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Host-derived probiotics *Enterococcus casseliflavus* improves resistance against *Streptococcus iniae* infection in rainbow trout (*Oncorhynchus mykiss*) via immunomodulation

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ABSTRACT

The present study evaluated the benefits of dietary administration of host-derived candidate probiotics *Enterococcus casseliflavus* in juvenile rainbow trout *Oncorhynchus mykiss*. Experimental diets were prepared by incorporating the microorganisms in the basal feed at 3 inclusion levels (*i.e.* 10^7 CFU g^{-1} of feed [T1], 10^8 CFU g^{-1} of feed [T2], 10^9 CFU g^{-1} of feed [T3]). The probiotic feeds were administered for 8 weeks, with a group fed with the basal diet serving as control. The effects on growth performance, gut health, innate immunity and disease resistance were evaluated.

Results showed that growth performance parameters were significantly improved in T2 and T3 groups. Activities of digestive enzymes such as trypsin and lipase were significantly higher in these two groups as well. Gut micro-ecology was influenced by probiotic feeding as shown by the significant increase in intestinal lactic acid bacteria and total viable aerobic counts in T2 and T3. Humoral immunity was impacted by dietary probiotics as total serum protein and albumin were significantly elevated in T3. The levels of serum IgM significantly increased in all probiotic fed groups at week 8; with the T3 group registering the highest increment. Respiratory burst activity of blood leukocytes were significantly improved in T2 and T3. Hematological profiling further revealed that neutrophil counts significantly increased in all probiotic fed groups. Challenge test showed that probiotic feeding significantly improved host resistance to *Streptococcus iniae* infection, specifically in T2 and T3 where a considerable modulation of immune responses was observed. Taken together, this study demonstrated *E. casseliflavus* as a potential probiotics for rainbow trout with the capability of improving growth performance and enhancing disease resistance by immunomodulation.

1. Introduction

Large-scale production of fish and shellfish is faced with both biotic and abiotic issues such as stress, diseases and deterioration of the environmental conditions that affect the production cycle [1]. Among these factors, outbreaks of infectious diseases are a major cause of serious economic losses; hence, various strategies are being developed to prevent or control the devastating effects of infectious diseases in an aquaculture facility. Immunostimulants, vaccines and probiotics are regarded to be effective strategies against infectious diseases and will ensure profitability and sustainability in aquaculture [2,3]. The increasing popularity of these disease control strategies became evident as alternatives to the use of antibiotics in aquaculture. It is now known that the use of antibiotics can lead to the development of antibiotic-resistant bacterial strains [4], which could alter the immune response of the host [5]. In addition, indiscriminate use of antimicrobials in disease prevention and growth promotion can bring about the emergence of

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drug-resistant microorganisms and leave antibiotic residues in the fish and in the environment [4]. Moreover, chemotherapy may also kill or inhibit the beneficial microflora in the digestive tract of the host.

Although vaccination is found to be effective in controlling several bacterial and viral diseases in fish, the use of vaccines in aquaculture is faced with issues on mass delivery; thus limiting its wide-scale use. On the other hand, the use of immunostimulants in fish has shown promise but commercial applications have been very limited [3]. A third viable approach is through the use of probiotics as biological control agents. Probiotics are live or dead, or a component of the bacteria that act under different modes of action in conferring beneficial effects to the host or to its environment [3,6]. These probiotics benefit the host by producing inhibitory compounds, competing for chemicals and adhesion sites, modulating and stimulating the immune function, and improving the microbial balance [7]. The use of probiotics, in human and animal nutrition is well documented [8] and recently, has been applied to aquaculture [3]. Scientific evidence is accumulating supporting various health benefits of probiotics in aquaculture including pathogen interference, immunostimulation, immunomodulation, maintenance of mucosal integrity, supplementing or even in some cases replacing the use of antimicrobial compounds, providing nutrients and enzymatic contributions, and improving water quality [3,9].

Several bacterial candidates, e.g., bacilli, lactic acid bacteria, and Pseudomonads have been evaluated as probiotics for aquaculture [3,10] but the search for new microorganisms that could be used as probiotics is continuously being undertaken [11]. This is because a particular probiotic candidate may be effective in controlling the bacterial pathogen in one species of fish, but may be ineffective in another species. There may never be a single probiotic candidate that could be used in all host species, because the physical, chemical and physiological conditions of the host and/or environment could influence these properties, which eventually affect the efficiency of a particular probiotics [3,11]. Hence, in this study a host-derived bacterium, *Enterococcus casseliflavus* was evaluated of its potential as a probiotic candidate.

Enterococci are currently used as probiotics in human and animal health [12]. The present study aimed to evaluate the efficiency of using *E. casseliflavus* as a potential probiotics in the culture of rainbow trout, *Oncorhyncus mykiss*. The evaluation involved aspects of determining modulation of the hematological and innate immune activity, production of digestive enzymes of the host, modulation of the intestinal microflora and growth performance following oral administration of the probiotic candidate. Moreover, the survival rate and protective effects against a challenge infection *with Streptococcus iniae*, an important bacterial pathogen of rainbow trout [13,14] were conducted.

2. Materials and methods

2.1. Candidate probiotics

The candidate probiotics used in the study was previously isolated from the intestine of healthy rainbow trout kept at the Caspian Sea Ecology Research Center, Iran. Standard biochemical identification protocols and 16S rDNA sequencing revealed that the isolate was *Enterococcus casseliflavus* (NC0209951). The inhibitory activity of *E. casseliflavus* was tested against the target pathogen *Streptococcus iniae* (ATCC29178) by disc diffusion method [15,16]. Briefly, 100 μL (containing 1.2×10^6 CFU mL^{-1} bacterium) of a 48-h culture of the target pathogen was seeded on Mueller Hinton (MH) agar. Then, 100 μL of *E. casseliflavus* (containing 1.2×10^7 CFU mL^{-1} bacterium) grown in brain heart infusion broth for 48 h at 25 °C was added to sterile paper discs (5 mm in diameter) and were placed on the surface of MH agar previously inoculated with the target pathogen. The plate was incubated at 25 °C for 48 h. Inhibitory activity was determined by measuring the diameter of zone of inhibition in mm and compare with a positive control (Enrofloxacin, 10 μg).

2.2. Fish

Rainbow trout with an average weight of 38.3 ± 1 g were procured from a local aquaculture farm in Mazandaran province, Iran and transferred to the aquaculture facilities at the Caspian Sea Ecology Research Center (Sari, Iran). The fish were allowed to acclimatize for at least two weeks. During the acclimation period, the fish were provided with a commercial diet (Supplementary Table 1) 3 times a day at a ration corresponding to 3% body weight. After the acclimation period, the fish were inspected, sorted and only those apparently healthy were selected to be used for the feeding experiment. Selected fish were distributed to 12 2000-L fiber glass tanks with a stocking density of 30 fish per tank. Water quality parameters during acclimation and actual experiment were maintained as follows: dissolved oxygen at 7.9 ± 0.5 mg/L, pH at 7.5 ± 0.3, temperature at 16.4 ± 0.9 °C and electrical conductivity at 5348.5 ± 458 MM/cm. The fish were subjected to a 16L:8D photoperiod regime.

All fish handling procedures employed in the study were in accordance to the bioethical principles for animal experimentation at the Caspian Sea Ecology Research Center.

2.3. Preparation of probiotic diets

The probiotic bacteria were cultivated in trypticase soy broth (TSB, pH = 7.3) (Merck, Darmstadt, Germany) for 48 h at 25 °C. The basal diet was prepared in-house (Supplementary Table 1). Bacterial cultures were incorporated in the basal diets following an earlier described method [17]. Three probiotic diets with different final concentration of candidate microorganisms were prepared: **T1** (10^7 CFU g^{-1} of feed), **T2** (10^8 CFU g^{-1} of feed) and **T3** (10^9 CFU g^{-1} of feed). The basal diet served as control. Sterile procedures were adopted during the various steps of feed formulation and preparation. The prepared diets were vacuumed-packed and stored at 4 °C until use. Prior to the actual preparation of experimental diets, several trials were conducted to optimize the incorporation success of the probiotic bacteria and their viability in the diet over a period of time. Two batches of diets were prepared to ensure that fish received equal amount of probiotics in the duration of the experiment.

The experimental diets were manually delivered 3 times a day at a ration corresponding to 3% body weight for a period of 8 weeks. Each experimental group was represented by three randomly allocated replicate tanks.

2.4. Sampling strategies

Feeding was restricted 24 h prior to sample collection. Three fish were randomly collected from each of the triplicate tank of the four experimental diet groups and were anesthetized with clove oil (80 mg L^{-1}, Sigma Aldrich, Steinheim, Germany) before sample collection. Microbiological, hematological and immunological parameters were determined at weeks 4 and 8 of the feeding period while the rest of the parameters were determined only at the end of the feeding trial.

Blood was collected from the caudal vein. One portion was transferred to a tube containing heparin (Sigma) for respiratory burst assay and hematological analysis. The remaining half was