Supplementation Diet Containing Probiotics, Herbal And Azadirachtin On Hematological And Biochemical Changes In *Cirrhina mrigala* Against *Aphanomyces invadans*

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Abstract

Cirrhina mrigala (63 ± 2 g) were intramuscularly (i.m.) administered with 2.3 x 10^7 CFU ml^{-1} of *Aphanomyces invadans* (isolate B99C). The hematological and biochemical parameters were studied in the experimental and control groups for 30 days. In infected untreated group (I), the white blood cell count (WBC: $10^6 mm^{-3}$) was significantly increased ($P < 0.05$) from the control, while no change was seen in groups fed probiotics, herbal and azadirachtin supplementation diets. Similar trend was noted in the haemoglobin (Hb: g/dL) and haematocrit (Hct: %) levels. Interestingly, infected fish fed probiotics, herbal and azadirachtin supplementation diets, did not differ ($P > 0.05$) from the control. A similar trend prevailed in the percentage of lymphocytes (LYM), monocytes (MON), eosinophils (EOS), and neutrophils (NEU). The total protein (TP: g dl^{-1}), glucose (GLU: mg dl^{-1}), calcium (CAL: mmol l^{-1}), and cholesterol (CHO mmol l^{-1}) levels were affected ($P < 0.05$) in the infected group. The present study suggests that the administration of probiotics, herbal, and azadirachtin supplementation diets for 30 days protects the hematological and biochemical parameters in *C. mrigala* from *A. invadans*.

Keywords: *Cirrhina mrigala*; *Aphanomyces invadans*; hematological parameter; biochemical parameter; herbal; probiotic; azadirachtin.

1. Introduction

Asian aquaculture is characterized by an enormous diversity of species, with several dozen marine and freshwater species being farmed. Over the past two decades, Epizootic Ulcerative Syndrome (EUS) is a serious condition affecting a large number of wild and cultured freshwater finfish species. The etiological agent is *Aphanomyces invadans* (also called *A. invaderis* and *A. piscicida*) and a variety of syndromes have been attributed, namely, granulomatous mycosis (GM), epizootic ulcerative syndrome (EUS), red sore disease (RSD), and ulcerative mycosis (UM) [1] in both estuarine and freshwater fish (wild and cultured). The geographical distribution of these *Aphanomyces* infections includes the eastern United States, Australia, Japan, South and Southeast Asia [2], recently in India [3]. The actual economic losses in the aquaculture industry worldwide are estimated to be in excess of US$9 billion per year, which is roughly 15% of the value of the world's farmed fish and shellfish production.

Currently, no effective prophylactic measures are available for EUS disease, although adding lime and salt to the ponds has been used [4]. Hydrogen peroxide and malachite green may have potential as a fungicidal treatment against this pathogen [5,6]. Vaccinations against this fungus are also being explored [7]. Application of these fungicides provides only partial recovery from the disease; besides, accumulation of fungicide residues not only leads to pollution but also consumer's reluctance. At present, many farmers still focus more on treatment than prevention. The prevention and treatment of these infectious diseases by applying products from plant compounds appears as a possible alternative. Hence, the interest in plant compounds as a potential and promising source of pharmaceutical agents has increased during the last few years [8].

Fish should be fed with a balanced diet as nutritional deficiency can have an adverse impact on disease resistance [9]. Many plants have been used in disease prevention by incorporation into fish feeds; in China about 10 herbs are commonly used to treat diseases like enteritis, gill rot, white head and white mouth disease [10]. The HIRM (Herbal Immunoregulation Mixture) is a combination of

several traditional Chinese medicines that can effectively activate the immune system and therefore have immense potential as immunomodulators [11,12] and in stabilizing the improved immune status. Recently, *Azidirachta indica* leaf extracts have been used to protect the blood parameters and increase immunity in fish against *A. invadans* [9,13,14]. Similarly, probiotics enhanced the immune system in fish [15]. The present study aims to assess the hematological and biochemical profile of infected fish after administration of diet containing probiotics, herbal, and azadiractin in *C. mrigala* against *A. invadans*.

2. Methods

2.1. Fish

Cirrhina mrigala (63 ± 2 g; mean ± SD; n = 750) were obtained from a commercial fish farm in Tiruchirapalli, Tamilnadu, India, during March 2006. Fish were transported alive in plastic bags containing water enriched with oxygen. They were acclimatized for 2 weeks in circular cement tanks (200 x 100 x 80 cm) filled with chlorine free tap water and provided with continuous aeration using electric air pumping compressors; 50% of the water was exchanged daily to remove waste feed and faecal materials. During the experiment, water temperature 18 ± 2°C, pH 6.91 ± 1.44, salinity 0.25 ± 0.05 ppt, dissolved oxygen concentration 6.63 ± 7.78 mg^{-1}, and photoperiod 14L : 10D, were maintained. Prior to the experiment, fish were fed with a standard feed intake twice a day at 0900 and 1500 h at a rate of 3% of their body weight.

2.2. Experimental design

The fishes were divided into five groups of 50 fish in each in triplicate. All the groups, except for the control, were administered (2.3 x 10^7 CFU ml^{-1} in autoclaved pond water, APW) spore suspensions of *A. invadans* (isolate B99C) intramuscularly (i.m.) using 1 ml tuberculin syringe with 24-gauge needle into the flank of the fish just below the anterior part of the dorsal fin. The control group received 50 µl of APW without challenge *A. invadans* and given with normal diet (C). On the 7^{th} day, infected fishes were fed with normal diet (I), probiotics diet (P), herbal diet (H), and azadirachtin diet (A). After this, water flow was suspended and individuals were anaesthetized with a buffered (pH 7.0; NaHCO3) tricaine methanesulphonate solution (TMS, 330 mg l^{-1}). Food was withheld for the first 24 h of each experiment and after 24 h fish were sacrificed.

2.3. Aphanomyces invadans strain and growth conditions

Aphanomyces invadans (isolate B99C) isolated from EUS infected carp, *Cirrhinus reba*, was obtained from the Department of Aquaculture, Sterling University, Scotland, UK [13]. The fungi were grown in glucose-peptone yeast (GPY) broth or Griffin's GY agar (Sigma) for 3 days at 20°C; zoospore suspensions were produced by washing mycelium mats five times with sterile distilled water and incubating overnight at 20°C in autoclaved filtered pond water (APW), diluted 1:2 with distilled water having pH 6.4 [16]. The resulting zoospores were enumerated using a Neubauer haemocytometer according to Willoughby and Roberts [17]. Fungal extracts were prepared by adding double strength GPY broth medium (Willoughby and Roberts 1994) to the zoospore suspensions, culturing them for 2 days at 20°C, then extracting the mycelial mats using extraction buffer as given in Lilley et al. [18]. Fungal extracts were stored frozen at -70°C until used.

2.4. Preparation of herbal extract

Azadirachta indica leaves were collected from the Bharathidasan University campus in March 2006. The leaves were collected and washed in sterile distilled water. They were shade-dried, powdered and stored at –20°C until further use. The extraction was done following the methods of Harikrishnan and Balasundaram [19]. Ten grams of the leaf powder placed into sterilized 100 ml conical flasks with 100 ml of solvent (ethanol) and mixed well. The flasks were tightly covered with aluminium foil, kept for 7 days at room temperature and agitated daily to ensure complete extraction. The extract was then filtered through sterile muslin cloth. The filtrate was collected and the solvent was evaporated using a rotary vacuum evaporator (Buchi SMP). The residue obtained after evaporation was mixed with sterile ethanol separately at 0.25% (w/v) in a sterilized screw-cap glass container for further use.

2.5. Probiotic bacteria

The probiotic bacterial strain of *Lactobacillus rhamnosus* was obtained from Intercare (Mehsana, Gujarat) [20]. The bacterium was cultured in MRS broth [21] by cultivating for 48 h at 30°C and subsequently preserved in 50% glycerol at –80°C and kept as stab culture for further use. A pure colony was taken for inoculation of seed cultures of 50 ml each and incubated at 30°C for 24 h before mass culture in MRS broth. After 1 day of culturing, bacteria were harvested by centrifuging at 16,500 × *g* for 10 min and washing 3 times with sterile peptone water (0.85% NaCl and 0.1% polypeptone).

2.6. Preparation of standard diet and supplementation diets

The formulated fish feed was prepared in the laboratory using soybean and fish meal as the protein sources. The prepared standard feed (g/kg) was composed of 18% fish meal, 18% groundnut oil cake, 16% sesame oil cake, 16% soya flour, 16% rice bran, 16% tapioca powder and 0.5% vitamin and mineral mix (w/v) with an approximate composition of 39% protein, 24% carbohydrate, 11% lipid and 9% ash (Table 1). To prepare the probiotics enriched diet, the required amount of bacterial suspension was sprayed into the feed slowly, mixing part by part in a drum mixer, after which it was air-dried under sterile conditions for 12 h. The viability of the incorporated bacterial cells in the feed was assessed by spreading onto triplicate plates of TSA (Becton, Dickinson and Company), MRS agar (Merck) and blood agar (BA, Nissui). The colony count was done after incubation at 30°C for 48 h. The bacterial count of the feed was taken at this point and twice during the trial and averaged 2.45×10^9 CFU g^{-1}. The pellets were dried in an oven at 30°C for 18 h, packed and stored in a freezer at –20°C until used. The final concentration of live *Lactobacillus rhamnosus* in the probiotic feed pellets before the feeding trial was 2.45×10^9 CFU g^{-1}. The supplemented diets were prepared by adding herbal or extracted azadirachtin (each at 0.2%) to standard feed. Feeding was started after the challenge with live *A. invadans* on day 7.

2.7. Hematology and blood biochemistry

Blood samples (0.5 ml) were drawn via the caudal vessels or a cardiac puncture using a heparin-coated needle and syringe (Houston 1990) on 30th day. Six fish from each group were randomly selected for hematological and biochemical analysis. White and red blood cells (WBC: 10^4 mm^{-3} and RBC: 10^6 mm^{-3}) were counted with a haemocytometer using Dacie's diluting fluid [22]. A 1:100 dilution was made by measuring 10 mm^3 of blood with a Thoma pipette into 0.99 cm^3 of diluent. Haematocrit was determined using a microhaematocrit reader (Compur M1100, Lab-Center, Madrid, Spain) and the values were expressed as the percentage of erythrocytes. The haemoglobin concentration was estimated by spectrophotometry (540 nm) using the cyanomethahaemoglobin method with Drabkin's reagent [22]. Leucocytes were counted from one or more blood slides of each group. Each slide was systematically surveyed under a 400x microscope to avoid re-encounting fields until 100 leucocytes were enumerated. Leucocytes were identified as lymphocytes (LYM), monocytes (MON), eosinophils (EOS) and neutrophils (NEU) following Yasutake and Wales [23] and Houston [24]. After reading the Hct, the packed erythrocytes were discarded and the plasma was stored at -12°C. Subsequently, the biochemical indices including the total protein (TP: g dl^{-1}), glucose (GLO: mg dl^{-1}), and cholesterol (CHO: mmol l^{-1}), were determined spectrophotometrically with a Hitachi 704C instrument. The calcium content (CAL: mmol l^{-1}) was determined with flame emission photometry [25].

2.8. Statistical analysis

Experimental data are presented as mean ± SE and were analyzed with 1-way ANOVA followed by Tukey's test to compare the means between individual treatments in SPSS at a significance level of $P < 0.05$.

3. Results

3.1. Primary hematological parameters

The hematological indices of the experimental groups are presented in Fig. 1. The white blood cells (WBC) of infected untreated (I) group were increased significantly ($P < 0.01$) after 30 days than in the control group. This value did not increase ($P > 0.05$) in the probiotics, herbal and azadirachtin supplementation diet treated groups. The red blood cells (RBC), haemoglobin (Hb), and haematocrit (Hct) of infected untreated group were significantly lower from the control values ($P < 0.01$). Infected fish after administration with probiotics, herbal, and azadirachtin supplementation diet did not show significant change ($P > 0.01$).

3.2. Differential leucocytes

The differential leucocyte i.e. lympocytes (LYM), monocytes (MON), eosinophils (EOS), and neutrophils (NEU), percentage in the infected untreated (I) group was reduced ($P < 0.05$) during the infection compared to the control group. However, infected fish treated with probiotics, herbal, and azadirachtin supplementation diet did not show any effect ($P > 0.05$) after 30 days (Fig. 2).

3.3. Biochemical profile

The total protein (TP), glucose (GLU), calcium (CAL), and cholesterol (CHO) level in the infected untreated (I) significantly decreased ($P < 0.05$) from the control values. On the other hand, infected fish after administration with probiotics, herbal, and azadirachtin supplementation diet did not show an increase ($P > 0.05$) (Fig. 3).

4. Discussion

In aquaculture, chemotherapeutic agents such as commercial antibiotics and disinfectants (chemicals) are commonly employed for disease management, although this is not advisable due to high cost, environmental hazards, and the antibiotic resistance developed by many pathogens [26]. In the present study, three different supplementation diets i.e. probiotics, herbal and azadirachtin were tested for hematological and biochemical changes in *C. mrigala* against *A. invadans*. The evaluation of hematological parameters can be useful for the diagnosis of pathological and physiological status in fish [27-29]. The blood appears to be mediated by a reduction in the number of circulating white blood cells (WBCs), particularly lymphocytes [30], and/or the suppression of their activity [31]. Both the degree of virulence of the infective agent and the degree of susceptibility of the host are important in determining whether a pathogenic challenge results in disease.

Table 1. Composition of diets for *Cirrhina mrigala*.

Component Composition	Concentration (%)
Standard diet	
Fish meal	18
Ground nut oil cake	18
Sesame oil cake	16
Soya flour	16
Rice bran	16
Tapioca flour	16
Vitamin[a] and mineral[b] mix	0.5
Additions to standard diet	
Lactobacillus rhamnosus	0.2
Herbal	0.2
Azadirachtin	0.2

[a]Composition (g 100 g–1 premix): thiamin hydrochloride (0.72), riboflavin (1.21), pyridoxine hydrochloride (0.48), cyanocobalamin (0.06), ascorbic acid (60.40), niacin (4.83), calcium pantothenate (1.21), inositol (24.15), biotin (3.62), folic acid (0.18), β-aminobenzoic acid (0.60), vitamin A acetate (0.97), vitamin D3 (0.97) and vitamin K3 (0.60)

[b]Composition (g 100 g–1): NaCl (1), $MgSO_4 \cdot 7H_2O$ (15), $NaH_2PO_4 \cdot 2H_2O$ (25), KH_2PO_4 (32), $Ca(H_2PO_4) \cdot 2H_2O$ (20), $FeC_6H_5O_7 \cdot H_2O$ (2.5), $ZnSO_4 \cdot 7H_2O$ (1.2), $MnSO_4 \cdot 5H_2O$ (0.6), $CuSO_4 \cdot 5H_2O$ (0.1), $CoCl_3 \cdot 6H_2O$ (0.0035), KIO_3 (0.0105) and cellulose (1.586)

The results presented in this study have revealed an interesting pattern showing that the number of RBCs significantly decreased ($P < 0.05$) in the infected untreated group compared to the control group. The RBC abnormalities, including viral inclusions, haemoglobin cysts and haemoparasites [32], are linked to nutritional status [33]. The reduced size of the erythrocytes implies that there is shorter mean diffusion path for oxygen; in addition to a higher concentration of erythrocytes, this change would make the oxygen uptake over the gills and oxygen release at the different tissues more efficient [34]. The haemolytic activity in fish serum against heterologous RBCs is considered to reflect significant components of natural defense mechanisms [35,36]. In the present study, infected fish after administration with probiotics and azadirachtin enriched diet were restored ($P > 0.05$) the WBC and RBC after 30 days, to the control values. Recently, many plants and their compounds have been used in disease prevention like enteritis, gill rot, white head and white mouth disease by incorporation into fish feeds [10] and they effectively activate the fish immune system [11,12]. On the other hand, herbal treatment restored the altered hematological parameter against bacterial and fungal disease in fishes [13,37].

The haemoglobin (Hb) concentration in the infected untreated fish was down significantly ($P < 0.05$) over the control value for 30 days, but the Hb level of probiotics, herbal, and azadirachtin diet treated groups did not show any change ($P > 0.05$) compared to control group. The decreased Hb trend may be a result of the swelling of the RBCs as well as poor mobilization of Hb from the spleen to other hemopoeitic organs in *Ictalurus punctatus* [38]. These data support the present finding that the significant decrease in RBCs and Hb content is possibly due to hypochromic microcytic anemia caused by *A. invadans*. Scott and Rogers [38] also reported that the significant increase in Hb in stressed fish leads to elevated oxygen carrying capacity of the individual erythrocytes in *I. punctatus*.

Zanjani et al. [39] assumed that Hb is related to the oxygen requirement and may act as a control mechanism in erythropoiesis in teleost fish.

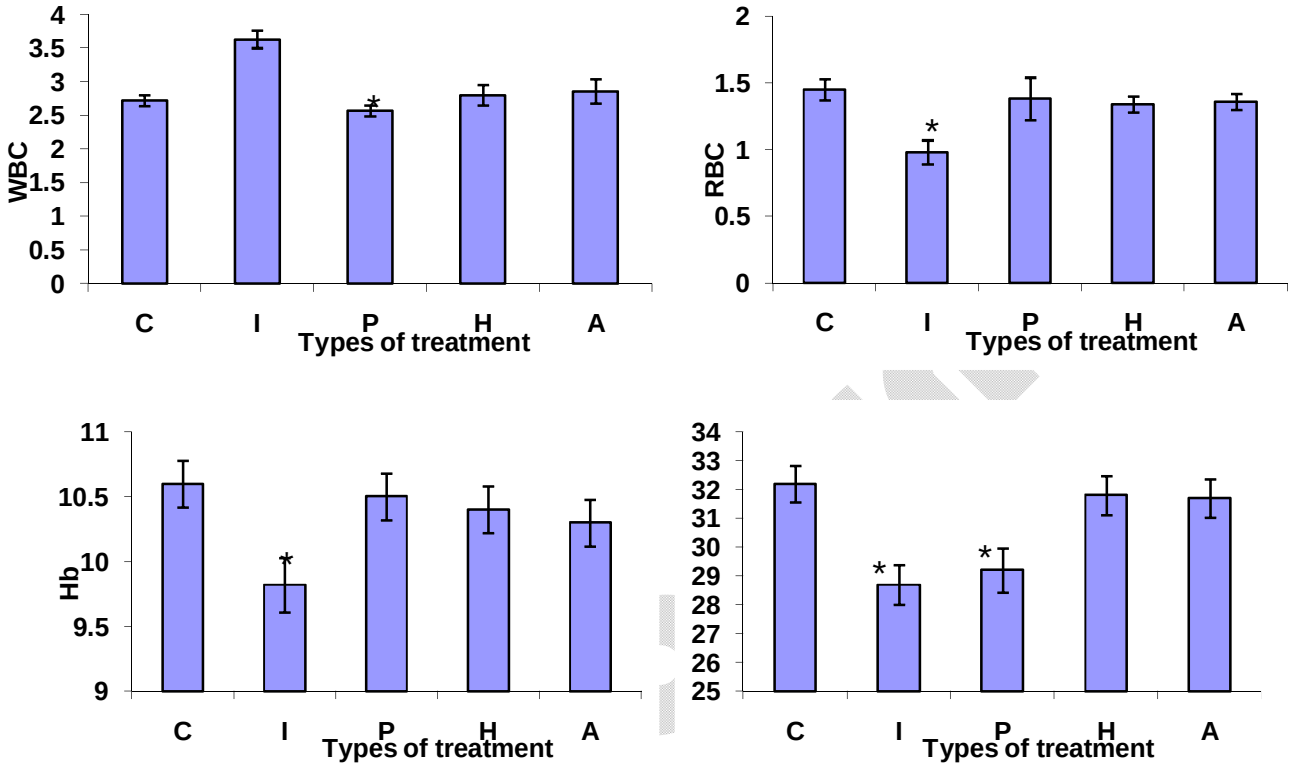
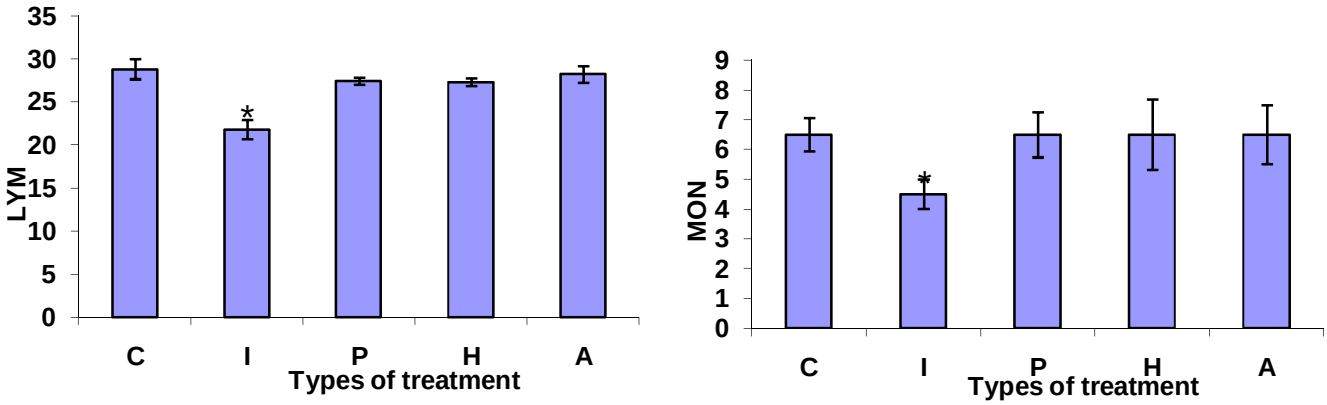


Fig. 1. Changes in the hematological parameters after administration with probiotics, herbal, and azadirachtin supplementation diets in *C. mrigala* against *A. invadans*. WBC: while blod cells, RBC: red blod cells, Hb: haemoglobin, Ht: haematocrit, C: control, I: infected untreated, P: probiotics diet, H: herbal diet, A: azadiractin diet.



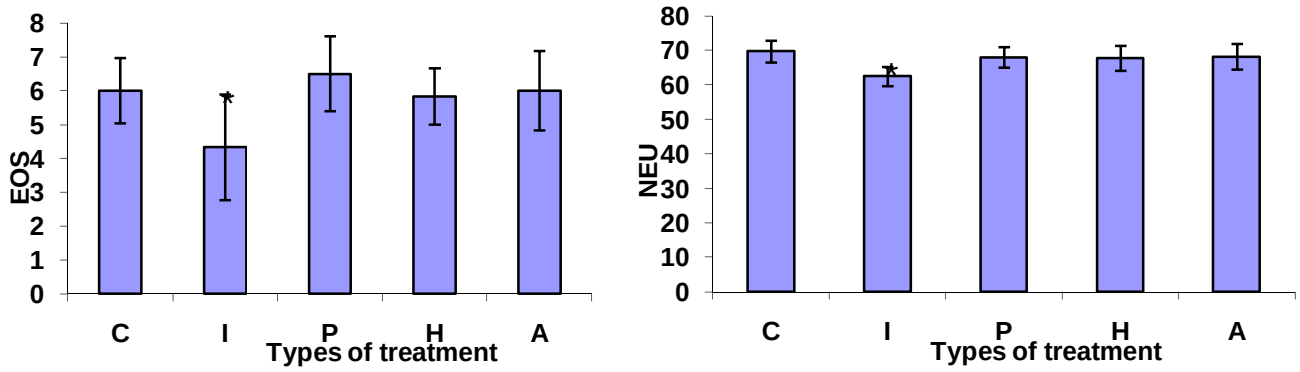


Fig. 2. Changes in the differential leucocytes after administration with probiotics, herbal, and azadirachtin supplementation diets in *C. mrigala* against *A. invadans*. LYM: lymphocytes, MON: monocytes, EOS: eosinophils, NEU: neutrophils, C: control, I: infected untreated, P: probiotics diet, H: herbal diet, A: azadiractin diet.

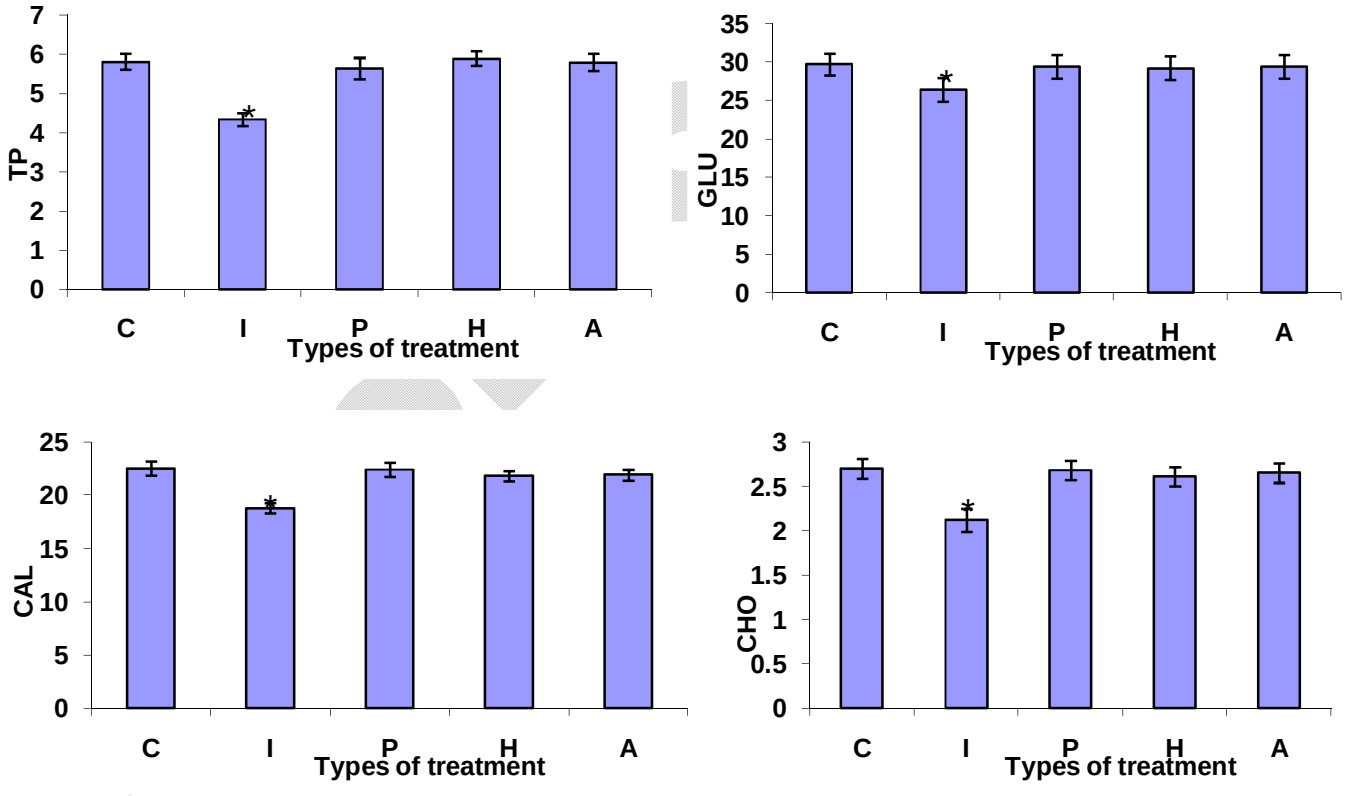


Fig. 3. Changes in the biochemical parameters after administration with probiotics, herbal and azadirachtin supplementation diets in *C. mrigala* against *A. invadans*. TP: total protein, GLU: glucose, CAL: calcium, CHO: cholesterol, C: control, I: infected untreated, P: probiotics diet, H: herbal diet, A: azadiractin diet.

In the present study, the infected untreated group had a decline in RBCs and Hb relative to the control, resulting in an anaemic condition. The smaller cells in this study were less susceptible to osmotic stress than the unexposed RBCs and could withstand greater osmotic swelling before rupturing [40]. The decreasing RBC, Ht, and Hb values indicate that RBCs are being destroyed by the leucocytosis in erythrocytic anaemia with subsequent erythroblastosis [41]. On the other hand, increase in Ht level has been reported as a result of oxygen deficiency [42]. In our experiments, the Ht level significantly decreased ($P < 0.05$) in infected untreated fish during the 30 days and increased in the diet treated groups. For instance, the pearl spot fish *Etroplus suratensis* when infected with EUS becomes anaemic and then suffers a significant reduction in RBC, Hb, and PCV levels [43]. In this study, infected untreated fishes eventually suffered a hypochromic, macrocytic anaemic condition that is attributable to the swelling of the RBCs, haemodilution and impaired Hb synthesis.

Neutrophils were also lower in *Aphanomyces*-infected *C. mrigala* and the same was suggested in a study of brown trout and rainbow trout [30,44]. Although erythrocytes, thrombocytes, eosinophils, basophils, lymphocytes, and monocytes are morphologically similar in reptiles, there are notable species differences in heterophils and azurophils [45]. Herein, neutrophil was also the main finding in *C. mrigala* diet treatment groups. Moreover, acute stress or different supplementary diet treatments in *C. mrigala* was found to decrease the number of circulating leucocytes in the present study but, in other studies, the number of these cells in the thymus and head kidney were found to increase [46].

Our paper indicates that the total protein level was significantly affected ($P < 0.05$) in the infected untreated group compared to the control. We observed no marked change ($P > 0.05$) in serum total protein, glucose, calcium, and cholesterol in the diet treated groups. Total plasma protein also increased due to the destruction of RBCs and the resultant release of cell contents into the blood stream [41]. Scott and Rogers [38] reported that the plasma protein value did not vary significantly ($P > 0.05$) from that of the control in infected fish. Yavuzcan Yildiz et al. [47] have reported higher total protein and lower Ht values in *Oreochromis niloticus* than those measured in our study. Similarly, in another study in *O. niloticus*, Hussein et al. [48] found higher total protein, glucose, cholesterol, and Ht. Rainbow trout and golden trout were also found to have lower calcium and total protein levels [49,50]; hybrid striped bass have higher total protein, chloride, and calcium levels as well [51].

Several herbal principles have been tested for their growth-promoting activity in aquatic animals [52,53]. The use of probiotics, herbals, and their active compounds in aquaculture is comparatively new, but they are becoming recognized as being important for disease control [13,54]. Probiotics in aquaculture can be effectively employed to help fish protect themselves and promote safe farming that would be less dependent on chemotherapy and vaccine for disease prevention [55]. The different hematological parameters of the present work revealed that these probiotics and herbal supplementation diets provide effective protection in *C. mrigala* against *A. invadans* infection.

5. Competing Interests

This work was financially supported by Brain Korea 21 programme of the Ministry of Education, South Korea. Otherwise, the authors declare that they do not have any competing interests.

6. Authors' Contributions

All authors contributed more or less equally to this research work.

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