientific, USA) at a speed of 150 rpm for an additional duration of 12 hours. Subsequently, the bacterial cultures underwent centrifugation (Eppendorf, Hamburg, Germany) at 8000 x g for 10 minutes at a temperature of 4°C, and the liquid portion above the sediment was removed. The cell pellets obtained were subjected to three washes using sterile 0.01 M phosphate-buffered saline (PBS; R&M Chemicals, Malaysia) and subsequently resuspended in 10 mL of the same buffer. To determine the bacterial concentration, the working culture was subjected to a series of

tenfold serial dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10}) in 1 mL centrifuge tubes (Merck, Darmstadt, Germany). From each dilution, 100 µL of the diluted culture was transferred onto TCBS agar containing 1.5% NaCl (w/v) and spread using a glass spreader. The plates (Sigma-Aldrich, Inc., Missouri, USA) were then incubated under the previously specified conditions. After the incubation period, bacterial colonies were counted using the plate count technique. The concentration of colony-forming units (CFU/mL) was calculated using the equation:

Concentration of colony-forming units (CFU/mL) =

(Number of colonies × dilution factor)/(Volume of culture plated) × 100.....(i)

Based on the total bacterial concentration, the working culture was subsequently diluted to the desired concentration for use in the study. Bacterial identification was performed by using PCR (2X PCR Mastermix, 1st BASE, Malaysia) with specified primers (Table 2). The PCR samples were subsequently analyzed by First BASE Laboratories Sdn. Bhd., Selangor, Malaysia, for DNA sequencing using the Sanger technique.

2.4 Storage Conditions of Live-Attenuated Vibrio harveyi Vaccine

The live-attenuated *Vibrio harveyi* vaccine was previously developed in our laboratory (Mohd-Aris *et al.,* 2019). In brief, the disease-causing *V. harveyi* strain Vh1 was combined with a modified *E. coli* SM10λpir containing the genetic modification plasmid. The colonies that exhibited resistance to chloramphenicol (CmR) and carried the *vhs* gene with the catalytic triad removed were examined, cultivated again, and identified as the live-attenuated *Vibrio harveyi* strain MVH-*vhs* (LAVh) vaccine. The live-attenuated *V. harveyi* bacterium was confirmed by conducting DNA sequencing, which revealed deletions at three specific places (D153, H123, and S228) on the *vhs* gene. These deletions differed from the pathogenic strain of *V. harveyi*, as shown in Table 3. Like the other bacterial strains in this investigation, the LAVh strain was cultivated in tryptic soy broth (TSB; Oxoid, Hampshire, UK) with the addition of 1.5% NaCl (w/v) and incubated at 30°C with agitation at 150 rpm for a duration of 12 hours. Following incubation, the bacterial cultures underwent centrifugation at 8000 x g under 4°C, and the liquid portion above the sediment was removed. The cell pellets were rinsed thrice with sterile 0.01 M phosphate-buffered saline (PBS) and reconstituted in 10 mL of the same buffer. Afterward, the bacterial suspension was spread over TCBS agar with 1.5% NaCl (w/v) to achieve concentrations ranging from 10^1 to 10^7 CFU/mL. The LAVh vaccinations were then classified based on their condition of preservation, as indicated in Table 4. The freshly prepared LAVh vaccine was administered within 24 hours after incubation, whereas the stale LAVh vaccine was kept at a temperature of 4°C for 24 hours before being utilized. Meanwhile, the freeze-dried LAVh vaccine was subjected to a freeze-drying procedure according to the methodology outlined by Nazarudin *et al.* (2013), and before usage, the freeze-dried vaccine was reconstituted with 0.01 M PBS at room temperature.

2.5 Vibrio spp. *Median Lethal Dose (LD_{50}) in Zebrafish*

A total of 405 zebrafish (*D. rerio*) were subjected to LD_{50} determination by challenging them against three distinct bacterial species (*Vibrio harveyi*, *V. alginolyticus,* and *V. parahaemolyticus*) at three different dosages (10^5, 10^6, and 10^7 CFU/mL) in triplicate. In addition, 45 fish were administered with 0.01 M PBS as an unchallenged control by injection. Before injection, all fish were rendered unconscious using tricaine methanesulfonate (MS-222; Sigma Aldrich, St. Louis, MO, USA) at a dosage of 100 mg/L. The fish were subsequently administered an intraperitoneal injection of either the bacterial suspension or PBS using a sterile Terumo® 1.0 cc/mL hypodermic syringe equipped with a 27 G x ½" needle (Terumo Corporation, Philippines) with an injection volume of 10 µL. Every group was kept in a 25-liter tank and observed daily for any deaths or vibriosis infection symptoms. The fish were given a daily meal equivalent to 2% of their body weight. The tanks were aerated continuously for 144 hours without any water exchange. Excess feed and waste were regularly removed by siphoning. The moribund fish were analysed using bacterial re-isolation and identification with PCR using specified primers (Table 2) to verify the cause of death.

2.6 Vaccination Regiment and Safety Assessment

A total of 400 zebrafish were evenly divided among four groups, with 100 fish in each group. The fish in Groups 1, 2, and 3 were given different storage conditions of live-attenuated *V. harveyi* strain MVH-*vhs* (LAVh) vaccinations: fresh LAVh, stale LAVh, and freeze-dried LAVh vaccines, respectively. The fish in Group 4 were assigned as non-vaccinated control, receiving 0.01 M PBS during the vaccination period. The fish in Groups 1, 2, and 3 were immunized with 10 *µL* of 1 x 10^6 CFU/mL of the LAVh vaccines, specific to their assigned vaccine storage conditions group in triplicate, adhering to the suggested vaccination dose by Mohd-Aris *et al.* (2019). On the other hand, the

control group, which did not receive the vaccine, was administered with a 10 *µL* injection of 0.01 M PBS (Table 4). The fish were fed twice daily and kept in a 25-litre tank with continuous aeration and salinity of 0 ppt. The experiment lasted for 28 days, during which, every week, two fish and water samples were collected from each group. Before sampling, the fish were sedated with MS-22 at 100 mg/L. Subsequently, the fish were dissected to examine the abdominal cavity for abnormalities or pathological conditions. Afterward, a sterile cotton swab was employed to isolate the bacterial colonies from the fish kidney. Subsequently, the swabs were streaked onto a TCBS agar plate to facilitate the growth of individual colonies. The plates were then incubated at 30°C for 12 hours. The development of LAVh was seen as small, round, and cream-colored colonies. A colony was randomly chosen and then underwent species confirmation using PCR with the specified primers listed in Table 2.

2.7 ELISA Antibody Analysis

Fourteen days after vaccination, five fish from each vaccinated and non-vaccinated group were selected for serum ELISA antibody analysis using the optimized methods outlined in a recent study by Mohamad *et al.* (2022). The microtiter plate wells (Techno Plastic Product, Trasadingen, Swtizerland) were individually covered with 100 µL of *Vibrio harveyi, V. alginolyticus,* and *V. parahaemolyticus* antigens. The plate was then left to incubate (Major Science, CA, USA) overnight at a temperature of 4°C. Excess cells were removed, and the wells were blocked with 100 µL of PBS containing 1% bovine serum albumin (BSA; Sigma-Aldrich, Inc., Missouri, USA) for 1 hour at 25°C. Following removing the blocking solution, the wells were washed three times with a washing buffer (PBS with 0.05% Tween-20) (R&M Chemicals, Malaysia). Subsequently, the wells were incubated at 25°C for 1 hour with 100 µL of diluted fish serum (1:300) (Aquatic Diagnostics Ltd., Scotland, UK). The detection of

Table 1. Bacterial strains used in this study.

***Vibrio* spp.**	**Relevant characteristic**	**Source**	**Reference**
Vibrio harveyi strain Vh1	Complete serine endoprotease gene (*vhs*)	Laboratory collection	(Mohd-Aris *et al.,* 2019)
Vibrio harveyi strain MVh-vhs	3-base deletion of DNA sequence on deficit serine endoprotease gene (*vhs*)	Laboratory collection	(Mohd-Aris *et al.,* 2019)
Vibrio alginolyticus strain Va2	A strain of *V. alginolyticus*	Laboratory collection	(Nehlah *et al.,* 2017)
Vibrio parahaemolyticus strain FORC_008	A strain of *V. parahaemolyticus*	Laboratory collection	(Mohd-Aris *et al.,* 2019)

Table 2. Primers used for species identification of different *Vibrio* spp.

Primers	**Primer sequence (5'–3')**	**Tm (°C)**	**Size (bp)**	**Reference**
Serine protease (*vhs*)	F:ATGAAAAAACCATTGCTTGCGTTAAC R:TTAGCGGATAACGAGGTAAACCG	61.0	1368	(Mohd-Aris *et al.,* 2019)
Gyrase sub-unit B (gyrB)	F:GAGAACCCGACAGAAGCGAAG R:CCTAGTGCGGTGATCAGTGTTG	51.0	332	(Mohd-Aris *et al.,* 2019)

Table 3. Deletion bases in catalytic sites of serine proteases gene (vhs).

Target bases for deletion	**Gene sequence (5' to 3')**
Serine (S228)	CAATTAACAGTGGTAACtccGGTGGCGCTT
Histidine (H123)	ATCGTAACGAACTATcacGTTATCAAAGGCGC
Aspartate (D153)	GAGACGAGATGTCAgacATTGCCTTGCTTAAG

antibody binding to the antigen was performed (after washing the wells thrice with washing buffer) by using 100 µL of monoclonal antibody against zebrafish IgM (Aquatic Diagnostics Ltd., Scotland, UK). The antibody was diluted at a ratio of 1:1000 and incubated for 2 hours at a temperature of 25°C. Subsequently, after washing the wells thrice with washing buffer, 100 µL of conjugated rabbit anti-goat IgM-horseradish peroxidase (Nordic Immunological Laboratories, Susteren, Netherlands) (diluted at a ratio of 1:1000) was added into the wells and incubated for 1.5 hours at 25°C. The color was generated by adding 100 µL of the chromogenic reagent TMB (Sigma-Aldrich, Inc., Missouri, USA) and allowing it to react for 30 minutes. The reaction was then halted by adding 50 µL of 0.16 M sulfuric acid (H_2SO_4) (R&M Chemicals, Malaysia). The plates were subsequently analysed at a wavelength of 450 nm using a microplate reader (Thermo-Fisher Scientific Massachusetts, USA.

Cumulative percent of mortality (CPM) = (total number of fish mortality)/(total number of fish) × 100.....(ii)

Relative percent of survival (RPS) = 1-(average CPM in the treatment group)/(average CPM in the control group) × 100.....(iii)

The median effective dose for 80% survival (ED_{80}) was established after determining the relative percent survival, followed by a probit regression analysis.

2.9 Analysis Data

Statistical analyses were conducted using PSS 25.0 (SPSS Inc., USA). The probit regression analysis approach following Pillai *et al.* (2021) was used to determine the LD_{50} and ED_{80} values of *Vibrio* spp. and the vaccine treatments, respectively. Using ANOVA and Tukey's HSD for multiple comparisons,

Table 4. Vaccination and treatment groups

Group	Designated	Description
1	Fresh LAVh	The live attenuated *Vibrio harveyi* vaccine (LAVh) that were used within 24 hours after it had been freshly prepared. Denotes the industry's capacity to produce vaccines at the location of production.
2	Stale LAVh	The live attenuated *Vibrio harveyi* vaccine (LAVh) that has been prepped for 24 hours and stored at a temperature of 4°C before use. Denotes the procurement and delivery of vaccines.
3	Freeze-dried LAVh	The live attenuated *Vibrio harveyi* vaccine (LAVh) that has undergone freeze-drying and was stored at a room temperature of 25°C for a duration of 1 month before its utilization. Denotes the process of storing and transporting goods over extended periods in various industries.
4	0.01M PBS, 25°C (Control)	Depict the control group that has not received vaccinations.

2.8 Vaccine Effective Dose of 80% Survival by Challenge Test Against Multiple Vibrio spp.

After 15 days post-vaccination, the fish were given 10 µL of the bacterial pathogens from *Vibrio harveyi, V. alginolyticus,* and *V. parahaemolyticus* according to their LD_{50} dosage (refer to 2.4) by intraperitoneal injection. The fish were then monitored for 144 hours in a 25-litre tank with 24-hour aeration and salinity set at 0 ppt. The fish were fed twice daily, equivalent to 2% of their body weight. The sick and dead fish were removed and documented. After that, Reed and Muench's (1938) formula was used as follows to determine the relative percentage of survival (RPS) of each vaccination group:

the survival rates and post-vaccination antibody levels were compared in all groups. Statistical significance was determined when the *p*-value was less than 0.05.

3. Results and Discussion

3.1 Results

3.1.1 Lethal dose of Vibrio spp.

The zebrafish were infected with three distinct bacterial species (*Vibrio harveyi*, *V. alginolyticus*, and *V. parahaemolyticus*) at three different dosages (10^5, 10^6, and 10^7 CFU/mL) using the caudal vein injection technique. Probit regression analysis with 95% confidence intervals was used to determine the LD_{50}

values of *Vibrio alginolyticus, V. parahaemolyticus* and *V. harveyi* in zebrafish. The results showed that 100% fish mortality was observed at 120 hours post-infection (hpi) with 10^7 CFU/ml of all tested bacteria. Lower bacterial concentrations at 10^5 and 10^6 CFU/ml did not lead to 100% mortality in the challenged fish. In *V. parahaemolyticus*, the mortality was lower at 10^5 and 10^6 CFU/ml compared to other tested bacteria, with the maximum mortality at 13.3%. Figures 1A–1C present the Kaplan-Meier survival curves illustrating these outcomes. Based on the probit regression analysis, the LD_{50} for *V. harveyi* and *V. parahaemolyticus* was determined at 1 x 10^6 CFU/mL, while *V. alginolyticus* was determined at 1 x 10^5 CFU/mL.

3.1.3 Antibody level detection

Results show that there was a significant difference ($p < 0.05$) in the expression of antibody levels against the *V. alginolyticus* antigen two weeks after vaccination with 1 x 10^6 and 1 x 10^5 CFU/mL of LAVh vaccine in all tested storage conditions compared with the control unvaccinated group (Figure 3A). The groups vaccinated with fresh and stale LAVh had the highest antibody levels, followed by the ones vaccinated with freeze-dried LAVh. However, at a 1 x 10^4 CFU/mL vaccination dosage of the LAVh vaccine, both freeze-dried and non-vaccinated controls performed similarly with similar antibody levels.

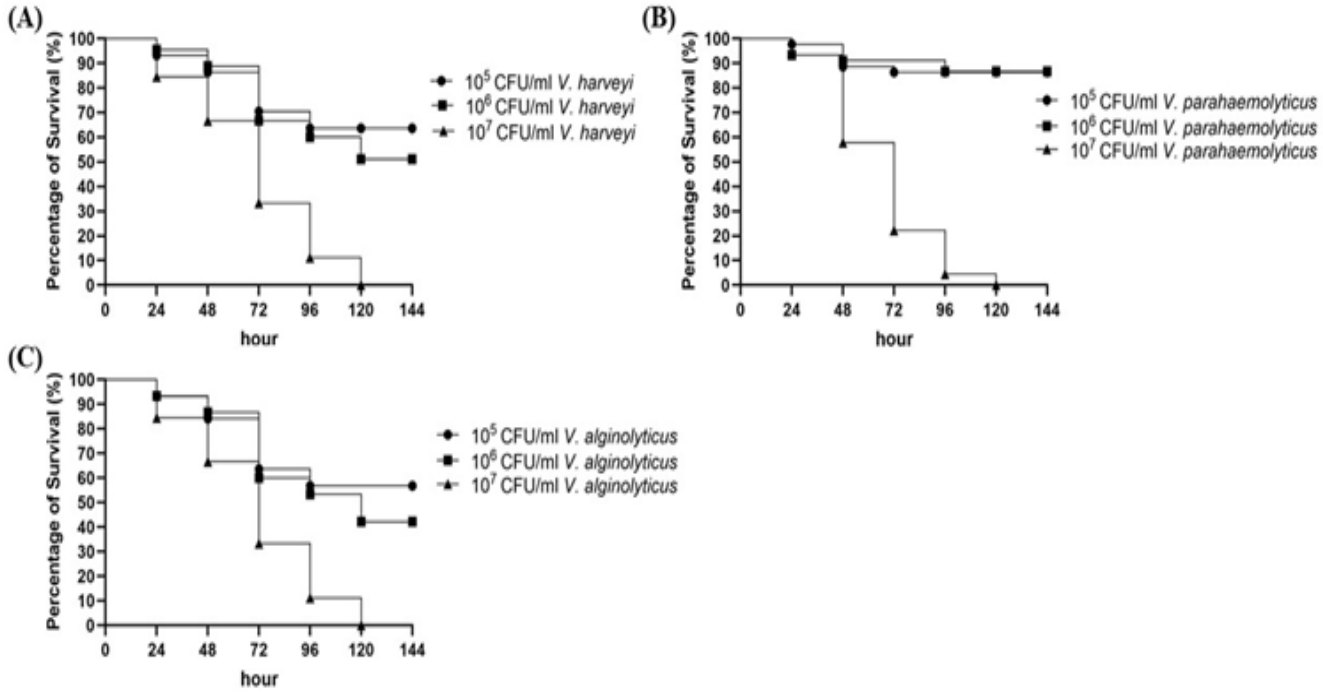

Figure 1. LD50 estimation with Kaplan-Meier survival curves of zebrafish, *Danio rerio* (n=45) against *Vibrio alginolyticus* (A), *V. parahaemolyticus* (B) and *V. harveyi* (C). Fish were intraperitoneally (i.p.) injected with 10 µL of bacteria for each dosage and monitored for 144 hours.

3.1.2 Vaccine stability and clearance period

No mortality was detected in the vaccinated or the unvaccinated group, with fish in all groups showing no unusual behavior or symptoms. The water samples collected for this investigation showed no indication of the LAVh vaccine leaking from the host (Table 5). On the other hand, the host showed the presence of LAVh through PCR detection from day 0 post-vaccination (Figure 2) until day 14 post-vaccination before being cleared off. No LAVh were detected in the fish host at 21 and 28 days post-vaccination (Table 5).

In contrast, antibody levels against *V. parahaemolyticus* were significantly higher in all vacci nated groups at all tested dosages than in the unvaccinated control group (Figure 3B). At a vaccination dosage of 1 x 10^4 CFU/mL, there were no significant differences ($p < 0.05$) in the expression of antibody levels between all LAVh storage conditions. The stale vaccine displayed a significant disparity ($p < 0.05$) in antibody levels at a vaccination dosage of 1 x 10^5 CFU/mL ($p < 0.01$) compared to other vaccine storage conditions and unvaccinated controls, while fresh and

freeze-dried LAVh vaccines showed no significant differences ($p > 0.05$). Notably, at a vaccination dosage of 1 x 10^6 CFU/mL, freeze-dried vaccines performed similarly with fresh and stale freeze-dried LAVh vaccines, with likely similar antibody levels ($p > 0.05$). doses showed significantly higher expression against the non-vaccinated control ($p < 0.05$). The highest antibody levels were observed in the fresh and stale LAVh at 1 x 10^4 and 1 x 10^5 CFU/mL of the LAVh vaccine. However, at a 1 x 10^6 CFU/mL vaccine dosage,

Table 5. The detection of LAVh in samples taken from both the fish host and water. The absence of growth of LAVh on the TCBS agar plate is indicated by (-), whereas the presence of growth of LAVh on the TCBS agar plate is indicated by (+).

Day	Live-attenuated *V. harveyi* isolation							
	Water sample				Fish host sample			
	Fresh LAVh	Stale LAVh	Freeze-dried LAVh	Unvaccinated control (PBS)	Fresh LAVh	Stale LAVh	Freeze-dried LAVh	Unvaccinated control (PBS)
0	-	-	-	-	+	+	+	-
7	-	-	-	-	+	+	+	-
14	-	-	-	-	+	+	+	-
21	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-

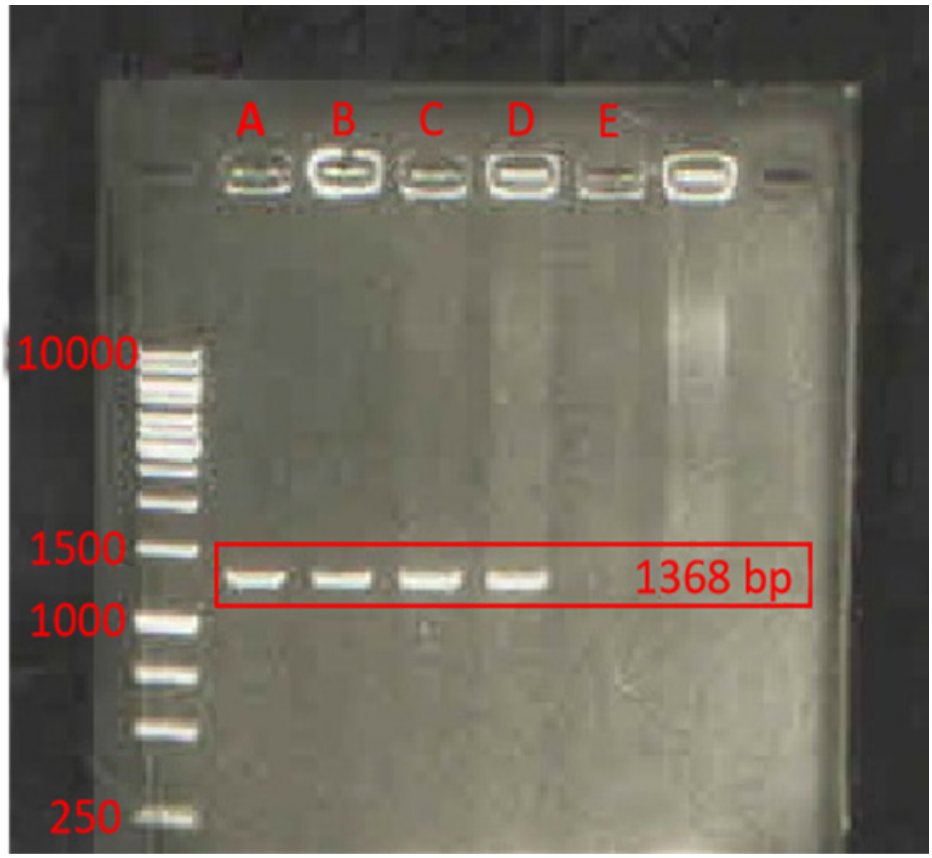

Figure 2. The identification of the vhs gene in colonies obtained from swabs taken from the peritoneal cavity of zebrafish 24h after vaccination on day 0. A) Positive control (LAVh colonies), B) fresh LAVh, C) stale LAVh, D) freeze-dried LAVh, and E) Unvaccinated control (PBS).

Regarding antibody levels in the fish serum against the *V. harveyi* antigen, all three vaccination stale LAVh showed the highest antibody, followed by freeze-dried and fresh vaccines.

3.1.4 LAVh effective dose of 80% survival (ED_{80})

The relative percent survival of LAVh-vaccinated adult zebrafish was determined post-challenged with different *Vibrio* species and the vaccine's ED_{80} were evaluated according to the vaccine's storage conditions. In particular, the group that received the fresh deaths were observed when using a vaccine dose of 1 x 10^6 CFU/mL against *V. alginolyticus* and 1 x 10^5 CFU/mL against *V. harveyi*. Both vaccine doses provide complete protection against *V. parahaemolyticus*.

Similar to other LAVh vaccine storage conditions, the group that received freeze-dried LAVh vaccine had the lowest survival rate when given a vaccine dose of 1 x 10^4 CFU/mL. Specifically, they had survival rates of 70%, 66.7%, and 76.7% against *V. alginolyticus*, *V. parahaemolyticus*, and *V. harveyi*, respectively. In contrast, no deaths were observed when exposed to *V. harveyi* at vaccine concentrations of 1 x 10^5 CFU/mL and 1 x 10^6 CFU/mL. Nevertheless, the dose required to achieve 80% survival (ED_{80}) was determined to be 1 x 10^4 CFU/mL through Probit regression analysis.

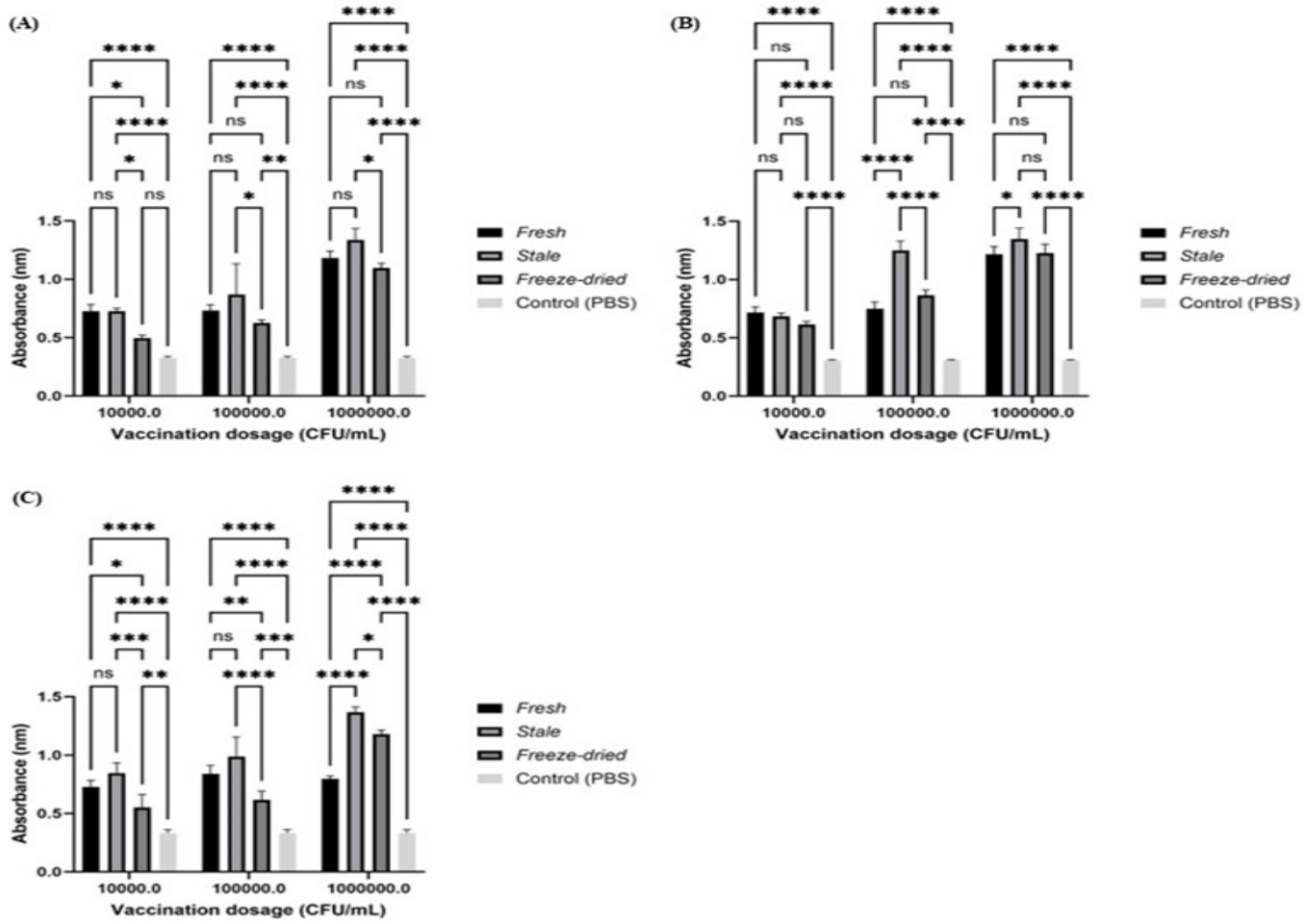

Figure 3. The serum antibody levels of zebrafish against antigens from (A) *V. alginolyticus*, (B) *V. parahaemolyticus*, and (C) *V. harveyi*, two weeks after vaccination. Every treatment was conducted with 5 fish per treatment. Only the upper half of the standard deviation bars is displayed. The asterisks denote statistically significant differences: *($p < 0.05$), **($p < 0.01$), ***($p < 0.001$), and ****($p < 0.0001$), between each treatment.

LAVh vaccine had the lowest overall survival rate at a vaccine dose of 1 x 10^4 CFU/mL, with survival rates of 70%, 76.7%, and 96.7% against *V. alginolyticus*, *V. parahaemolyticus*, and *V. harveyi*, respectively. Notably, no deaths were observed at a vaccine dose of 1 x 10^6 CFU/mL against *V. alginolyticus* and *V. parahaemolyticus*. However, only one death was observed against *V. harveyi* (Table 6).

Likewise, the group of stale LAVh-vaccinated fish had the lowest survival rate at a vaccine dose of 1 x 10^4 CFU/mL. Specifically, they exhibited survival rates of 60%, 60%, and 90% against *V. alginolyticus*, *V. parahaemolyticus*, and *V. harveyi*, respectively. No

3.2 Discussion

Zebrafish are an appropriate animal model for

investigating infection and immune responses. Researchers have extensively utilized zebrafish to study functional biology, pathogenicity, oncology, and the development of drugs and vaccines (Bailone *et al.*, 2020). Therefore, the fish were deemed the most suitable model for investigating aquatic diseases due to their rapid reproductive cycle, low maintenance requirements, and small physical stature (Jørgensen, 2020). Several studies have investigated the LD_{50} of pathogenic *Vibrio* spp. in various fish species, including zebrafish. In our study, the LD50 for *Vibrio alginolyticus* in zebrafish was determined to be 1×10^5 CFU/mL, consistent with the findings of Zhang *et al.* (2022). using the *V. alginolyticus* strain HY9901. Similarly, our research determined an LD_{50} of 1×10^6 CFU/mL for *V. parahaemolyticus*, aligning with the LD_{50} reported by Vinoj *et al.* (2015). Additionally, we found the LD_{50} of *V. harveyi* in adult zebrafish to be 1×10^6 CFU/mL, which differs from the LD_{50} of 4.31×10^5 CFU/fish reported by Huang *et al.* (2022) for zebrafish.

Table 6. LAVh effective dose of 80% survival (ED80)

Group	Vaccine dose (CFU/mL)	Relative percent of survival (%)			ED_{80} (CFU/mL)
		V. alginolyticus	*V. parahaemolyticus*	*V. harveyi*	
Fresh LAVh	$1x 10^6$	100.0	100.0	93.4	$1 x 10^4$
	$1 x 10^5$	78.7	78.7	100.0	
	$1 x 10^4$	36.2	51.1	93.4	
Stale LAVh	$1x 10^6$	100.0	100.0	86.6	$1 x 10^4$
	$1 x 10^5$	29.8	100.0	100.0	
	$1 x 10^4$	14.9	78.7	80.0	
Freeze-dried LAVh	$1x 10^6$	100.0	100.0	100.0	$1 x 10^4$
	$1 x 10^5$	78.7	78.7	100.0	
	$1 x 10^4$	36.2	29.9	54.0	
Unvaccinated control	0.01M PBS	0.0	0.0	0.0	

These discrepancies in LD_{50} values likely result from variations in the *Vibrio* strains used, as well as differences in the size and culture conditions of the host. The LD_{50} values determined in this study were subsequently used in experimental challenges to assess the effective dose and efficacy of the LAVh vaccine under different storage conditions.

Teleosts, such as zebrafish, can reliably receive small doses of vaccine through the intraperitoneal route, effectively protecting the host for a prolonged period (Du *et al.*, 2022). Administering small amounts of LAVh would guarantee minimal environmental effects and prevent the transfer of genes between organisms. Compared to studies utilizing immersion delivery, administering the substance directly into the peritoneal cavity of the host at a high concentration is likely to entail certain risks and result in increased mortality (Mondal and Thomas, 2022). While it is desirable to have a strong antibody response following vaccination, a high number of bacteria in the host can have negative consequences over time. An optimal approach would involve adjusting the concentration of the vaccine concerning the production of antibodies, aiming to achieve a sufficient amount of antibodies capable of effectively countering the highlighted risks. Prior to its assessment of commercial marine fish, the zebrafish model played a pivotal role in producing empirical data for vaccine development (Smyrli *et al.*, 2022).

Live-attenuated vaccines preserve the functional properties of the antigen, allowing it to express complete protein markers and identification signals without causing illness in the host (Laith *et al.*, 2019; Liu *et al.*, 2018). The present investigation involved the creation of a genetically altered variant called LAVh by deliberately removing its serine protease (*vhs*) gene. This gene is responsible for the occurrence of hemorrhagic septicemia in hosts that are exposed by the pathogen. The deliberate deletion was intended to specifically affect the catalytic triad site of the serine protease gene, resulting in the LAVh strain becoming a potent vaccine for triggering a cell-mediated immune response (Chin *et al.*, 2020; Mohd-Aris *et al.*, 2019).

In this study, we focused on examining three storage conditions of the LAVh vaccines to enhance the live-attenuated vaccine's reliability. Specifically,

the goal was to improve its shelf life and durability during transportation from vaccine processing plants to rural farms. By assessing the impact of extended storage on the capacity to provide immunological defense, the modified versions of LAVh vaccines can be utilized in remote regions with limited access while still maintaining the ability to offer biological protection against *Vibrio* spp. infection. The vaccination dosage range was selected based on meticulous consideration of previous studies conducted on LAVh by Mohd-Aris *et al.* (2019) and Chin *et al.* (2020). The studies concluded that the ideal range for achieving host safety and effective antibody production through intraperitoneal injection was between 1 x 10^4 and 1 x 10^6 CFU/mL. It was noted that a higher concentration of 1 x 10^8 CFU/mL resulted in the host's death, whereas lower concentrations reduced the effectiveness of protection. Furthermore, the efficacy of the live-attenuated vaccine administration can be impacted by several factors, including the type of fish, its size, age, and the temperature during vaccination (Chin *et al.*, 2020; Mohd-Aris *et al.*, 2019; Soto *et al.*, 2014).

Hosts that received live-attenuated vaccines might endure a regulated, mild infection, which enhanced their ability to recognize antigens and produce antibodies. Notably, live-attenuated vaccines might have inherent risks, such as the potential to induce systemic symptoms and cause mild infections in the host (Mondal and Thomas, 2022). In addition, live attenuated vaccines possess the capacity to release a live strain of the vaccine into the surrounding environment. One of the crucial factors to consider is the existence of bacteria in the natural aquatic ecosystem and their capacity to cause infections in humans and aquatic animals (Monir *et al.*, 2020). Nevertheless, this study demonstrated that our live-attenuated vaccines posed no risk to the host, as no deaths were observed in either the vaccinated or unvaccinated groups. Neither group of fish exhibited any abnormal symptoms or behaviours. Furthermore, there was no evidence of any leakage of live-attenuated *V. harveyi* vaccine into the water during the entire duration of this study.

In the zebrafish model, it has been observed that the vaccine dosage influences the production of antibodies. Specifically, higher vaccine doses within safe limits lead to greater antibody production. This phenomenon could be attributed to the increased abundance of antigens that the fish take up as the dosage of the vaccine increases (Sudheesh and Cain, 2016). Notably, even at the minimal dosage of 1 x 10^4 CFU/mL, antibody levels were substantially increased compared to the control group. These findings suggest that even this lower dosage effectively stimulated immunity against the specific *Vibrio* spp. antigens. The findings demonstrate that the LAVh vaccine confers protection against disease, even when administered in low doses. This could be attributed to the high affinity between the antibodies and the surface antigen presenters, which facilitates the recruitment of phagocytes for complement-mediated opsonization or lysis. Like this study, Ye *et al.* (2016) proved that administering a live attenuated *Vibrio anguillarum* vaccine to zebrafish exhibited elevated levels of antibodies. Subsequently, the antibodies were transmitted to the offspring through vaccinated broodstock. In addition, Zhang *et al.* (2014) discovered that zebrafish that were administered the live attenuated *V. anguillarum* vaccine exhibited a progressive enhancement in their specific antibody response against *V. anguillarum* over a period of 28 days following vaccination with booster vaccinations prolonged this immune response and successfully shielded the zebrafish from vibriosis.

This study observed that antibody levels reached their highest point at higher dosages when using stale LAVh compared to fresh and freeze-dried versions. The observed rise in antibody production may be due to the age of the bacteria used for inoculation, as older bacteria are likely to exhibit a wider range of outer membrane proteins (OMPs) than younger ones. This improves the ability of the antibodies generated in response to invading antigens to bind more effectively. The maximum levels of antibodies in all storage conditions of the LAVh vaccine highlight the capacity of live-attenuated vaccines to stimulate strong and long-lasting immune responses, thereby aiding in the prevention of diseases in aquaculture. However, additional research is required to gain a more comprehensive understanding of the efficacy of the stale live-attenuated vaccines in interacting with the immune responses of fish.

Typically, bacterial vaccine production primarily involves the development of strain-specific vaccines that specifically target particular pathogens. The LAVh vaccines in this study demonstrated multiple protection against different *Vibrio* spp. These findings were evident from the survival rate of 80% among fish exposed to pathogenic *V. harveyi*, *V. alginolyticus*, and *V. parahaemolyticus*. The challenge test for ED_{80} utilized the LD_{50} dose of the pathogenic strain as a benchmark, thereby establishing a standardized criterion for evaluation. The relative percentage of survival (RPS) of the freeze-dried LAVh at a lower dosage (1 x 10^4 CFU/mL) was lower than the fresh and stale LAVH. This difference may be attributed to the loss of viable bacteria during the freeze-drying and rehydrating procedures before introducing them into

the zebrafish host model. Nevertheless, when administered at a higher dosage of 1 x 10^6 CFU/mL, the overall survival rate of adult zebrafish in the freeze-dried vaccine group did not show any significant variation compared to the fresh and stale vaccines in the challenge test against *V. harveyi*, *V. alginolyticus*, and *V. parahaemolyticus*. This discovery highlights the possible feasibility of using freeze-drying to preserve the vaccine for future uses.

4. Conclusion

The LAVh vaccine shows high potential for managing vibriosis and improving the long-term viability of aquaculture practices in areas with limited resources. The accessibility, effectiveness, and simplicity of its administration make it a valuable tool in the ongoing effort against infectious diseases in aquatic environments. These findings might potentially help to protect the livelihoods of aquaculture farmers and ensure the continued growth of the industry. An essential requirement for aquaculture farmers on offshore and remote farms is a dependable vaccine that can be readily utilized and stored in large quantities while maintaining optimal antibody levels for extended durations. From this study, the LAVh vaccine has shown its ability to be used in a stale condition and stored as a freeze-dried powder, effectively protecting the fish receiving it. In addition, the LAVh vaccine has the ability to provide cross-protection to its host, resulting in an 80% survival rate for the entire population when exposed to pathogenic strains of *Vibrio* spp. The wide range of protection provided by this vaccine enhances the ability of aquaculture systems to withstand various disease challenges, thereby improving the overall health and productivity of aquatic environments. In addition, the LAVh vaccine emerges as a versatile and reliable tool for disease management in aquaculture, offering a sustainable way to prevent diseases and ensuring the continued success of aquaculture operations in the long run. The demonstrated effectiveness, user-friendly nature, and ability to protect against multiple strains highlight the importance of this method in addressing vibriosis and other infectious risks in aquaculture environments.

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Authors' Contributions

MHNM-S and AM were responsible for data collection, manuscript drafting, and figure design. MHNM-S, MA-S, MNAA, MSM, MZ-S, and ISMY contributed to the conception of the main ideas and provided critical revisions to the article. All authors have reviewed and contributed to the final manuscript.

Conflict of Interest

The author affirms that the study was conducted without any commercial or financial affiliations that might pose a conflict of interest.

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