Effects of dietary supplementation of potential probiotic *Pseudomonas aeruginosa* VSG-2 on the innate immunity and disease resistance of tropical freshwater fish, *Labeo rohita*

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1. Introduction

Aquaculture has emerged as one of the most promising and fastest-growing industries, and provides high-quality animal protein, raises nutritional levels, and generates income and employment around the globe [1]. Indian aquaculture production mainly consists (~70%) of 3 major carps (*Labeo rohita*, *Catla catla*, and *Cirrhinus mrigala*) [2]. The global production of *L. rohita* was approximately 1.2 million tons in 2005, out of which nearly 1 million tons was contributed by India [2]. Bacterial infections are one of the most important causes of disease problems in Indian aquaculture [3]. *Aeromonas hydrophila* is the most common pathogen, and it can easily spread through accidental abrasions [4]. This bacterium causes haemorrhagic septicemia, which is characterized by the presence of ulcers, abscesses, exophthalmia, abdominal distension, small superficial lesions, local haemorrhages, particularly in the gills and opercula [4,5].

One of the most promising methods of disease control in aquaculture is the strengthening of defence mechanisms in fish through prophylactic administration of immunostimulants [6]. Probiotics play important roles as immunostimulants and antimicrobial agents [7,8]. Probiotics are live microbial or cultured product feed supplements that beneficially affect the host by producing inhibitory compounds, competing for chemicals and adhesion sites, modulating and stimulating immune function, and improving microbial balance [7,9]. In aquaculture, probiotics have been used to control diseases, enhance specific and non-specific immunity, provide nutrients and enzymatic functions, and improve water quality [10].

Particularly, species of *Bacillus*, *Lactococcus*, *Saccharomyces*, and *Lactococcus* are being used as immunostimulants in aquaculture practice [7,10–14]. Furthermore, the immunostimulatory effects of these bacteria against *A. hydrophila* infection in fish have been demonstrated [14–17]. In vitro antagonistic activity of the cellular components of *Pseudomonas* species against *A. hydrophila* has been reported [18]. However, the immunomodulatory effects of *Pseudomonas aeruginosa* on the major Indian carp *L. rohita* have not been reported. Recently, we isolated a potential probiotic,
P. aeruginosa VSG-2, from the gut of the tropical freshwater fish rohu, <i>L. rohita</i> [19].<sup>1</sup> <i>P. aeruginosa</i> VSG-2 and its cellular components inhibit the growth of the fish pathogen <i>A. hydrophila</i> in vitro [19]. Furthermore, VSG-2 exhibits good tolerance to acid and bile, and adhesion to intestinal mucus [Unpublished data]. Hence, we hypothesized that <i>P. aeruginosa</i> VSG-2 may act as an immunostimulant against <i>A. hydrophila</i> infection in <i>L. rohita</i>. Therefore, we evaluated the effects of dietary administration of <i>P. aeruginosa</i> VSG-2 on the innate immune responses of <i>L. rohita</i> and its resistance against <i>A. hydrophila</i> infection.

2. Materials and methods

2.1. Bacterial strains

The potential probiotic bacterium <i>P. aeruginosa</i> VSG-2 was previously isolated from the gut contents of the tropical freshwater fish <i>L. rohita</i> [19]. The bacterium was grown in brain heart infusion broth for 24 h at 37 °C. Cell density was calculated from OD<sub>600</sub> values and correlated with colony forming unit (cfu) counts using serial dilution and spread plating on tryptone soya agar (TSA). The quantified bacteria were maintained at 4 °C in a suspended form and were used for seed preparation as required.

2.2. Diet preparation

A basal diet comprising 39% groundnut oil cake, 34% rice bran, 20% soybean meal, 5% fish meal, and 2% mineral and vitamin mixture (Every 250 g of mineral-vitamin mixture provided vitamin A, 500,000 IU; vitamin D3, 100,000 IU; vitamin B2, 0.2 g; vitamin E, 75 units; vitamin K, 0.1 g; calcium pantothenate, 0.25 g; nicotinamide, 0.1 g; vitamin B12, 0.6 mg; choline chloride, 15 g; calcium, 75 g; manganese, 2.75 g; iodine, 0.1 g; iron, 0.75 g; zinc, 1.5 g; copper, 0.2 g and cobalt, 0.045 g) was prepared. Proximate analysis of the basal feed performed according to the AOAC (Association of Official Analytical Chemists) method [20] revealed 37.8% crude protein, 9.4% crude lipid, and 12.3% ash. The basal diet was used as control diet. In the 3 experimental diets D-I, D-II, and D-III, probiotic <i>P. aeruginosa</i> VSG-2 suspension was added at a final dose of 1 x 10<sup>7</sup>, 1 x 10<sup>8</sup>, and 1 x 10<sup>9</sup> cfu g<sup>-1</sup> respectively. To achieve accurate final concentrations of the diet, the bacterial suspension was slowly added to dough, with gradual mixing in a drum mixer. The experimental diets were air-dried in a drying cabinet using an air current. The amount of <i>P. aeruginosa</i> in each diet was determined at 0, 30, and 60 days of storage by spread plating on TSA. <i>P. aeruginosa</i> levels decreased by 5–12% and 25–35% over 30 days and 60 days of storage, respectively. Therefore, fresh diets were prepared after 30 days to ensure high probiotic levels in the diets.

2.3. Experimental design

Healthy rohu (<i>L. rohita</i>) showing no signs of disease (gross and microscopic examination of skin, gills, and kidney tissues of representative samples), with no previous history of parasitic infections, and having a mean body weight of 60 g were obtained from a local fish farm in Thanjavur, Tamil Nadu, India and acclimatised to laboratory conditions for 2 weeks in 500-L plastic quarantine tanks at 28 ± 2 °C. All the fish were fed with control diet during the acclimatisation period. About 20% of the water in all tanks was exchanged daily and 100% of the water was exchanged once a week. The basic physico-chemical parameters of the water were measured every week [21]. The O<sub>2</sub> and ammonia concentrations ranged from 6 to 7.5 mg L<sup>-1</sup> and 0.5–1 ppm, respectively, and pH ranged from 7.0 to 8.0 throughout the study period.

The fish were randomly divided into 4 experimental groups with three replicates in each. Tank capacity was 200 L and each tank contained 15 fish. Fish were fed one of 4 diets (Control, D-I, D-II, or D-III). The feed rate was 3% of body weight per day, and equal rations were provided at 09.00 and 17.00 h for 60 days. The amount of diet consumed was determined by daily recovery of excess feed, which was then dried and weighed [22]. Daily feed was adjusted every 15 days by batch weighting after 24 h of starvation.

2.4. Analysis and measurements

2.4.1. Sample preparation

Sampling was scheduled at day 30 and day 60 after probiotic feeding. At each time point, 3 fish were randomly removed from each tank after batch weighing and thus, a total of 9 fish were collected per treatment for immunological assays. Blood samples were collected from the caudal vein using a 2-ml syringe after anaesthetising the fish with MS222 (Sigma–Aldrich, St. Louis, MO, U.S.A.). The blood samples were transferred into Eppendorf tubes. Following centrifugation (2000 g, 10 min, 4 °C), serum was collected and stored at −20 °C until use. Head kidney macrophages were isolated from 6 fish in each group using the method of Secombes [23] with previously described modifications of Geng et al. [12]. Cell viability was evaluated using the trypan blue exclusion test and cell density was determined in a haemocytometer. Harvested cells were adjusted to 1 x 10<sup>7</sup> cells mL<sup>-1</sup> for the assay.

2.4.2. Lysozyme activity assay

Serum lysozyme activity was measured according to the method described by Ellis [24]. One unit of lysozyme activity was defined as the amount of enzyme producing a decrease in absorbance of 0.001 min<sup>-1</sup> mL<sup>-1</sup> serum.

2.4.3. Alternative complement pathway activity assay

Alternative complement pathway activity (ACh<sub>50</sub>) was determined and calculated using the method of Yano et al. [25]. The volume of serum producing 50% haemolysis (ACh<sub>50</sub>) was determined and the number of ACh<sub>50</sub> U mL<sup>-1</sup> was calculated for each group.

2.4.4. Respiratory burst activity

The respiratory burst activity of phagocytes was measured using the nitroblue tetrazolium (NBT, Sigma–Aldrich) assay, according to the method of Secombes [23] with previously described modifications [12]. Colour development was measured at 630 nm with a spectrophotometer. KOH/DMSO was used as blank.

2.4.5. Phagocytic activity assay

Phagocytic activity of head kidney macrophages was determined using the previously described method of Ai et al. [26]. The number of phagocytic cells per 100 adhered cells was microbiologically determined. Phagocytic activity (PA) was calculated using the formula:

\[ PA = \left( \frac{\text{phagocytic leucocytes/total leucocytes}}{100} \right) \times 100. \]

2.4.6. Superoxide dismutase assay

Serum superoxide dismutase (SOD) activity was determined with an enzymatic assay method using a reagent kit (Randox, Crumlin, U.K.), as described by Sun et al. [22]. One unit of SOD

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activity was defined as the amount of enzyme necessary to produce a 50% inhibition of the NBT reduction rate measured at 550 nm.

2.4.7. IgM levels
Serum total IgM levels were measured with an Enzyme-linked Immunosorbent assay (ELISA) using a commercial kit (Cusabio, Wuhan, China), as described by Sun et al. [22]. ELISA plates were read at 450 nm using a plate reader. Negative controls consisted of samples without the biotin antibody. The mean absorbance of the negative controls for each plate was subtracted from the optical density at 450 nm.

2.5. Challenge study

*Abra hydrophila* MTCC 1739 was grown in nutrient broth and incubated at 37°C for 24 h. The seven-day lethal dose 50 (LD50), determined by intraperitoneal injection of graded doses of *A. hydrophila* (10⁵, 10⁶, 10⁷, and 10⁸ cfu per fish) into 20 fish, was 10⁷ cfu/mL.

At the end of the feeding trial, 9 fish each from the treatment groups were injected i.p. with 100 μl PBS containing 10⁷ live *A. hydrophila*. For negative control, a group of 9 fish were injected with PBS. The survival percentage in each group was recorded up to the 10th day of challenge.

2.6. LD₅₀ of *P. aeruginosa* VSG-2 in mice

Twelve-week-old male BALB/c mice were obtained from Sri Venkateshwara Enterprises, Bangalore, India. Mice were lightly anaesthetised with halothane (Nicholas Piramal, Mumbai) in a glass desiccator and challenged with 1 × 10⁵, 1 × 10⁶, 1 × 10⁷, 1 × 10⁸, 1 × 10⁹, 1 × 10¹⁰ cfu/mL of *P. aeruginosa* VSG-2 suspensions prepared as described in Section 2.1. The bacterial preparations were administered drop wise through the external nares using a micropipette (Eppendorf) fitted with a fine tip. The mice were housed in cages and fed with standard rat feed pellets and water ad libitum. Mice were monitored for 30 days and the development of disease signs and mortality were recorded. The LD₅₀ value was determined according to Reed and Muench [27].

2.7. Statistical analysis

One way analysis of variance (ANOVA) was used to analyse the data. Multiple comparisons were performed with Tukey's test to analyse the differences between treatments [11,28]. All statistical analyses were performed using the OriginPro software (version 8; OriginLab Corporation, Northampton, U.S.A). The level of significance was set at *P* < 0.05 and the results are expressed as mean (S.E.M).

3. Results

3.1. Challenge test

The challenge test (*n* = 3 for each dietary treatment) revealed that long-term oral administration of probiotic-supplemented feed enhanced the resistance of *L. rohita* to bacterial infection (Fig. 1). Significantly higher post-challenge survival rates (*P* < 0.05) were observed in the fish groups fed diets containing 10⁷ g⁻¹ (*P. aeruginosa* 66.66%) and 10⁹ g⁻¹ *P. aeruginosa* (55.55%), respectively (Fig. 1). The fish fed with control diet exhibited the lowest survival rate (i.e. 11%), followed by the fish fed a diet containing 10⁵ g⁻¹ *P. aeruginosa* (34.34%). Typical symptoms of haemorrhagic septicaemia (i.e. haemorrhages on the ventral surface of the body and at the base of the pectoral and pelvic fins, opercula, and swollen reddish vent)

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3.6. SOD activity

The SOD activities of fish fed diets containing 10^5, 10^7, and 10^9 cfu g⁻¹ of *P. aeruginosa* were not significantly different from that of the control group after 30 days of feeding (Fig. 2). However, the SOD activities of fish fed diets supplemented with 10^7 and 10^9 cfu g⁻¹ of *P. aeruginosa* were significantly higher than that of the control group (12% and 14%, respectively) after 60 days of feeding.

3.7. IgM levels

After 30 days of feeding, serum IgM levels of fish fed *P. aeruginosa*-supplemented diets were significantly higher than that of fish fed the control diet (Fig. 3), and the highest IgM level was observed in fish fed a diet containing 10^9 cfu g⁻¹ of *P. aeruginosa* (9.83 ± 0.03 mg mL⁻¹). After 60 days of feeding, the IgM levels of the treatment groups were significantly higher than that of the control group, and the lowest IgM level (4.53 ± 0.03 mg mL⁻¹) was observed in fish fed a diet containing 10^7 cfu g⁻¹ of *P. aeruginosa*.

3.8. LD₅₀ of *P. aeruginosa* VSG-2 cells in mice

The 30-day LD₅₀ of *P. aeruginosa* VSG-2 in BALB/c mice, calculated according to the Reed and Muench method [27] after recording the mortality at each dilution, was 10¹⁰ cfu/mL.

4. Discussion

Stimulation of non-specific host defense mechanisms using specific biological compounds, called immunostimulants, enhances the disease resistance and growth of the hosts [29]. The innate immune system, comprising physical barriers, and cellular and humoral components, serves as a defense weapon in invertebrates [30]. Live bacteria in probiotics and prebiotics, known as immunostimulants, act as alternatives to antibiotics and chemicals, and function as alarm molecules to activate the immune system [31]. The beneficial effects of probiotics as immunostimulants have already been studied in several freshwater fish (e.g., *L. rohita* [32], *Oreochromis niloticus* [16], *Epinephelus bruneus* [8], *Rachycentron canadum* [12,13], and *Onchorhynchus mykiss* [33]). To the best of our knowledge, the present study is the first to evaluate the potential of probiotic *P. aeruginosa* VSG-2 isolated from the gut of *L. rohita* as an immunostimulant in *L. rohita*. Among the tank water parameters examined, the concentration of ammonia was slightly high and ranged from 0.5 to 1 ppm. The same range of ammonia concentration was reported in a recent study with *Cyprinus carpio* [34].

As a first line of defense, various serum peptides, such as lysozyme, antibodies, and complement factors, inhibit adhesion and colonisation of microorganisms, leading to the prevention of infection and disease [35]. Lysozyme disrupts bacterial cell walls by splitting glycosidic linkages in the peptidoglycan layers [35]. In the present study, fish fed diets supplemented with different levels of *P. aeruginosa* showed significantly higher lysozyme activities after 60 days of feeding than the control group. At present, information on the probiotic effects of *P. aeruginosa* on serum lysozyme activity in aquatic animals is limited. However, positive effects of other probiotic bacteria (e.g., *Lactobacillus* spp., *Bacillus subtilis* etc.) on the serum lysozyme activity in various aquatic animals have been reported [11–13,17]. In contrast, the serum lysozyme levels of *O. mykiss* significantly declined after 2 weeks of probiotic feeding [33].

Complement, which is the major humoral component of innate immune responses, plays an essential role in alerting the host immune system to the presence of potential pathogens as well as in their clearance. The complement cascade is initiated by 1 or a combination of 3 pathways, namely the classical, alternative, and lectin. Among them, the alternate complement pathway (ACP) is very active in fish compared to mammals [36]. In our study, serum ACP activities of the treated groups were significantly higher than that of control group throughout the study period. The present results are in complete agreement with the findings of previous studies [12,13,17,28]. In contrast, Sun et al. [22] reported that the serum complement C4 levels of *Epinephelus coioides* fed diets containing *Bacillus clausii* and *Bacillus pumilus* were lower than that of the control group after 60 days of feeding.

Respiratory bursts are produced by phagocytes to attack invasive pathogens during phagocytosis and have been widely used to evaluate host defence capabilities against pathogens; however,
Invertebrates, the phagocytic process is followed by the production of highly microbicidal reactive oxygen molecules, such as superoxide anion \((\text{O}_2^-)\), hydrogen peroxide \((\text{H}_2\text{O}_2)\), and hydroxyl radicals \((\text{OH})\) [39]. SOD catalyses the dismutation of the highly reactive \((\text{O}_2^-)\) to the less reactive \(\text{H}_2\text{O}_2\) and functions in the main antioxidant defense pathways in response to oxidative stress [40]. In the present study, dietary supplementation of \(P.\ aeruginosa\) at \(10^5, 10^7, 10^9\) cfu g\(^{-1}\) had no significant effects on serum SOD activities after 30 days of feeding; however, the SOD activities improved slightly after 60 days of feeding. Interestingly, SOD activities of \(E.\ coioides\) fed diets supplemented with \(10^5, 10^7, 10^9\) cfu kg\(^{-1}\). \(S.\ cerevisiae\) were lower than that of the control group after 1 and 2 weeks of feeding; however, the SOD activities increased after 4 weeks of feeding [28]. In contrast, Son et al. [11] found that dietary administration of \(Lactobacillus\ plantarum\) for 4 weeks significantly decreased the SOD activity in \(E.\ coioides\).

Serum immunoglobulins are major components of the humoral immune system, and IgM is the main immunoglobulin in fish [41]. Probiotic supplements stimulate immunoglobulin production in fish [11,32,42]. In the present study, the serum IgM levels of fish fed \(P.\ aeruginosa\)-containing diets were significantly higher than that of the control group at 30 days of feeding; however, the IgM levels decreased thereafter and were lower than control levels at 60 days of feeding. These findings are consistent with those of Sun et al. [22] who reported that supplementation of \(B.\ subtilis\) or \(P.\ putida\) induced greater serum immunoglobulin levels in \(E.\ coioides\) until 30 days of feeding; thereafter, the immunoglobulin levels decreased in the experimental groups. Panigrahi et al. [42] reported that \(Lactobacillus\ rhamnosus\) JCM 1136-supplemented diets induced higher immunoglobulin levels in the plasma of rainbow trout until 20 days, and thereafter, the immunoglobulin levels decreased in all probiotic-fed groups. In light of our results and earlier reports, we suggest that the stimulation of immunoglobulin levels is a short-term phenomenon attributable to probiotics.

In our study, dietary administration of \(P.\ aeruginosa\) at \(10^7\) and \(10^9\) cfu g\(^{-1}\) for 60 days significantly increased fish survival rates (44% approx.) in \(L.\ rohita\) challenged with \(A.\ hydrophila\). Earlier studies showed that dietary supplementation of probiotic bacteria significantly increases the disease resistances in rohu [17,32], rainbow trout [42], tilapia [16], cobia [12,13], and groupers [11,22,28]. Further, probiotic bacteria enhance the resistance of fish to \(A.\ hydrophila\) infection [14,16,17]. The higher survival of fish may be due to the ability of probiotic bacteria, particularly \(P.\ aeruginosa\), to out-compete other bacteria for nutrients and space and exclude other bacteria through metabolite production [7]. The effects of dietary probiotic supplementation on host growth and pathogen resistance may be related to the species of aquatic organisms, the feeding duration and dosage, and the origin of the probiotic strain.

In order to designate a culture as a probiotic, it is necessary to evaluate the safety and pathogenicity of the strain in the host and mammalian system [7]. \(P.\ aeruginosa\) VSG-2 did not produce any harmful effects in rohu \((L.\ rohita)\). Furthermore, the high \(LD_{50}\) of \(10^{10}\) cfu in BALB/c mice also indicates that it is safe for mammals.

In conclusion, the present study demonstrated that dietary supplementation of \(P.\ aeruginosa\) VSG-2 can improve the innate immunity and survival of \(L.\ rohita\) against \(A.\ hydrophila\) infection. To elevate the non-specific immunity of \(L.\ rohita\), dietary \(P.\ aeruginosa\) VSG-2 administration at \(10^7\) cfu g\(^{-1}\) is an optimal dose. In addition, the high \(LD_{50}\) value in BALB/c mice indicates the safety of the strain in mammalian systems. Further experiments should be conducted using other pathogenic bacteria and other fish species to establish the probiotic potential of gut-dominant \(P.\ aeruginosa\) VSG-2 as an immunostimulant.
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