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Streptomyces Extract Supplemented Heat-Killed Vaccine Treatment Effectively Suppress the *Vibrio Anguillarum* Infection in Tilapia *(Oreochromis niloticus)* Fish

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Abstract: This study was carried out to assess the effect of *Streptomyces* sp. VITMK3 derived cell-free supernatant on tilapia fish (*Oreochromis niloticus*) infected with *Vibrio anguillarum*. *Streptomyces* showed inhibitory activity against *V. anguillarum* was characterized by molecular taxonomy and chemotaxonomic methods. It was identified as *Streptomyces* species and was designated as *Streptomyces* sp.VITMK3. *Oreochromis niloticus* was divided into six experimental groups. Groups A and B served as the negative and positive control, respectively. Groups C and E were vaccinated with heat-killed and formalin-killed vaccines of *V. anguillarum*. Groups D and F were vaccinated with lyophilized cell-free supernatant of *Streptomyces* sp. VITMK3, along with the heat-killed and formalin-killed vaccines respectively. A booster dose was also given after 14 days. Following the treatment, all the groups, excluding the negative control, were infected with *V. anguillarum* by intraperitoneal injection. Groups D and F showed higher survival of 86.6% and 73.33%, respectively, when compared to groups C and E (59.9% and 53.55%). Hemorrhagic spots, tail, and fin rot were observed in positive control. These results suggest that the isolate could be used as a potential bacterial antagonist, probiotic, and immunostimulant to control *V. anguillarum* infection in *Oreochromis niloticus*.

Keywords: Streptomyces sp. VITMK3; *Vibrio anguillarum; Oreochromis niloticus;* heat-killed vaccine; formalin-killed vaccine.

1. Introduction

Aquaculture supports and assists the constantly increasing global fish food demand. Carp and tilapia are the major groups of farmed fish, and they are an ideal menu addition due to their year-round supply, reasonable price, wild and delicious flavor, and flexibility in preparation. There has been a marked increase in tilapia farming, which contributes to increasing global aquaculture production. The 44% increase in production since the start of the decade till 2014 illustrates the economic importance of tilapia [1]. The rapid development of aquaculture has led to the intensive rearing of captive fish, which causes disease outbreaks resulting in huge economic losses [2]. This is evident in *Oreochromis niloticus* cultivation under stressful conditions (high fish density and poor water quality). They are at high risk of infection by pathogens such as *Vibrio*, *Aeromonas*, *Edwardsiella*, *Pseudomonas*, etc.[3]. Bacterial diseases are the major limiting factors in the rearing of captive fish [4]. *Vibrio* species (*Vibrio harveyi*, *V. parahaemolyticus*, and *V. campbellii*) are responsible for causing severe

vibriosis in shrimp culture farms with increased mortality (50–100%) and vibrio infections in humans [5]. Vibriosis not only affects marine aquaculture systems but also affects wild fish populations [6]. *V. anguillarum*, an opportunistic pathogen, continuously threatens fish rearing worldwide. Vibriosis in tilapia is caused by *V. anguillarum*, a Gram-negative bacterium that causes severe epidemic vibriosis with clinical signs of hemorrhage and septicemia of fish [7]. Antibiotics are the most commonly adopted method to treat and control infection caused by *V. anguillarum*. Antibiotic abuse has led to increased bacterial resistance and issues regarding food safety and environmental pollution [8,9]. More and more antimicrobial-resistant *Vibrio* spp. have been reported recently [10]. Frequent use of antibiotics has also resulted in fish immunosuppression [11]. The European Union has banned the use of antibiotics in aquaculture due to their side effects [12]. Therefore, it is necessary to look for various agents, such as natural growth promoters, pathogen inhibitors, and immunostimulators [13]. Vaccination with inactivated antigens and the addition of probiotics in the diet can decrease the usage of chemicals and antibiotics in aquaculture [14]. The use of different vaccines, probiotics, and antimicrobial peptides to combat *V. anguillarum* has been reported [15]. Previous reports on the protective effect of various types of vaccines include heat-killed and formalin-killed vaccines against *V. anguillarum* [16]. Actinomycetes are a group of Gram-positive, branching microorganisms with exquisite ability to produce several secondary metabolites with numerous biological activities [17]. The secondary metabolites produced by actinomycetes are precursors for many of the commercially available antibiotics in the market [18]. Actinomycetes have been reported for their antibacterial activity against *Vibrio* spp.[19]. The administration of an immunostimulant can activate non-specific defense mechanisms and specific immune responses along with a vaccine [20]. Actinomycetes have been previously studied for their use as immunostimulants [21]. Several antivibrio compounds have been reported from actinomycetes [22]. Antimicrobial peptides have also been recently used to control vibriosis in shellfish hatcheries [23]. Biological control methods are considered to be effective, economical, and environmentally safe [24]. The administration of actinomycetes along with heat-killed and formalin-killed vaccines is studied to understand the immunostimulant activity of the isolates to control vibriosis in tilapia.

2. Materials and Methods

2.1. Isolation and screening of actinomycetes.

The terrestrial soil sample collected from Amirthi forest, Tiruvannamalai District, Tamil Nadu, India, was subjected to pre-heat treatment (45°C) in a hot air oven for two days. The pre-treated sample was subjected to serial dilution and spread onto Actinomycetes Isolation Agar (AIA), then monitored for growth of actinomycetes, and a single colony was streaked on AIA for further use. The actinomycete cultures were screened for their antagonistic activity using the cross streak method, which streaked the actinomycete culture vertically on the AIA plate. Upon necessary growth of actinomycete, *V. anguillarum* was streaked in perpendicular and was incubated at 30°C for 16 hours. Isolates showing a zone of inhibition were subjected to secondary screening by the well diffusion method. *Streptomyces* isolates showing strong antibacterial activity were inoculated to Tryptone Yeast Extract (ISP 1) broth and incubated for the growth of *Streptomyces* for a period of seven days. *V. anguillarum* was swabbed onto Muller Hinton Agar (MHA), and 6 mm wells were punched using a sterile cork borer. Cell-free supernatant (100 µL) obtained from *Streptomyces* was added to the wells and

incubated for 16-24 hours at 37°C, and zones of inhibition developed were measured. The isolate showed a maximum zone of inhibition, referred to as the potential isolate.

2.2. Characterization of the potential Streptomyces isolate.

The selected *Streptomyces* were inoculated on an AIA medium and observed for their aerial mass color, production of soluble pigment, and reverse side pigments. The cell wall of the isolate was studied using Gram staining and spore morphology using a scanning electron microscope (SEM). The utilization of various carbon sources such as arabinose, inositol, mannitol, rhamnose, sucrose, sorbitol, raffinose, and xylose as a source of energy was studied by the method of the International Streptomyces Project [25]. The whole cell hydrolysate was used to determine the cell wall amino acids by thin-layer chromatography. ISP 1 media was used to grow Streptomyces, and the harvested cells were processed for genomic DNA extraction by phenol-chloroform method. Universal primers 27F-5'AGAGTTTGATCMTGGCTCAG3' and 1492R-5'TACGGYTACCTTGTTACGACTT3' were used for the amplification of 16 S ribosomal RNA genes of *Streptomyces* by polymerase chain reaction (PCR). The obtained PCR product was purified using a Nucleospin ® PCR clean-up gel extraction kit (MACHEREY – NAGEL GmbH & Co. KG, Germany). The 16S rDNA nucleotide sequence of the purified DNA was sequenced by an ABI3730xl sequencer (Applied Biosystems, USA). The 16S rDNA sequence was searched through the National Centre for Biotechnology Information (NCBI) sequence database for similarity using a BLAST search. A phylogenetic tree was constructed using the data by the neighbor-joining method using Clustal X and MEGA 6.06.

2.3. Experimental fishes and ethical approval.

Tilapia (*O. niloticus*) weighing 10-15 g were obtained from Ram Raghu fish bank, Walaja, Vellore District, Tamil Nadu, India and were used for the study. Fishes were maintained at 24°C in freshwater 500 L tanks with continuous aeration. During the acclimatization and experimental periods, all animals were fed twice with boiled fish meat every day. After acclimatization for a week, fish were placed in separate tanks for experimental purposes. All the experiments using fishes are carried out in OIE Reference Laboratory for Aquatic Animal Health (Aquatic Biotechnology Laboratory, C. Abdul Hakeem College, Melvisharam- 632 509, Tamil Nadu, India) and approved by the Institutional Animal Ethical Committee.

2.4. Development of vaccines and preparation of extract from Streptomyces.

V. anguillarum was cultured at 30°C for 16 hours in nutrient broth. The cells were collected by centrifugation (10000 rpm for 10 minutes at 4°C) and washed with sterile saline (1% w/v NaCl). The washed cells were suspended in saline, resulting in 1 mL approximately containing 10^9 CFU. The washed cell suspension was used to prepare heat-killed and formalin-killed vaccines by incubating at 60° C for 120 minutes and adding 0.1% formaldehyde, followed by incubation at room temperature for 24 hours, respectively. Excess formaldehyde was removed by washing the cells with sterile saline. Both the preparations were checked for their usability as a vaccine by seeding them on a TCBS agar plate and observing any growth. The heat-killed and formalin-killed vaccines were stored at 4°C until further use. The potential isolate was grown in tryptone yeast extract broth (ISP No. 1 Medium) by submerged

fermentation in an orbital shaker maintained at 150 rpm at 30°C for seven days. The cell-free supernatant separated from the cells using Whatman No.1 filter paper was freeze-dried and stored until further use.

2.5. Vaccination and experimental challenge.

O. niloticus (10-15 g body weight) was used in the experiment to evaluate the efficacy of the vaccine. Fishes were divided into six groups (5 fishes per group) for challenging experiments. Group A was treated as negative control, and Group B was injected with phosphate buffer saline (PBS) (positive control). Group C was injected intraperitoneally with heat-killed vaccine suspended in PBS (30 µL containing 1.4 $x10^7$ cells). Group D was injected intraperitoneally with lyophilized cell-free supernatant (LCFS) (30 µg) of the isolate per fish along with the heat-killed vaccine. Group E was injected with formalin-killed vaccine suspended in PBS (30 µL containing 1.4 $x10^7$ cells) intraperitoneally. Group F was injected intraperitoneally with 30 µg of LCFS of the *Streptomyces* isolate per fish along with the formalin-killed vaccine. All the experiments were carried out in triplicate. A booster dose of the vaccine was injected intraperitoneally after 14 days. Fourteen days post-vaccination, all the groups, excluding Group A were injected intraperitoneally with $1.2x10^6$ cells of *V. anguillarum* in 0.1 ml of PBS. After the experimental challenge, all fishes were observed every day up to 30 days.

3. Results

3.1. Isolation and screening of Streptomyces isolates.

A total of 15 isolates were obtained from the terrestrial soil samples of Amirthi forest. A few isolates exhibited promising activity against V. anguillarum using the cross streak method. In the secondary screening method, the isolate, which revealed a prominent zone of inhibition (16 mm), was chosen for further studies.

3.2. Characterization of Streptomyces isolates.

The potential *Streptomyces* isolate showed good aerial and substrate mycelium formation on the AIA medium, which was used as the optimal medium for storage and further use. An SEM image of the Streptomyces showed that the spore surface was smooth and had a looped (*Retinaculi aperti*) structure (Figure 1).

It also grows well on ISP No. 1 medium and was used as the medium for submerged fermentation to produce cell-free supernatant. The aerial spore mass of the isolate was found to be reddish grey in color with the reverse side, and diffusible pigments were produced on peptone yeast extract iron agar. The isolate was found to be Gram-positive. Glucose, mannitol, fructose, arabinose, sucrose, and glycerol were observed as optimal carbon sources for maximal growth of the isolate. LL-DAP with glycine were present as cell wall amino acid, representing the isolate belonged to cell wall type I. 16S r DNA sequencing of the isolate yielded 1277 nucleotides. A BLAST search of the sequence showed 99 % similarity to *Streptomyces fradiae* (Figure 2).

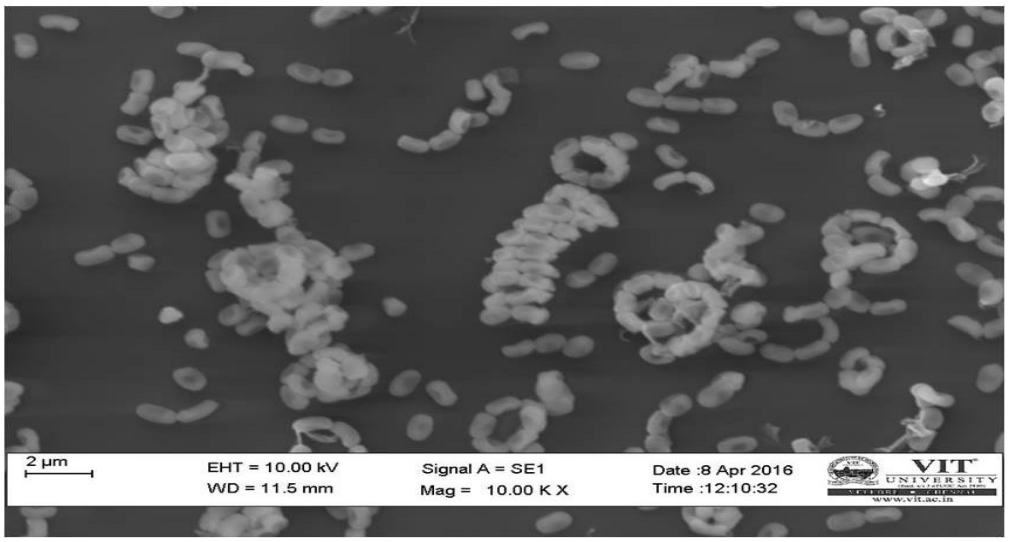

Figure 1. SEM image of *Streptomyces* sp. VITMK3.

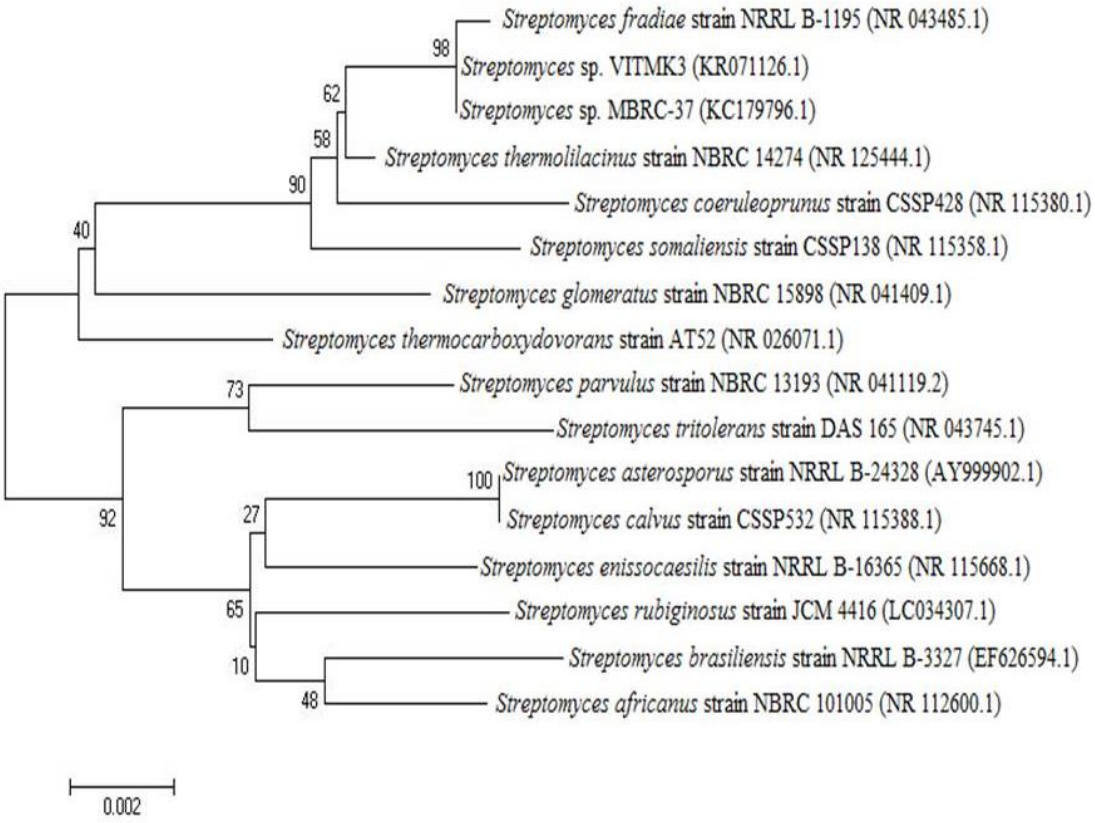

Figure 2. Phylogenetic tree of *Streptomyces* sp. VITMK3.

Clear exclusion of the *Streptomyces* sp. VITMK3 from all other sequences in the phylogenetic tree suggests that the isolate was genetically different from the reference sequences obtained from the BLAST search. The 16S DNA nucleotide sequence of the *Streptomyces* was deposited in the GenBank under the accession number KR071126. Based on molecular taxonomy and phylogeny, the isolate was designated *Streptomyces* sp.VITMK3.

3.3. Survival of test fishes after infection.

Fish groups vaccinated with the heat-killed and formalin-killed vaccines (Group C and Group E) showed survival of 59.9 ± 6.6% and 53.55 ± 6.6%, respectively, after 30 days.

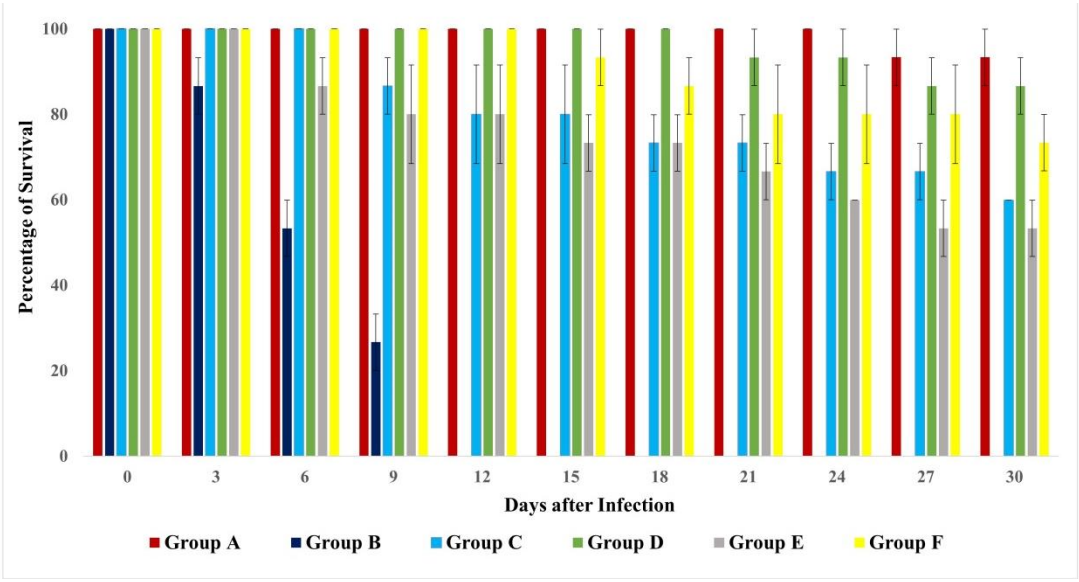

Figure 3. Survival of tilapia fish at different time intervals after challenging with *V. anguillarum* (Group A – Negative Control; Group B – Positive Control; Group C –Heat-Killed Vaccine; Group D – Heat-Killed Vaccine along with *Streptomyces* sp. VITMK3; Group E – Formalin-Killed Vaccine and Group F – Formalin-Killed Vaccine along with *Streptomyces* sp. VITMK3).

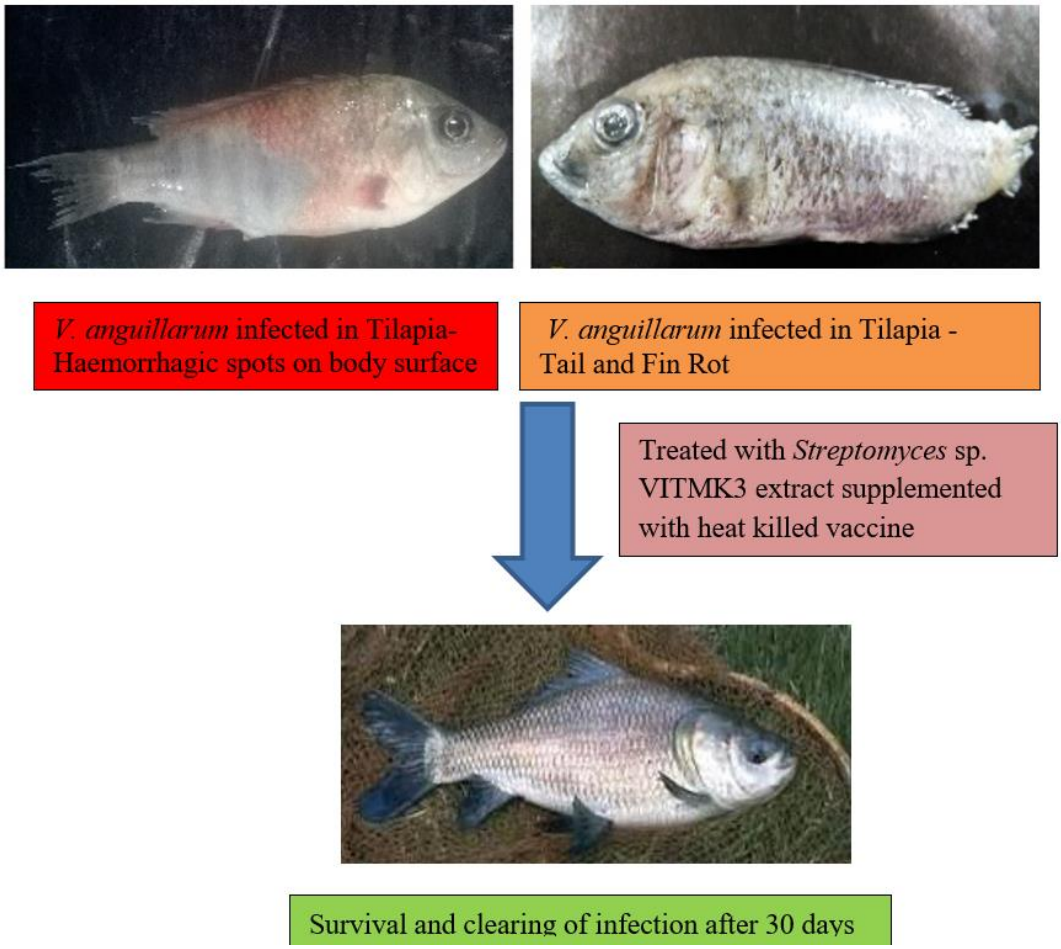

Figure 4. Survival of *V. anguillarum* infected tilapia fish after treatment with *Streptomyces* sp. VITMK3 extract supplemented with heat-killed vaccine.

Fish groups vaccinated with heat-killed vaccine and lyophilized cell-free supernatant of *Streptomyces* sp. VITMK3 (30 µg/fish), formalin-killed vaccine, and lyophilized cell-free supernatant of *Streptomyces* sp. VITMK3 (30 µg/fish) showed a higher survival of 86.6 ± 6.6%

and 73.33 ± 6.6% respectively after 30 days. Since the pathogen did not challenge the negative control, no mortality was recorded in this group. In positive control, 100% mortality was observed in ten days. The survival percentage of fish in all the groups is given in Figure 3. The survival and infection clearing was observed in *V. anguillarum*-infected tilapia fish after 30 days of treatment with a heat-killed vaccine and lyophilized cell-free supernatant of *Streptomyces* sp. VITMK3 (30 µg/fish) (Figure 4).

4. Discussion

Vibrosis caused by *V. anguillarum* and *V. harveyi* is considered to be a huge threat to marine aquaculture due to its severe pathogenicity and associated complications on fish species [26]. In general, younger and smaller fish do not acquire immunity after vaccination by any method, and the size is also important for vaccination. In general, 21 days old tilapia is immune-competent [7]. The animals used for our experiments are of moderate size and age and are thus assumed to be immune-competent. It was reported that the relative percentage of survival (RPS) of animals was very low in tilapia through bath immunization method of vaccination compared to that of the intraperitoneal injection method[21]. The following factors strongly influence the efficiency of the vaccine: the mode of administration, dosage, use of an adjuvant, environment, and water temperature [27]. This implies that a detailed knowledge of the infectious pathogen and the host response is necessary to develop an efficient vaccine. Vibriosis in tilapia is due to *V. anguillarum* and *V. vulnificus* infection in brackish water, and in freshwater, it is due to *V. cholera* and *V. paraheamolyticus* infection [3]. In *Salmon gairdneri* (rainbow trout), the heat that killed *V. anguillarum* injected with Freund's complete adjuvant showed good agglutinin titer compared to the formalin-killed bacteria with or without adjuvant [28]. Similar results were obtained in our study, where the animals vaccinated with the heat-killed vaccine showed a higher survival percentage when compared to the formalin-killed vaccine. Heat-killed *V. anguillarum*, when intraperitoneally injected in Atlantic cod, *Gaus morhia* enhances the serum antibacterial activity against *V. anguillarum*. This study also supports the idea that intraperitoneal injection is one of the most successful modes of vaccine administration [29]. The inactivated whole cell of *V. anguillarum* has been used as part of a multivalent vaccine in Japanese flouder, which exhibited a better relative survival percentage (RPS) when challenged with *E. tarda* and *V. anguillarum* [30]. Actinomycetes were reported to possess antagonistic activity against *Vibrio* spp. Actinomycetes isolated (51.2%) from marine sediment from a shrimp farm showed antibacterial activity against pathogenic *Vibrio* spp. [31]. It was reported that the decreased *Vibrio* count leads to increased *Streptomyces* cell concentration [32]. The probiotic use of *Streptomyces* on the growth and prevention of disease of black tiger shrimp, *Penaeus monodon* (Fabricius), has already been reported. A marine actinomycete, *Nocardia brasiliensis* produced a bioactive metabolite that showed antagonistic activity against *Vibrio damsela* [33]. Actinomycetes strain A66 was reported to be a potential organism against biofilms produced by *V. anguillarum* [19]. Inhibition of fish and shellfish pathogens, *Aeromonas hydrophila*, *Serratia* sp., and *Vibrio* spp. by polyene compounds derived from marine sponge-associated *Streptomyces* has already been reported [34].

Streptomyces rubrolavendulae M56, when co-cultured it, forms bio granules and significantly excludes the pathogenic *Vibrio* spp.[35]. *Streptomyces* isolated from the shrimp culture pond was reported to be effective against *V. parahaemolyticus, V. alginolyticus*, and *V. harveyi* [36]. Actinomycetes RL8 and V4 isolated from the nearshore sediment in Cuba have been reported to possess high inhibitory activity against *V. parahaemolyticus, V. harveyi*, and

V. vulnificus [37]. A proteinaceous compound extracted from soil actinomycetes have shown to be active against fish pathogens *Aeromonas hydrophila*, *V. alginaticus*, and *V. harveyi* [38]. *Streptomyces* sp. NHF165-derived actinonin inhibited the growth of *V. anguillarum* [39]. The crude ethyl acetate extract of *Streptomyces carpaticus* MK-01 showed a significant range ($P<0.05$) of antibacterial activity against gram-positive fish bacterial pathogens (*S. iniae* and *S. parauberis*) than Gram-negative bacteria, *E. tarda*, *V. anguillarum*, *V. furnissii*, *V. fluvialis*, and *V. ichthyoenteri* [40]—the EA extract prepared from *Streptomyces* sp. VITNK9 exhibited antibacterial activity against, *A. caviae* (15.33 mm), *A. hydrophila* (17.66 mm), *E. tarda* (18.33 mm), *V. anguillarum* (14.33 mm), and *V. harveyi* (14.33 mm) [41].

A component from *Streptomyces olivaceogriseus* sp. nov. was used to develop an immunostimulant, FK565, which is a synthetic lactoyl tetrapeptide [42]. This immunostimulant conferred protection in rainbow trout when it was injected one day before infecting with *A. salmonicida* [43]. A similar approach was reported in one of the recent studies, where an herbal immunostimulant and a recombinant protein were used, resulting in an increased RPS of shrimp. The RPS of the shrimp fed with recombinant VP28 (50 µg/g of feed) was found to be 68.8%. When the immunostimulant (500 µg/g of feed) was fed along with the recombinant VP28 (50 µg/g of feed), the survival rate increased to 84.4% [44]. Copper oxide nanoparticles (CuO NPs) synthesized using actinomycetes have been shown to be very effective against fish bacterial pathogens (*E. tarda*, *A. caviae*, *A. hydrophila*, and *V. anguillarum*) [45]. Marine *Streptomyces* S073-derived anti-vibrio compound dibutyl phthalate (DBP) was reported to be effective against *Vibiro parahaemolyticus* [46]. The cytotoxic compound cytotoxic actinonin produced by *Streptomyces* sp. NHF165 showed activity against the aquaculture pathogen *Vibrio anguillarum* [47]. Phenol,2,4-Bis(1,1-Dimethylethyl) derived from *Bacillus licheniformis* was reported to be effective against *V. parahemolyticus* [48]. Compounds extracted from endophytic fungus *Penicillium* sp. Z2230 viridicatol, cyclopenol, and cyclopenoin have been reported to be potent anti-Vibrio activity at micromolar concentrations [49]. Antimicrobials from microalgae and cyanobacteria have also been used to control *V. anguillarum* infection [50]. It is suggested that antibiotics, probiotics, bacteriophages, antimicrobials from natural sources, and vaccines be used to control vibriosis [51].

5. Conclusions

The results of our study revealed that the LCFS prepared from *Streptomyces* sp. VITMK3, when administered along with the heat-killed or formalin-killed vaccines, confers better protection than the vaccines alone to tilapia when infected with *V. anguillarum*. Based on the results, it can be concluded that the administration of *Streptomyces* LCFS, along with vaccines, proved to be very effective in combating vibriosis in tilapia. This study also provides a novel approach for developing a new vaccine for better control and effective management of vibriosis in tilapia fish.

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Conflict of Interest

No conflict of interest is declared.

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