



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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ABSTRACT

The effects of dietary *Pseudomonas aeruginosa* VSG-2 supplementation on innate immunity and protection against *Aeromonas hydrophila* infection were evaluated in *Labeo rohita*. Fish were fed for 60 days with control diet or 3 experimental diets containing *P. aeruginosa* VSG-2 at 10^5 , 10^7 , and 10^9 cfu g⁻¹, respectively. Various innate immune parameters were examined at 30 and 60 days post-feeding. Fish were challenged with *A. hydrophila* 60 days post-feeding and mortalities were recorded over 10 days post-infection. Dietary supplementation of *P. aeruginosa* VSG-2 significantly increased serum lysozyme and alternative complement pathway (ACP) activities, phagocytosis, and respiratory burst activity in head kidney macrophages of *L. rohita* throughout the experimental period. Superoxide dismutase (SOD) activity significantly increased after 60 days in the groups fed diets containing 10^7 and 10^9 cfu g⁻¹ *P. aeruginosa*. Serum IgM levels were significantly higher in the treatment groups than in the control group after 30 days of feeding; however, the opposite result was observed at 60 days. Moreover, fish fed diets containing 10^7 and 10^9 cfu g⁻¹ *P. aeruginosa* had significantly higher post-challenge survival rates against *A. hydrophila* infection. Further, *P. aeruginosa* VSG-2 was found to be safe for mammals. These results indicate that dietary *P. aeruginosa* VSG-2 supplementation at 10^7 cfu g⁻¹ can effectively improve innate immunity and disease resistance in *L. 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 septicaemia, 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*, *Lactobacillus*, *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,

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P. aeruginosa VSG-2, from the gut of the tropical freshwater fish rohu, *L. rohita* [19]. *P. aeruginosa* VSG-2 and its cellular components inhibit the growth of the fish pathogen *A. hydrophila* in vitro [19]. Furthermore, VSG-2 exhibits good tolerance to acid and bile, and adhesion to intestinal mucus [Unpublished data]. Hence, we hypothesized that *P. aeruginosa* VSG-2 may act as an immunostimulant against *A. hydrophila* infection in *L. rohita*. Therefore, we evaluated the effects of dietary administration of *P. aeruginosa* VSG-2 on the innate immune responses of *L. rohita* and its resistance against *A. hydrophila* infection.

2. Materials and methods

2.1. Bacterial strains

The potential probiotic bacterium *P. aeruginosa* VSG-2 was previously isolated from the gut contents of the tropical freshwater fish *L. rohita* [19]. The bacterium was grown in brain heart infusion broth for 24 h at 37 °C. Cell density was calculated from OD₆₀₀ 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 feed 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 *P. aeruginosa* VSG-2 suspension was added at a final dose of 1×10^5 , 1×10^7 , and 1×10^9 cfu g⁻¹, 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 blower at 38 °C until moisture levels were around 10%. After air-drying, the diets were broken up and sieved into pellets of appropriate size and stored at -20 °C until use.

The amount of *P. aeruginosa* in each diet was determined at 0, 30, and 60 days of storage by spread plating on TSA. *P. aeruginosa* 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 (*L. rohita*) 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 O₂ and ammonia

concentrations ranged from 6 to 7.5 mg L⁻¹ 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 weighing 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 1×10^7 cells mL⁻¹ 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⁻¹ mL⁻¹ serum.

2.4.3. Alternative complement pathway activity assay

Alternative complement pathway activity (ACH₅₀) was determined and calculated using the method of Yano et al. [25]. The volume of serum producing 50% haemolysis (ACH₅₀) was determined and the number of ACH₅₀ U mL⁻¹ 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 microscopically determined. Phagocytic activity (PA) was calculated using the formula:

$$PA = (\text{phagocytic leucocytes} / \text{total leucocytes}) \times 100.$$

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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