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Feeding pyridoxine prevents *Saprolegnia parasitica* infection in fish *Labeo rohita*

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ABSTRACT

A 60-day experiment was carried out to delineate the role of dietary pyridoxine (DP) in *Labeo rohita* fingerlings in modulating immunity and prevention of fungal infection. Two hundred and seventy fingerlings were randomly distributed into three treatments in triplicates. Three iso-caloric and iso-nitrogenous purified diets were prepared with graded levels of pyridoxine. Three experimental groups were C (0.0% DP), T1 (0.01% DP) and T2 (0.02% DP). The role of dietary pyridoxine in modulating immunity and prevention of fungal infection was assessed by haemato-immunological parameters like erythrocyte counts (EC), leucocyte counts (LC), haemoglobulin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), nitro-blue tetrazolium (NBT), phagocytic activity, albumin, globulin, total plasma protein, albumin/globulin and by challenge study with *Saprolegnia parasitica*, where relative percentage survival (RPS) were recorded. Hb, PCV, MCV, MCH, NBT, total plasma protein, albumin, globulin contents, lysozyme and phagocytic activity was significantly ($P < 0.05$) higher in DP fed group. Significantly ($P < 0.05$) higher RPS was recorded from T2 group fed with 0.02% DP for 45 days. Hence, DP has the capacity to stimulate nonspecific immunity and increase resistance to *S. parasitica* infection in *L. rohita* fingerlings.

1. Introduction

Aquaculture has been expanded, intensified and diversified globally [1] as fastest growing food producing sector to meet the increased demand for quality protein. Asia is the leader in aquaculture production, where Indian major carps, rohu (*Labeo rohita*) is the most preferred fish and share about 35% of the Indian major carps production [2] However, the species is a host for many pathogens for which many chemotherapeutants as well as antibiotics are used for their treatments. But the use of antibiotics and cytotoxic drugs has been widely criticized for their negative impacts like development of drug resistance, accumulation in the tissues, and immunosuppression [3]. Unlike antibiotics and live vaccines, most of the nutraceuticals do not show any adverse impact on fish and environment [4–13]. Thus, they can be used as alternative medicines for treating and managing fish diseases [14].

Saprolegnia is a most abundant oomycete of aquatic origin are present in water and cause great losses to aquaculture and fisheries around the world [15–19]. Earlier, *Saprolegnia* was classified as pathogenic fungi [19,36] and considered to be opportunist facultative parasite [20] saprotrophic and necrotrophic [21], however, recent findings categorise them as oomycete [16]. *Saprolegnia* invades epidermal tissues visible as white or grey patches of filamentous mycelium on fish [21,22], generally beginning on the head or fins [20,23] and can spread over the entire surface of the body. *Saprolegnia* infection has cotton-like appearance that radiates out in a circular, crescent-shaped or whorled pattern. The zoospores of *Saprolegnia* may be transmitted by fish eggs, wild fish, water sources, and equipment [21].

A number of workers have reported *Saprolegnia* infection in fishes; salmonids [22,24], channel catfish [25], pike [26], elver and suckers [27], roach, orfe, carp, tench, lamprey, sturgeon, barramundi, tilapia, and mullet [21], *Labeo rohita, Catla catla, Channa striatus, C. punctatus, Clarias batrachus, Mystus cavasius, M. seenghala, Tilapia mossambica* [28], kissing gourami, guppy, swordfish and platyfish [23,27], *Anabas testudineus* [29]. However, among

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several species of *Saprolegnia*, *S. parasitica*, in particular, appears to be largely responsible for such infections, Saprolegniasis [30,31].

Saprolegnia generally invades fish that have been stressed or otherwise have weakened immune systems [21]. A number of chemicals such as malachite green [21,31], formalin [32], hydrogen peroxide [33,34], sodium chloride at high concentrations [34,35], etc. are available for treatment of *Saprolegnia* infection but a few are approved for use in aquaculture. Formalin treatment [36–38] is currently the most used method against saprolegniasis in fish eggs in fish farms. Heikkinen et al. [39] showed that strong UV irradiation of inlet water decreased *Saprolegnia* infection on eggs. Several studies to find replacements for malachite green (banned in European Union in 2001) have been performed [40], but no better substitute has yet been found.

Many researchers have reported many functions of pyridoxine (vitamin B_6) including pyridoxal and pyridoxamine for absorption and metabolism of amino acids and development of red blood cells [41], coenzymes for transaminases [42], control the biosynthesis of neurotransmitters like GABA, dopamine and serotonin (5-HT), development and function of the central nervous system (CNS) [43] and anti-stress function [12,44,45]. Chen et al. [46] has reported that dietary pyridoxine plays an important role in stimulating the immune responses by enhancing phagocytic activity and respiratory burst activity of abalone, *Haliotis discus hannai*. However, this vitamin has a protective effect against pathogenic *Vibrio anguillarum,* and *Aeromonas hydrophila* in Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum) fingerlings [47] and *Labeo rohita* fingerlings [12]. However, in one of our pilot study (data not shown here) revealed that pyridoxine has the antifungal property (*in-vitro*) against *S. parasitica*. Hence, with all these backdrop information the present study was envisaged to elucidate the possibility of using dietary pyridoxine for protecting *L. rohita* fingerlings against pathogenic *S. parasitica.*

2. Materials and methods

2.1. Experimental animals

The experiment was carried at the laboratory of Department of Aquatic Health and Environment, College of Fisheries, Lembucherra, Tripura. Apparently healthy *L. rohita* fingerlings (average length 12.67 ± 1.5 cm and average weight 22.56 ± 7.5 g) were collected from the college fish farm. The fish were allowed to acclimatize in circular fiber reinforced plastics (FRP) tanks (Plasto Craft, Mumbai) of 500 L under the laboratory condition for 15 days and then used for the experiments. Fishes were provided with adequate aeration and fed with control diet. Satiation level feeding was done twice a day and 30% of water was exchanged daily. Deep tube well water was stocked in 1000 L FRP tanks with aeration and was used for setting the experiment. FRP tanks of 500 L size were used for the experiment. Round the clock aeration was provided.

2.2. Feed preparation and proximate composition

Three iso-nitrogenous(35.16–35.95% crude protein) and iso-caloric (1766.85–1794.61 kJ 100 g^{-1}) purified diets were prepared with graded levels (0, 100 and 200 mg pyridoxine/kg) of pyridoxine (Himedia Laboratories, Mumbai, India) (Table 1). Inclusion rate was decided based on the earlier findings of Akhtar et al. [12,13]. Proximate composition of the experimental diets were analyzed (Table 1) following standard method [48].

2.3. Experimental design and feeding diet

The acclimatized rohu fingerlings (n = 270) were randomly distributed into three distinct experimental groups in triplicates following a completely randomized design (CRD). Randomly selected thirty fish were stocked in circular FRP tanks of 500 L capacity. Five fingerlings were randomly sampled on 15, 30 and 45 d of feeding from each tank without replacement. The control group was fed without pyridoxine whereas the remaining groups were fed with either 0.01% (T1) and 0.02% pyridoxine (T2) for 45 days. For the challenge experiment, remaining 10 fishes from each treatment including control were innoculated with the spores of *S. parasitica* after 45 days of feeding. The water used for the experiment were analysed for various environmental parameters such as temperature, pH, dissolved oxygen (DO), total alkalinity and total ammonia-N following APHA [49]. The water quality parameters such as pH, temperature, dissolved oxygen (DO), total alkalinity, and total ammonia-N were found in the range of 7.0–7.5, 26.5–28.0 °C, 5.2–5.6 mg l^{-1}, 103–110, and 0.01–0.03 mg l^{-1}, respectively, during the experiments, which are in the optimum range required for rohu [50].

2.4. Collection of blood and plasma

At different sampling days (0, 15, 30 and 45 d), each fish was anesthetized with clove oil (Merck, Germany) at 50 μl of clove oil per litre of water before collecting blood samples. Five fish from each tank were used for blood collection and pooled samples from each tank were used for analysis. Blood was drawn from the caudal vein of fish by using 1.0 ml hypo-dermal syringe and 24 gauge needles [51]. The collected blood was immediately transferred into two vials, where vials were coated with thin layer of EDTA, an anticoagulant. One for using blood to measure cell counts, packed cell volume, haemoglobin, and NBT and other for plasma collection. Collection of plasma was done by centrifuging blood samples at 3500 rpm for 15 min at 4 °C and stored at −20 °C until use.

2.5. Culture of Saprolegnia parasitica

2.5.1. Isolation of fungal strains from diseased fish

Isolation of fungal strains were done following Lilley et al. [52]. Briefly, diseased fish samples were brought to the laboratory; fish were killed by decapitation and pinned, to a dissecting board with the lesions uppermost. Then the fish along with the board were brought under laminar air flow. Using a sterile fine pointed forceps and scalpel blade, the muscles pieces were removed. Using aseptic technique, the muscle pieces were placed into a petridish containing GP (glucose-peptone) PenStrep broth (composition: glucose: 3 g L^{-1}, peptone: 1 g L^{-1}, $MgSO_4{\cdot}7H_2O$: 0.128 g L^{-1}, KH_2PO_4: 0.014 g L^{-1}, $CaCL_2.2H_2O$: 0.029 g L^{-1}, $FeCl_3{\cdot}6H_2O$: 2.4 mg L^{-1}, $MnCl_2{\cdot}4H_20$: 1.8 mg L^{-1}, $CuSO_4{\cdot}5H_2O$: mg L^{-1}, $ZnSO_4{\cdot}7H_2O$: 0.4 mg L^{-1} autoclaved at 121 °C for 15 min, cooling to 50 °C and added penicillin-K: 250 units ml^{-1} and streptomycin sulphate: 200 μg ml^{-1}. Inoculated media were incubated at 20 ± 2 °C and after 24 h the emerging hyphal tips were repeatedly transferred to fresh plates of glucose peptone agar (Composition: glucose peptone broth + technical agar: 12 g L^{-1} autoclaved at 121 °C for 15 min, cooling to 50 °C and added penicillin-K: 250 units ml^{-1} and streptomycin sulphate: 200 μg ml^{-1}) until cultures were free from bacterial contamination. Confirmation of *Saprolegnia parasitica* was done following Soderhall et al. [53], Willoughby [23], Lilley et al. [52] and Eissa et al. [54].

2.5.2. Inducing sporulation in fungal culture

Sporulation was induced following Lilley et al. [52]. Briefly, an agar block of actively growing mycelium was placed on a petri-dish containing glucose peptone yeast (GPY) broth (Composition: glucose peptone broth + Yeast extract: 1.5 g L^{-1}) and incubated for