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Effects of Dietary Blend of Algae Extract Supplementation on Growth, Biochemical, Haemato-Immunological Response, and Immune Gene Expression in *Labeo rohita* with *Aeromonas hydrophila* Post-Challenges

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Abstract: In this study, the effects of a mixed algal blend (*Chlorella vulgaris*, *Euglena viridis*, and *Spirulina platensis*) at different levels were evaluated on growth, hematological immune responses, and expression of immune genes in *Labeo rohita* against post-challenges of *Aeromonas hydrophila*. Fish samples were fed a diet containing different levels of mixed blend algal (0, 0.01, 0.02, 0.04 and 0.08% of basal diet). At the end of the feeding period, the fish were challenged with *A. hydrophila* and fish mortality was recorded over a 14-days period. To evaluate the serum biochemical (albumin, globulin), hematological parameters (Hb, RBC and WBC) and immune parameters (neutrophil activity, lysozyme activity, myeloperoxidase activity, antiprotease activity, ceruloplasmin activity, and bactericidal activity), as well as the expression of immune genes (NKEF-B, Lysozyme C and G, TNF α, TLR22, β2M, and β-actin), fish were sampled on Day 7, 14, 21 and 28. Fish were challenged with virulent *A. hydrophila* 30 days post-feeding and mortalities were recorded over 30 days post-infection. Results demonstrate that fish fed with a mixed algal blend showed that total body weight gain, specific growth rate, total serum protein, globulin, total hemoglobin content, white blood cells, neutrophil, lysozyme, bactericidal, myeloperoxidase, and antiprotease activity in dietary algae blended application was higher than in the control ($p < 0.05$). According to the results, relative expression of target genes showed significant increases of 0.02 to 0.04% in the treatment group compared to the control group ($p < 0.05$). At the end of the 30-day exposure to *A. hydrophila*, the fish that received the mixed algal blend had a significantly higher rate of survival than the control group, with the highest survival rate recorded in the 0.02% mixed algal blend ($p < 0.05$). According to the effective results of the mixed algal blend on stimulating the immune system and increasing fish resistance to *A. hydrophila*, it is recommended to use 0.02 to 0.04% of this mixed algal blend in rohu, *L. rohita* diets.

Keywords: mixed algae; rohu; *Aeromonas hydrophila*; immune gene expression

1. Introduction

The association between fish health and feed content is a significant issue in fish farming. Diets with naturally functioning elements that can enhance fish immunity to illness are especially significant [1,2]. Additionally, the application of herbal immunostimulants to manage disease and boost the innate and acquired immune response of fish has been established in many countries as has the use of plant extracts and powders that have the potential to replace chemical medications [3]. These substances also have less negative side effects than chemical and synthetic substances, and when taken properly, they can even have positive side effects. In aquaculture systems, particularly with farmed aquatic species, plant-derived immunostimulants, such as seaweed as a substitute for antibiotics, can be crucial in boosting the immune system and preventing sickness. These stimulants increase fish illness resistance by boosting the non-specific immune system's effectiveness [4].

Previous studies assessed the inclusion of significant macroalgal species in the diets of aquaculture species, including *Ulva* [5], *Sargassum* sp. [6], *Porphyra* [7], and *Gracilaria gracilis* [1]. Due to their low levels of saturated fats, low-calorie content, high levels of carbohydrates, and anti-bacterial, antioxidant, antiviral, and antifungal biological properties, as well as their high levels of minerals, dietary fiber, vitamins, proteins, and polyunsaturated fatty acids, marine algae have been widely used as natural ingredients in the food and pharmaceutical industries [8]. Red algae appear to be one of the best macroalgae species for animal feeding because of their relatively high protein content and the presence of bioactive chemicals with a variety of structures that have tremendous promise for use in medicine and pharmaceuticals [9]. Algae and other plant-based feed additives can aid in fish growth [10], disease prevention [11], immune system bolstering [12], hunger stimulation and increased feed consumption [13], stress reduction [14], and digestion improvement by boosting the secretion of various digestive enzymes [15]. Additionally, they possess antiviral and antibacterial activities [16]. Algae and other plants are widely employed in the place of expensive antibiotics in the management of fish health because they are less expensive, environmentally benign, and have less adverse effects [17].

Three filamentous blue-green algae, *Spirulina platensis*, *Euglena viridis*, and *Chlorella vulgaris*, have the potential to be applied in aquafeed as growth and immune stimulants [18,19]. High-protein content can be found in dried *Spirulina* and *Chlorella* powder (up to 57% of dry weight). Gamma-linolenic acid (GLA), polysaccharides, phycobiliproteins, carotenoids, vitamins (particularly B12), and colors such as carotenoids and minerals are also abundant in it. *Spirulina*'s immune-boosting effects have also been documented in research on a variety of fish species [20]. The relevance of engaging non-specific immune mechanisms, the advantages of macro- and microalgae, and changes in immune gene expression as indicators of improved fish immune states have all been demonstrated by research [21]. The current study is, therefore, the first of its kind in India to examine the effects of a green microalgal extract blend on growth, biochemical, haematological, immunological responses and gene expression in rohu resistance to *A. hydrophila* challenge.

2. Materials and Methods

2.1. Collection and Culture of Algae

Spirulina platensis was grown in an enhanced medium [22]. The corresponding medium was sterilized for 20 min at 100 mL at 121 °C. Each beaker was a subculture with a 10 mL culture representing 10^7–10^8 colony-forming units/mL, incubated for 7 days and then used for 21 days as a 1 L sterilizing inoculum of the specific medium. The cultures were maintained in controlled laboratory conditions of 23 ± 2 °C. During the course of incubation, the container was supplied with sterile air using a pump to stir and controversially transmit the culture. The culture was filtered using a sterile cotton cloth and washed with distilled water to dispense the remaining nutrients. The cells were air-dried for 24 h at room temperature and then in an oven at 70 °C to obtain the adjusted weight.

Euglena viridis blooms were harvested from private fish farm ponds (0.2–2.5 ha), Chidamparam, Tamil Nadu, India with a plankton net. Under the magnification, samples

were observed as elongated dynamic mobile unicellular (movement of euglenoids) and dark greenish filaments, identified as *E. viridis* [23]. The collected samples were washed thrice with sterilized distilled water to confiscate suspended particles, and centrifuged at 1000 rpm using a macro-rotor (Sorvall™ WX, Thermo Fisher Scientific, Waltham, MA, USA). The pellet was extracted and dried at room temperature for 2–3 days.

Chlorella vulgaris was cultured in a glass container (20 L) of seawater. Seawater (30 ppt) was obtained, filtered and sterilized with Erd-Schreiber medium at 120 °C for 30 min (0.5 µm pore-size Millipore filters) [24]. Cultures were maintained with sufficient aeration and constant illumination at 24 ± 1 °C. Continuous-flow centrifugation at 10 L/h resulted in the algae for 40 min at 4 °C at 2000 g. The samples of dried cells were analyzed using the methods presented in [25].

2.2. Preparation of Experimental Diet

The three separate microalgae were individually dried for 7 days until the desired weight was achieved. In an electric mixer, each sample was finely powdered. The algal blend (*C. vulgaris*, *E. viridis* and *S. platensis*) was mixed uniformly at a proportion of 1:1:1. The mixed algal extract was made up to 1000 mL sterile distilled water with 100 g of the mixture dissolved or soaked. Securely protected with aluminum foil, conical flasks were kept at room temperature for 7 days and stirred periodically at regular intervals. The extract was filtered through a sterile muslin cloth and the solvent evaporated using a rotary vacuum evaporator. The residue collected was mixed separately with the normal diet (Table 1). In order to enhance the balanced diet, mixed extracts of 0, 0.01, 0.02, 0.04, and 0.08 % were slowly soaked into the feed, mixed part by part in a drum mixer under sterile conditions, further air-dried for 12 h. The pellets were dried in the oven at 30 °C for 18 h, packed and stored in the freezer at −20 °C until further use [26].

Table 1. Composition of feed and proximate composition of the diets and tissue of the experiment.

Experimental Diet (g/kg)					
Ingredients (g/kg)	**Control (0 %)**	**T1 (0.01%)**	**T2 (0.02%)**	**T3 (0.04%)**	**T4 (0.08%)**
Rice bran (g)	170	169	168	166	162
Soybean meal (g)	550	550	550	550	550
Fish meal (g)	50	50	50	50	50
Wheat flour (g)	20	20	20	20	20
Corn flour (g)	150	150	150	150	150
Vegetable oil (v/w)	25	25	25	25	25
Iodized salt (g)	10	10	10	10	10
Vitamin and minerals *	25	25	25	25	25
Mixed algal blend (%) (w/w)	Absent	0.01	0.02	0.04	0.08
Proximate Composition of Diets (%)					
Total protein	35.6 ± 4.54 ^{c}	35.2 ± 4.89 ^{b}	35.6 ± 5.33 ^{c}	35.2 ± 4.21 ^{b}	33.5 ± 4.49 ^{a}
Total carbohydrate	94.2 ± 5.35 ^{a}	94.4 ± 4.69 ^{a}	94.3 ± 5.55 ^{a}	94.2 ± 5.33 ^{a}	94.4 ± 5.39 ^{a}
Total lipid	120.4 ± 10.43 ^{a}	124 ±10.35 ^{b}	124.2 ±12.55 ^{b}	124.6 ± 9.85 ^{b}	124.9 ± 10.54 ^{b}
Moisture (%)	4.0 ± 0.89 ^{b}	3.5 ± 0.84 ^{a}	4.8 ±1.04 ^{d}	4.2 ± 1.90 ^{b}	4.3 ± 0.92 ^{c}
Ash (%)	7.5 ± 0.99 ^{a}	9.0 ± 1.40 ^{b}	9.4 ± 0.82 ^{b}	8.7 ± 1.04 ^{b}	9.1 ± 1.03 ^{b}
Dry matter (%)	87.4 ± 5.32 ^{a}	98.9 ±4.35 ^{b}	93.0 ± 5.43 ^{b}	91.3 ± 5.52 ^{b}	94.5 ± 4.37 ^{b}
Proximate Composition of Tissue (%)					
Total protein	216 ± 18.8 ^{a}	284 ± 18.54 ^{d}	233 ± 19.54 ^{b}	240 ± 19.54 ^{b}	258 ± 18.95 ^{c}
Total carbohydrate	143 ± 12.54 ^{a}	235 ± 21.64 ^{e}	213 ± 22.87 ^{d}	208 ± 23.98 ^{c}	194 ± 22.87 ^{b}
Total lipid	98 ± 10.84 ^{d}	77 ± 10.8 ^{b}	89 ± 9.95 ^{c}	69 ± 8.69 ^{a}	98 ± 10.84 ^{d}
Moisture (%)	25.7 ± 8.12 ^{b}	47.6 ± 7.95 ^{e}	39.2 ± 9.44 ^{c}	26.7 ± 5.87 ^{b}	43.2 ± 8.65 ^{d}
Ash (%)	26.8 ± 5.65 ^{b}	26.4 ± 6.84 ^{b}	29.9 ± 6.35 ^{c}	25.5 ± 6.78 ^{b}	21.3 ± 6.92 ^{a}
Dry matter (%)	89.7 ± 12.25 ^{b}	85.8 ± 13.68 ^{a}	98.6 ± 15.84 ^{e}	90.4 ± 16.32 ^{c}	94.8 ± 15.98 ^{d}

Table 1. *Cont.*

Experimental Diet (g/kg)					
Ingredients (g/kg)	**Control (0 %)**	**T1 (0.01%)**	**T2 (0.02%)**	**T3 (0.04%)**	**T4 (0.08%)**
Proximate Composition of Microalgae (%)					
Proximate composition	Different green micro-algae				
	Spirulina platensis	*Euglena viridis*	*Chlorella vulgaris*	Mixed algae blend	
Total protein	26.84	25.59	27.65	32.70	
Total carbohydrate	38.45	36.07	36.95	42.58	
Ether extract	6.54	6.85	7.22	8.24	
Ash (%)	7.14	8.20	7.65	8.65	
Dry matter (%)	78.52	78.25	78.96	78.65	

* Composition of supply for 1 kg dry weight: (Virbac Animal Health, India). Nicotinamide (1000 mg), cobalt (150 mg), copper (1200 mg), iodine (325 mg), iron (1500 mg), manganese (1500 mg), potassium (100 mg), selenium (10 mg), sodium (5.9 mg), sulfur (0.72 percent), zinc (9600 mg), calcium (25.5 percent), phosphorous (25.5 percent), vitamin A (7,000,000 IU), vitamin D3 (70,000 IU), vitamin E (250 mg) (12.75 percent).Values observed were expressed as mean ± standard error. $^{a-e}$ Means in the same row with different superscript differ significantly ($p < 0.05$).

2.3. Proximate Analysis

Standard methods were used to analyze the proximate feed and body carcass [25]. The moisture content was determined by drying the samples at 110 °C in a 24 h hot-air oven. The crude protein content was estimated using the micro-Kjeldahl method, and the crude lipid content was estimated using the Soxhlet extraction method. Samples were incinerated in a muffle furnace at 500 ± 50 °C for 10 h to estimate the ash content. For the crude lipid, DE fattened samples were estimated.

2.4. Experimental Design, Animal, and Maintenance

Rohu (*L. rohita*) fish (n = 600, with an average weight of 100.50 ± 2.50 g) exhibiting no symptoms of infection and no prior clinical pathogenesis from the private farm, Dimapur, Nagaland, India, were obtained for this study. In plastic tanks with a capacity of 600 L, fish were acclimatized using dechlorinated tap water for 15 days before the investigation. Together with the remaining feed and fecal matter, approximately 10 percent of water was removed daily. The fish were divided into 15 tanks, with triplicates being maintained for each tank containing 40 fish. Basal diets were fed to the control group and the treatment groups were fed for 28 days with 0.01%-T1; 0.02%-T2; 0.04%-T3; and 0.08%-T4. Throughout the experiment, all of the groups were fed their respective diets. Under a normal light regime (light/dark 12/12), 4 percent of the fish's body weight was fed twice daily at 10:00 and 18:00 h. Uneaten food and feces were removed daily, and 50 percent of the water was replaced with clean borewell water that was kept at the same temperature. Round clock aeration was provided. Physicochemical parameters remained within the optimum range throughout the experiment (dissolved oxygen: 6.5–7.4 mg/dL; pH: 7.2–8.1; ammonia: 0.12–0.25 mg/L; nitrite: 0.001–0.006 mg/L; nitrate: 0.02–0.06 mg/L). The experimental protocol and analysis were approved by the Animal Care and Use Institutional Ethics Committee (SJU/ZOO/348/2021.12.18), conducted in the Animal Research Center at St. Joseph University, Nagaland, India.

2.5. Blood Sample and Measurements

Collecting Blood and Separating the Serum

Fish were fed for 24 h before blood samples were taken from six randomly selected fish from each triplicate, for a total of eighteen fish from each treatment, at seven-day intervals (day initial, 7, 14, 21 and 28). Blood was collected from the caudal vein with a 1 mL tuberculin syringe fitted with a 24-gauge needle rinsed with heparin (15 unit mL^{-1}) and stored at 4 °C for use the next day after the fish were anaesthetized with 0.1 ppm MS-222. Without heparin, blood samples were collected, allowed to clot, centrifuged at 6000 g, and serum was collected

and centrifuged again. For the estimation of hematological, immunological, and biochemical parameters, serum was pooled into six groups based on volume [27].

2.6. Growth Measurements

Fifteen fish were randomly selected from each tank on Day 1 and 30 post-feeding and the batch was weighed to calculate the growth parameters. Growth performances were assessed in terms of total weight gain (TWG), specific growth rate (SGR), and feed conversion rate (FCR).

$TWG\ (g) = W_t - W_0$

$SGR\ (\%/d) = 100 \times (\text{Ln}\ W_t - \text{Ln}\ W_0)/t$

$FCR = FI/\ (W_t - W_0)$

Where W_t and W_0 are final weight and initial weight of fish, respectively; t is the duration of feeding (days); FI is feed intake [28].

2.7. Serum Biochemical Assays

The different serum samples collected earlier were analyzed for total protein, following the method of Bradford [29], albumin content, following the method of Doumas et al. [30], serum total protein, and globulin content (subtracting albumin from total protein), and the albumin-globulin (A:G) ratio was produced.

2.8. Hematological Parameters

The total count of red blood cells (RBC, 10^6 cells/cu mm^3) and white blood cells (WBC, 10^3 cells/cu mm^3) was determined using a hemocytometer. Haemoglobin concentrations (Hb g/dL) were determined spectrophotometrically at 540 nm by measurement of the cyanmethemoglobin method [26].

2.9. Immunological Assays

2.9.1. Respiratory Burst Assay (Neutrophil Activity)

Using the Stasiack and Bauman [31] method, the respiratory burst activity was determined. In brief, 100 µL of heparinized blood was placed in a 100 µL solution of 0.2 percent nitroblue tetrazolium (Sigma, St. Louis, MO, USA) and the final solution was homogenized for 30 min and incubated at 25 °C. Following incubation and the second homogenization, 50 µL of the solution was added to 1 mL of N, N-dimethyl formamide (Sigma, St. Louis, MO, USA). Again, this solution was homogenized and centrifuged at 3000 g over a period of 5 min. The optical density of the supernatant was set at 540 nm on a spectrophotometer (Biospectro SP-220, Curitiba, Brazil).

2.9.2. Lysozyme Assay

Lysozyme activity was determined according to the method with minor changes being made [32]. In turbidimetric assay, a substrate of 0.03% lyophilized *Micrococcus luetus* in a 0.05 mM sodium phosphate buffer (pH 6.2) was used. About 10 µL of fish serum was placed in 250 µL of bacterial suspension with a 'U' bottom microtiter plate in a duplicate well, and a reduction in absorption at 490 nm was determined after 0.5 and 4.5 min of incubation at 22 °C using a microplate reader. The decrease in absorbance was defined as one unit of lysozyme activity at 0.001 per min.

2.9.3. Ceruloplasmin and Myeloperoxidase (MPO) Assay

In the method described earlier, with minor modification, ceruloplasmin activity in serum was measured as p-phenylene diamine oxidase activity [26]. The amount of oxidase that catalyzed a decrease in absorbance of 0.001 min^{-1} at 550 nm was defined as one unit of ceruloplasmin. Using the described method [33], the total amount of myeloperoxidase activity was determined. A microplate reader recorded the OD at 450 nm.

2.9.4. Bactericidal Assay

An adequate amount of 100 µL serum and bacterial suspension (105 cfu/mL) were mixed and incubated for 1 h at room temperature, according to the standard serum bactericidal activity procedure [34]. Serum was replaced with sterile phosphate-buffered saline (PBS) for blank control. At a ratio of 1:10, the solution was diluted again with PBS. At 37 °C with 24 h of incubation, the diluted 100 µL suspension was poured on nutrient agar plates. The number of bacterial cells was determined by counting with nutrients the new colonies cultivated in the agar plates.

2.9.5. Serum Antiprotease Assay

According to Rao and Chakrabarti [35], the total antiprotease activity was determined with minor modifications. Approximately 10 µL of undiluted serum was initially incubated with duplicate trypsin solution (Trypsin bovine pancreas, 0.01 M TrisHCl, pH 8.2, HiMedia, India), followed by the addition of 500 µL of 2 mM BAPNA substrate (sodium-benzoyl-DL-arginine-p-nitroanilide HCL, HiMedia, India) to a 1 mL volume with 0.1 M Tris HCL (pH 8.2) incubated at 22 °C for 25 min. The reaction was stopped with 30 percent acetic acid, and optical density was read against the blank in a microplate reader at 415 nm. The antiprotease inhibitory capacity was expressed in terms of the percentage of inhibition of trypsin.

$$\text{Percent inhibition (\%)} = (\text{OD reference} \times \text{OD sample}/\text{OD reference}) \times 100 \quad (1)$$

2.10. Challenge Test

The seven-day lethal dose 50 (LD_{50}) for *A. hydrophila* (MTCC-1739) was 10^7 cfu mL^{-1} as determined. After the 28-day feeding trial, 15 fish from each treatment group (5 fish × 3 tanks) were injected intraperitoneally with 100 µL of PBS containing 1×10^7 live *A. hydrophila*. The challenged fish were kept under observation for 30 days and fed a basal diet. The survival percentage in each group was recorded up to 30 days post-challenge. Survival was calculated as: relative percentage survival (RPS) [36].

$$\text{RPS} = \text{No. of surviving fish after challenge}/\text{No. of fish injected with bacteria} \times 100 \quad (2)$$

2.11. Immune-Related Gene Expression Analysis

Ten fish (n = 10) were intraperitoneally injected with a virulent bacterial suspension at the termination of the feeding session. Following the bacterial injection, the fish were fed for 24 h in accordance with the feeding protocol (similar to the feeding period). All dead fish's spleens and head kidneys underwent microbiological tests, which revealed the presence of germs. Fish were tested for the expression of immune genes using existing techniques after the pathogenicity period.

Synthesis of cDNA, PCR and Expression Analysis

Total RNA was extracted from spleen and kidney cells using the total RNA kit (A&A Biotechnology Gdansk, Poland) according to the manufacturer's instructions. The total RNA concentration and purity were measured spectrophotometrically using a NanoDrip reader (Thermo Fisher Scientific) and RNA was stored at −20 °C [35].

A mixture of 5 µL of isolated RNA, 9.5 µL of water and 1 µL of p(dN)6 (Invitrogen, Bangalore, India) were denatured at a temperature of 65 °C for 5 min, and then transferred to an ice bed for 5 min. Then, 5 µL of reverse transcriptase (Fermentas, Poland), 2.5 µL of dNTP (2 mM Fermentas, Poland), 1.0 µL of ribonuclease inhibitor (10 u/µL, Fermentas, Poland), 1.0 µL of reverse transcriptase (200 u/µL, Fermentas, Poland) were added. The cDNA synthesis was performed at a temperature of 50 °C for 30 min in a Biometra thermocucler (Biometra, Goettingen, Germany). The reaction mixture was then maintained at a temperature of 70 °C for 15 min. Samples were fixed with 20 µL demineralized water and stored at −20 °C until future use [29].

The real-time PCR reaction for all cDNA samples was carried out using the Corbett apparatus. Pairs of carp-specific primers for β-actin, NKEF-B, TNF α, TLR 22, β2M and lysozyme C and G were used in the reaction (Table 2). The PCR was carried out in real time with the SYBR Green 1 dye, in thin-walled test tubes with a capacity of 100 µL. The DyNAmo HS SYBR Green qPCR Kit (Finnzymes, Poland) was used, allowing us to conduct a high-specificity reaction. About 20 µL of the reaction mixture consisted of the following components: 2 µL of the DNA matrix, 7.2 µL of water, 0.4 µL of each primer (final concentration 50 pM), 10 µL pf the Master Mix containing a Hot Start version of the modified polymerase Tbr (Thermus brockianus), buffer for the polymerase Tbr, dNTP, $MgCl_2$ and the intercalating SYBR Green 1 dye [35].

Table 2. Primers that are used for real-time quantitative PCR.

Target Gene	Primer Sequence (5′-3′)	Optimum Annealing Temperature (°C)	Size of PCR Amplification (bp)	Reference
NKEF-B	F-ACTGTGACCATCGAGTTC R-TGGGCAAGTAATTGCTG	49	285	Chen et al. [37]
Lysozyme–C	F-GCTGTGATGTTGTCCTATCTTC R-GTAACTTCCCCAGGTATCC	52.7	321	Huttenhuis et al. [38]
Lysozyme G	F-CTTATGCAGGTTGACAAACG R-GGCAACAACATCACTGGAGTAATC	53.5	249	Mohanty and Sahoo [39]
TNF α	F-CCAGGCTTTCACTTCAGG R-GCCATAGGAATCGGAGTAG	51.6	102	Gonzalez et al. [40]
TLR22	F-TCACCCCATTTCGAGGAATGTC R-GAAGGCGTCGTACTGGCTAACAT	56.0	520	Saurabh et al. [41]
β_2M	F-TCCAGTCCCAAGATTCAGGTG R-TGGTGAGGTGAAACTGCCAG	59.7	175	Mohanty and Sahoo [39]
β-actin	F-GACTTCGAGCAGGAGATGG R-CAAGAAGGATGGCTGGAACA	55.3	138	Mohanty and Sahoo [39]

2.12. Statistical Analysis

The data obtained in this study are presented as the mean ± standard error of the mean (SEM) and were analyzed using the SPSS (version 21) software. The significance of the differences was determined by one-way variance analyses for the quantitative data assessment. Differences were considered significant at $p < 0.05$ in the figures.

3. Results

In recent years, as fish production has increased, microalgae have emerged as a more cost-effective and environmentally friendly alternative source of nearly all the nutrients that fish require. The proximate composition of the mixed algal blend was 32.7% protein, 42.58% carbohydrate, 8.24% ether extract, 8.65% ash, and 78.65% dry matter (Table 1). The proximate composition of various feeds and tissues is shown in Table 1. In the experimental diet (0.02%), highest total protein, lipid, moisture content, ash, and dry matter were noted when they differed slightly from other experimental diets. Compared with control, the maximum protein, carbohydrate, and lipid content was found in 0.02 to 0.08% of the experimental groups. Table 3 shows the experimental animals' growth parameters in different groups. The T3-treated group (0.04%) had the highest weight gain, SGR, and FCR among the dietary algae blend supplementation groups ($p < 0.05$). At 0.01% integration (T1), the fed groups had the lowest FCR, which differed significantly ($p < 0.05$) from the other algae blend groups.

Over the course of the study, the total serum protein and albumin content were significantly higher in all treatments compared to the control group (Table 4). The 0.01% algae blend was most likely to result in a significant ($p < 0.05$) increase in serum protein and albumin content on the 21st day. Significant increases in serum globulin content, on the other hand, were seen only after 14 to 28 days of feeding. There was no difference in the albumin-globulin ratio between treatments over the entire experimental period, except on the 28th day, when the fish were fed a 0.04% diet of mixed algae had a significantly higher albumin-globulin ratio than the control group.

Table 3. Effects of inclusion of algae blend on growth performance traits in *Labeo rohita.*

Parameters	Control (0 %)	T1 (0.01%)	T2 (0.02%)	T3 (0.04%)	T4 (0.08%)
Initial body weight (g)	100.46 ± 1.38	100.19 ± 1.34	100.12 ± 2.26	100.10 ± 1.68	100.60 ± 1.98
Final body weight (g)	115.98 ± 4.65 ^{a}	118.77 ± 6.77 ^{a}	126.09 ± 5.3 ^{b}	130.41 ± 7.52 ^{c}	124.49 ± 8.68 ^{b}
Total weight gain (TWG)	15.51 ± 1.43 ^{a}	18.58 ± 1.64 ^{ab}	25.97 ± 2.56 ^{b}	30.30 ± 1.55 ^{c}	23.89 ± 1.98 ^{b}
Specific growth rate (SGR/d)	0.51 ± 0.03 ^{a}	0.60 ± 0.09 ^{b}	0.82 ± 0.02 ^{d}	0.94 ± 0.05 ^{e}	0.76 ± 0.03 ^{c}
Feed conversion rate (FCR)	1.65 ± 0.35 ^{b}	1.53 ± 0.21 ^{a}	1.55 ± 0.43 ^{ab}	1.56 ± 0.32 ^{ab}	1.55 ± 0.09 ^{b}

Values observed were expressed as mean ± standard error. $^{a–e}$ Means in the same row with different superscript differ significantly ($p < 0.05$).

Table 4. Effects of inclusion of algae blends on biochemical profiles in *Labeo rohita.*

Parameters	Treatment Days	Control (0 %)	T1 (0.01%)	T2 (0.02%)	T3 (0.04%)	T4 (0.08%)
Total serum protein (g dL^{-1})	Initial day	9.85 ± 0.24 ^{a}	10.98 ± 1.02 ^{a}	11.42 ± 1.49 ^{b}	10.53 ± 1.40 ^{a}	9.84 ± 0.98 ^{b}
	7	9.9 ± 0.12 ^{a}	11.42 ± 0.20 ^{b}	12.42 ± 0.19 ^{c}	13.34 ± 0.14 ^{d}	10.53 ± 0.16 ^{c}
	14	10.2 ± 0.11 ^{a}	12.54 ± 0.13 ^{b}	15.76 ± 0.09 ^{c}	14.76 ± 0.12 ^{d}	12.54 ± 0.11^{c}
	21	9.93 ± 0.84 ^{a}	13.76 ± 0.89 ^{b}	17.65 ± 0.88 ^{c}	15.59 ± 0.95 ^{d}	13.98 ± 0.96^{e}
	28	10.1 ± 1.2 ^{a}	12.76 ± 1.5 ^{b}	15.76 ± 1.6 ^{c}	16.65 ± 1.9 ^{d}	14.77 ± 1.2 ^{e}
Albumin (g dL^{-1})	Initial day	5.34 ± 0.9 ^{c}	6.34 ± 0.04 ^{a}	7.53 ± 0.5 ^{b}	8.0 ± 0.22 ^{a}	6.2 ± 0.25 ^{c}
	7	5.03 ± 0.43 ^{a}	7.34 ± 0.45 ^{b}	8.76 ± 0.38 ^{c}	10.34 ± 0.78 ^{c}	7.53 ± 0.58^{d}
	14	5.2 ± 0.35 ^{a}	7.98 ± 0.54 ^{b}	9.7 ± 0.33 ^{c}	8.65 ± 0.32 ^{d}	8.66 ± 0.06^{c}
	21	5.04 ± 0.33 ^{a}	8.7 ± 1.45 ^{d}	10.25 ± 1.64 ^{b}	8.59 ± 1.55 ^{b}	9.98 ± 2.54 ^{c}
	28	5.32 ± 0.54 ^{a}	7.76 ± 1.44 ^{b}	9.76 ± 1.21 ^{c}	9.65 ± 1.32 ^{e}	8.77 ± 1.03 ^{d}
Globulin (g dL^{-1})	Initial day	4.51 ± 0.93 ^{a}	4.64 ± 0.85 ^{b}	3.89 ± 0.75 ^{b}	2.53 ± 0.96 ^{b}	3.64 ± 0.99 ^{a}
	7	4.87 ± 1.24 ^{b}	4.08 ± 1.43 ^{a}	3.66 ± 1.29 ^{b}	3.0 ± 1.04 ^{d}	3.0 ± 1.02^{c}
	14	5.00 ± 1.2 ^{a}	4.56 ± 2.1 ^{b}	6.06 ± 2.2 ^{c}	6.11 ± 1.9 ^{d}	3.88 ± 1.8^{c}
	21	4.89 ± 1.4 ^{a}	5.06 ± 1.04 ^{b}	7.4 ± 1.8 ^{c}	7.0 ± 1.4 ^{d}	4.0 ± 1.05^{d}
	28	4.78 ± 0.9 ^{a}	5.0 ± 0.8 ^{b}	6.1 ± 0.5 ^{c}	7.0 ± 0.6 ^{d}	6.0 ± 0.9 ^{d}
A/G ratio	Initial day	1.18 ± 0.09 ^{b}	1.36 ± 0.08 ^{a}	1.93 ± 0.06 ^{a}	3.16 ± 0.04 ^{a}	1.70 ± 0.06 ^{b}
	7	1.03 ± 0.4 ^{a}	1.79 ± 0.06 ^{b}	2.39 ± 0.6 ^{b}	3.44 ± 0.2 ^{a}	2.51 ± 0.5^{b}
	14	1.04 ± 0.34 ^{c}	1.75 ± 0.4 ^{d}	1.60 ± 0.24 ^{b}	1.41 ± 0.53 ^{a}	2.23 ± 0.43^{b}
	21	1.03 ± 0.29 ^{c}	1.71 ± 0.21 ^{d}	1.38 ± 0.22 ^{b}	1.22 ± 0.21 ^{a}	2.49 ± 0.25 ^{b}
	28	1.11 ± 0.95 ^{a}	1.55 ± 0.89 ^{b}	1.62 ± 0.74 ^{b}	1.37 ± 0.98 ^{b}	1.46 ± 0.76 ^{a}

Values observed were expressed as mean ± standard error. $^{a–e}$ Means in the same row with different superscript differ significantly ($p < 0.05$).

Compared to the control group of fish on all testing period, fish given dosages of 0.02 to 0.8% of a mixed algal blend showed significantly ($p < 0.05$) higher Hb content on the 21st day post-feeding and a higher total RBC and WBC count (Figures 1–3). Due to the 28-day trial period's administration of various dosages of a mixed algal blend, the neutrophil activity was considerably raised ($p < 0.05$, Figure 4). Only on the 14th post-feeding was a significant ($p < 0.05$) increase in lyosozyme activity observed in the fish of the various treatment groups (Figure 5). Throughout the experimental period, serum MPO activity increased significantly ($p < 0.05$) on each of the assay days (Figure 6). Ceruloplasmin, serum bactericidal, and serum antiprotease activities increased significantly ($p < 0.05$) during the trial period on all assay days. Furthermore, the fish provided with a 0.02% diet for 14 days showed the highest levels of serum bactericidal activity, serum antiprotease activity, and ceruloplasmin activity (Figures 7–9). Immune index levels generally exhibited an upward increase in both the experimental and control groups.

Figure 10 indicates the percentage of survival of *L. rohita* under algal blend treatments. Most of the mortality during the mixed algal blend post-challenge period was attributed to the challenge infection with *A. hydrophila*; however, this mortality was also observed after the challenge, reaching 37 percent in the control diet group (Figure 11). Fish fed diets

with mixed algal diets were more tolerant, and mortality was only seen after 10 days of pathogen exposure. The fish fed diets containing 0.02 and 0.04% ($p < 0.05$) of mixed algae had the highest relative percentage survival rate (90 percent). The control diet of the fish fed exhibited 60% mortality. On moribund or dead fish, typical symptoms of hemorrhagic septicemia have been observed. Dead fish formed isolated colonies of *A. hydrophila.*

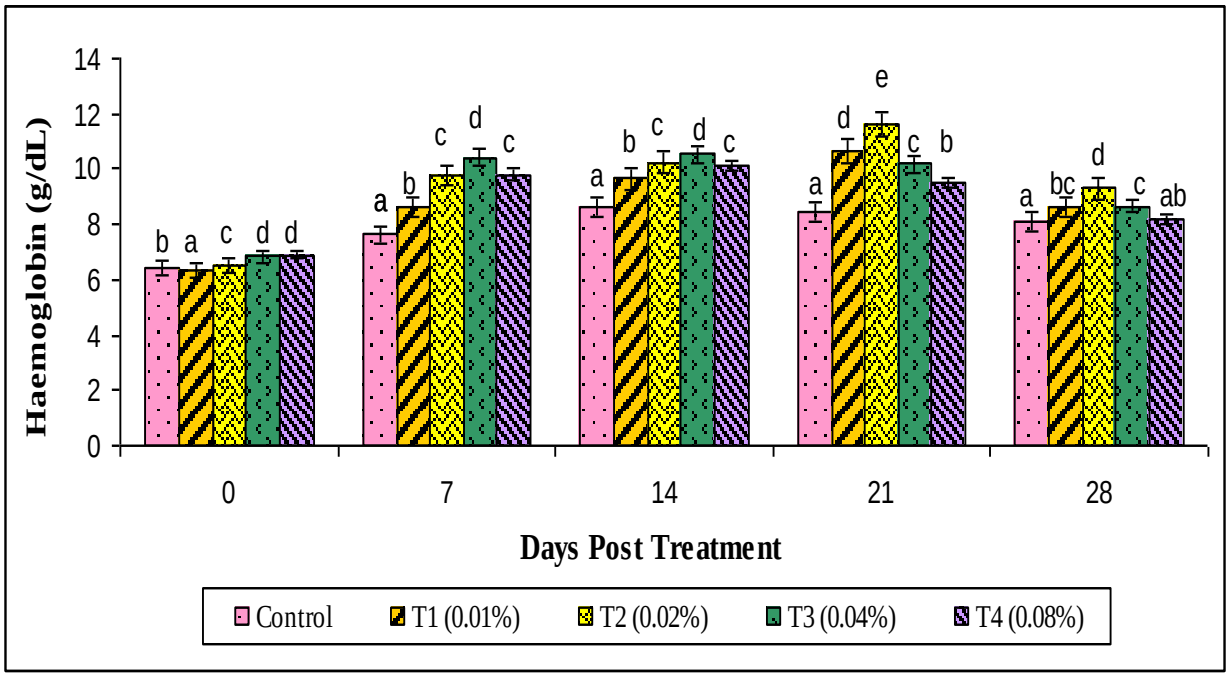

Figure 1. Total hemoglobin content (g/dL) fed with a mixed dietary algal supplement. The mean value differs significantly with different superscripts in each stage ($p < 0.05$). As mean ± standard error ($n = 6$), values are expressed.

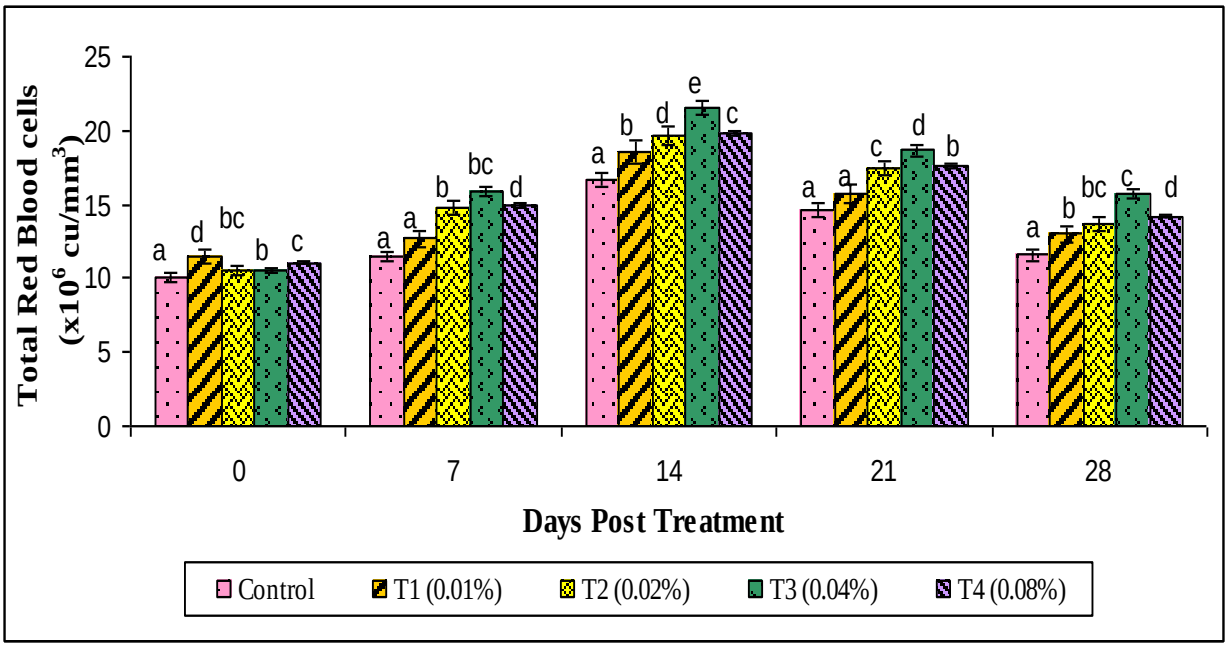

Figure 2. Total red blood cells (one million cells) fed with a mixed dietary algal supplement. The mean value differs significantly with different superscripts in each stage ($p < 0.05$). As mean ± standard error ($n = 6$), values are expressed.

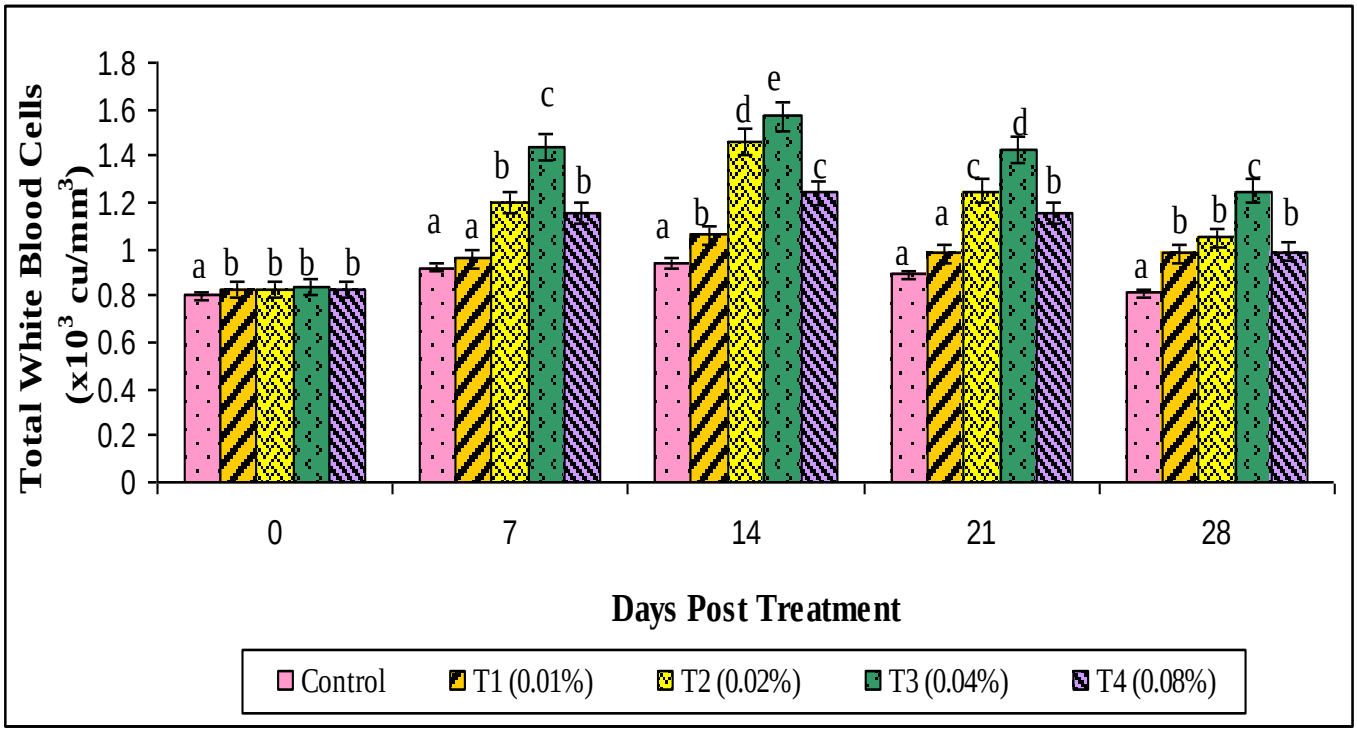

Figure 3. Total white blood cells (one thousand cells) fed with a mixed dietary algal Scheme 0. As mean ± standard error (n = 6), values are expressed.

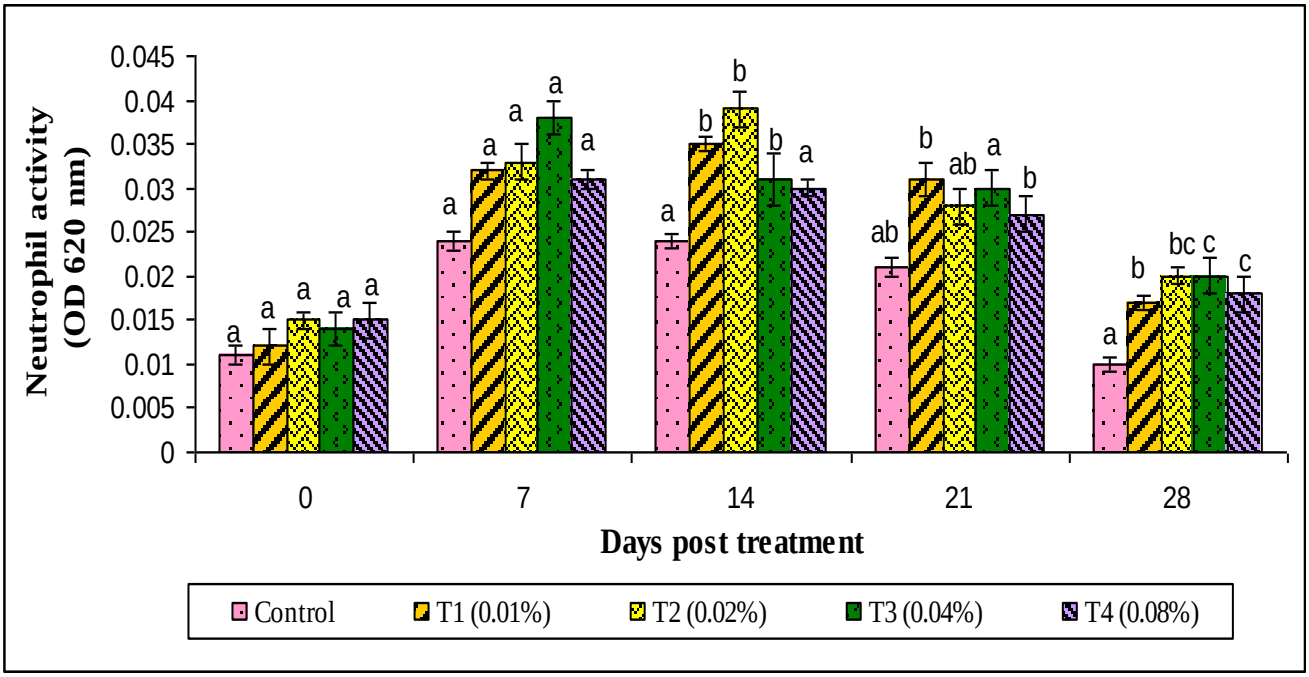

Figure 4. Neutrophil activity fed with dietary mixed algal supplement. The mean value differs significantly in columns with different superscripts ($p < 0.05$). As mean ± standard error (n = 6), values are expressed.

Changes in the level of expression of different immune-related genes were also observed in the head kidney and spleen at different time periods after the *A. hydrophila* challenge. The densitometric expression quantification of these genes revealed that the expression of NKEF-B, TLR 22, TNF-alpha, C, and G lysozyme types in *A. hydrophila*-infected

fish was significantly downregulated compared to control and compared to beta-actin transcripts. Infected fish, on the other hand, had higher levels of beta-microglobulin expression than control samples (Figure 12).

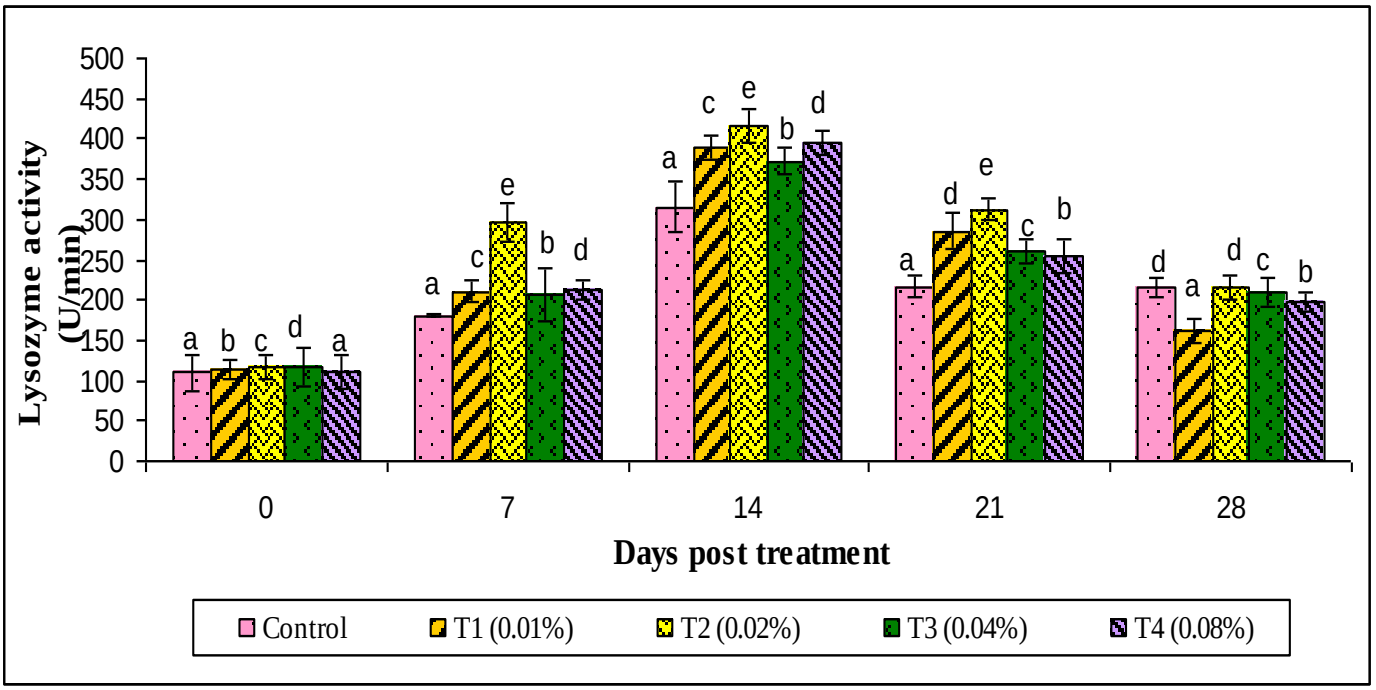

Figure 5. The activity of lysozyme *Labeo rohita* is fed with a mixed algal supplement diet. The mean value differs significantly in columns with different superscripts ($p < 0.05$). As mean ± standard error ($n = 6$), values are expressed.

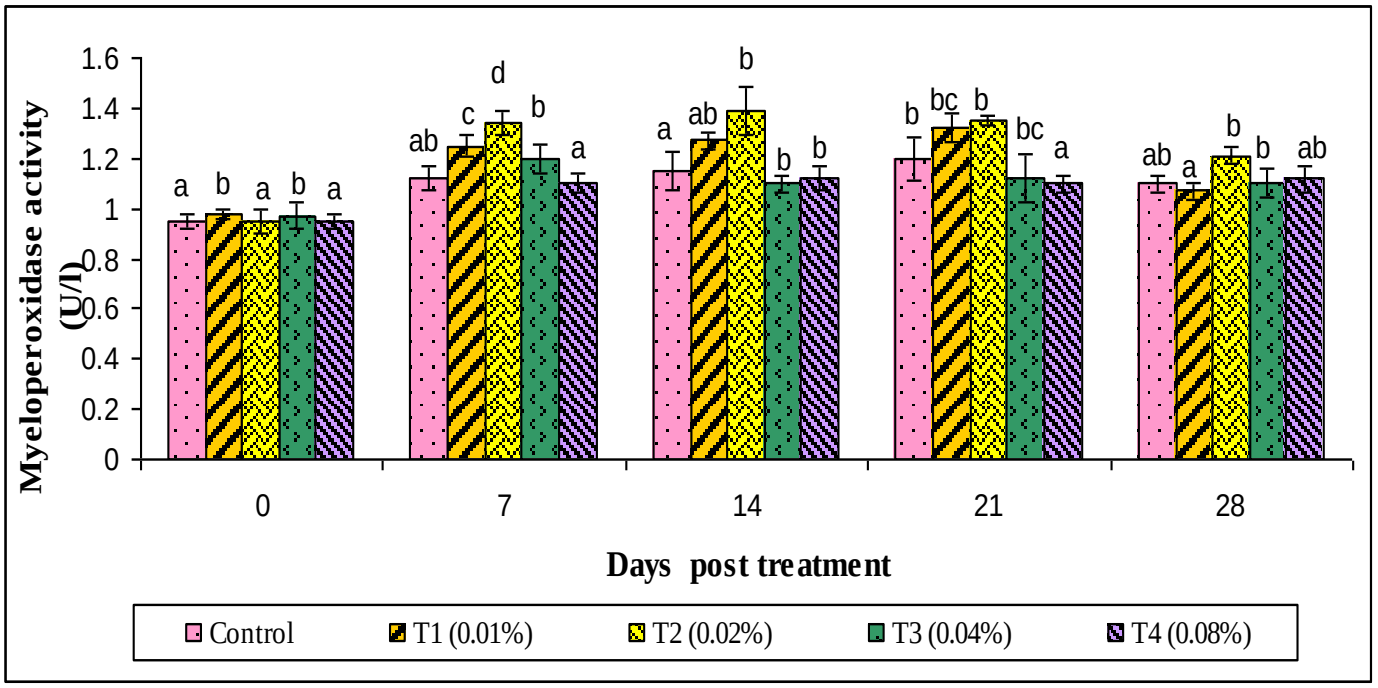

Figure 6. The myeloperoxidase activity fed with a diet of mixed algal supplements. The mean value differs significantly in columns with different superscripts ($p < 0.05$). As mean ± standard error ($n = 6$), values are expressed.

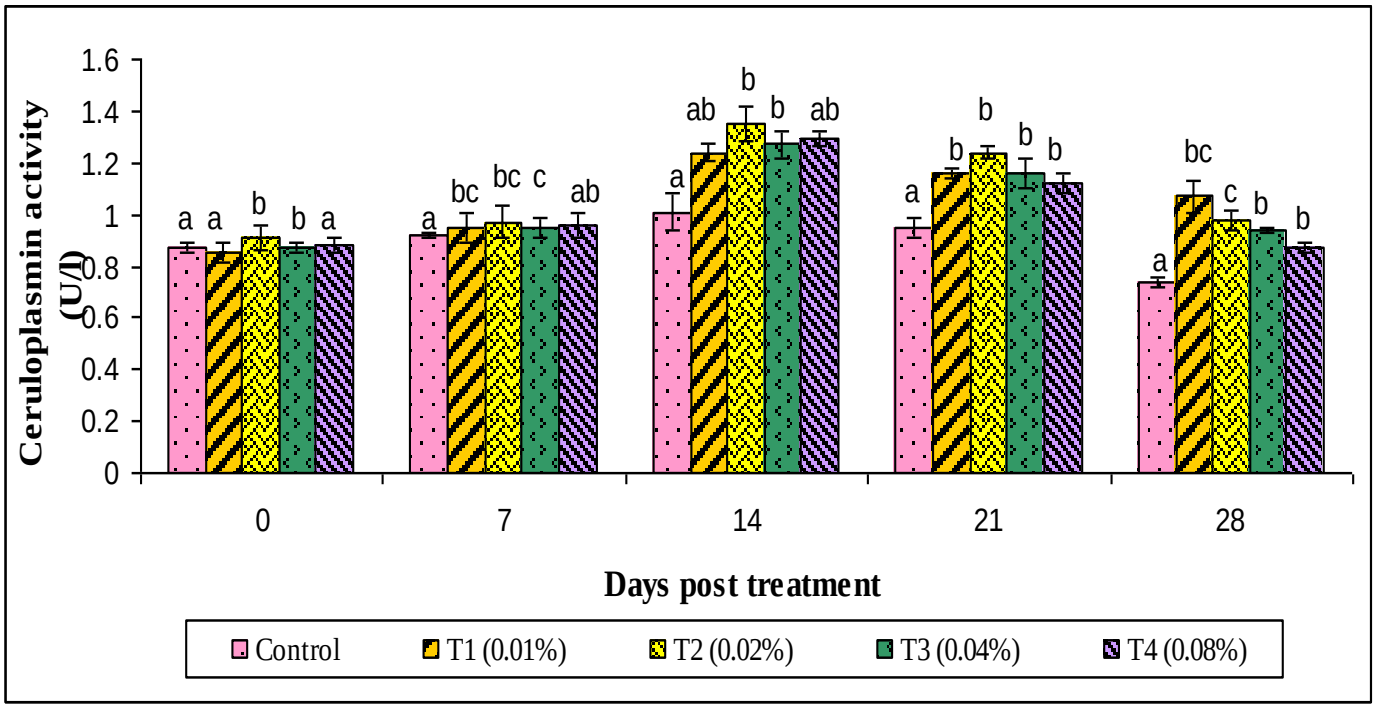

Figure 7. Ceruloplasmin activity, fed with mixed dietary algal supplement. The mean value differs significantly in columns with different superscripts ($p < 0.05$). As mean ± standard error ($n = 6$), values are expressed.

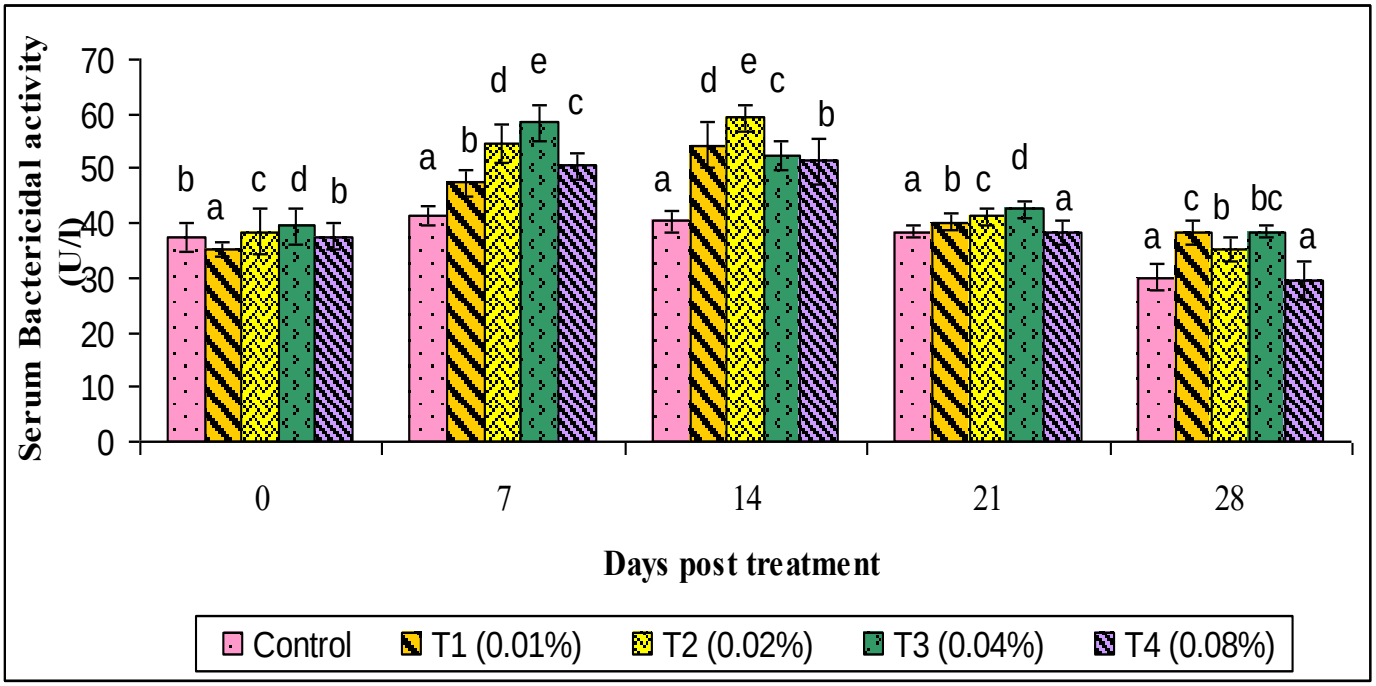

Figure 8. Serum bactericidal activity fed with a mixed dietary algal supplement. The mean value differs significantly in columns with different superscripts ($p < 0.05$). As mean ± standard error ($n = 6$), values are expressed.

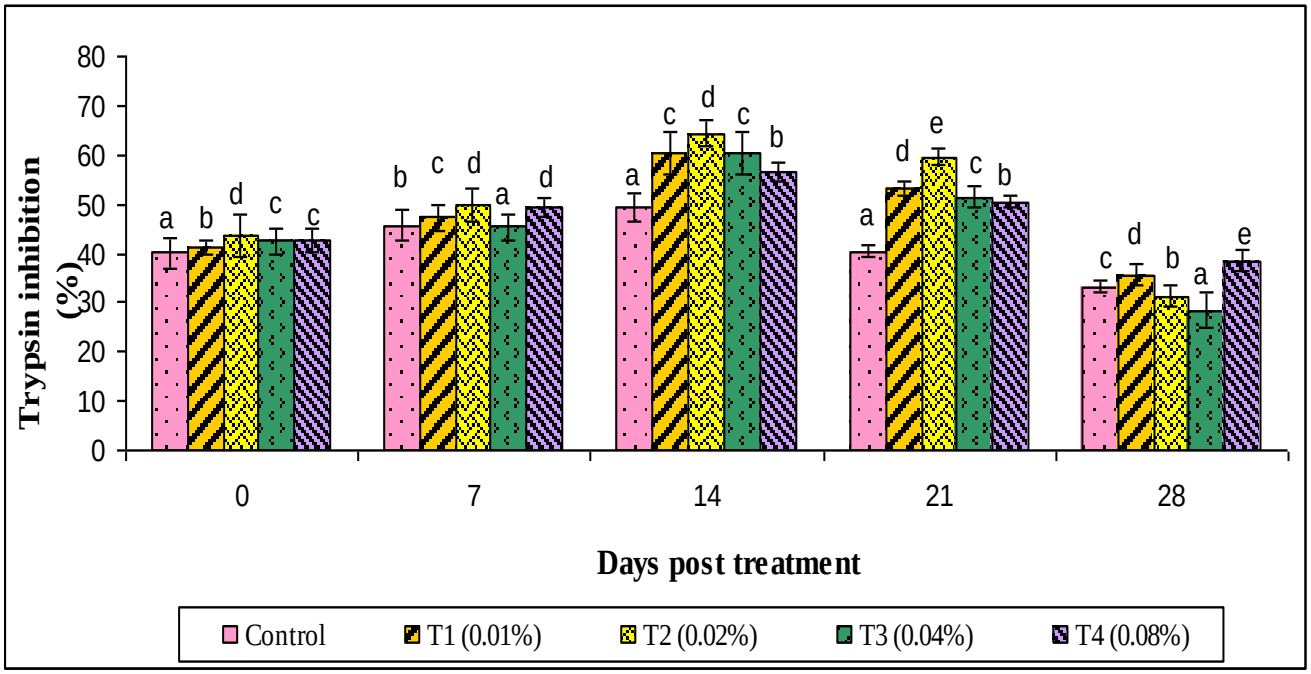

Figure 9. Serum antiprotease activity fed with a diet of mixed algal supplements. The mean value differs significantly in columns with different superscripts ($p < 0.05$). As mean ± standard error ($n = 6$), values are expressed.

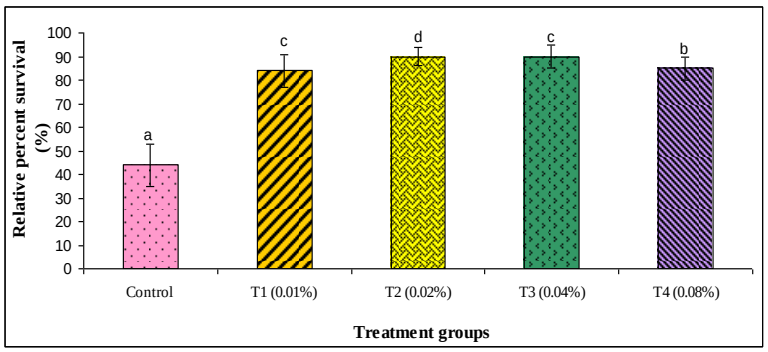

Figure 10. Effect of mixed algal blend after *Aeromonas hydrophila* challenge on survivability of *Labeo rohita*. The mean value differs significantly in columns with different superscripts ($p < 0.05$). As mean ± standard error ($n = 6$), values are expressed.

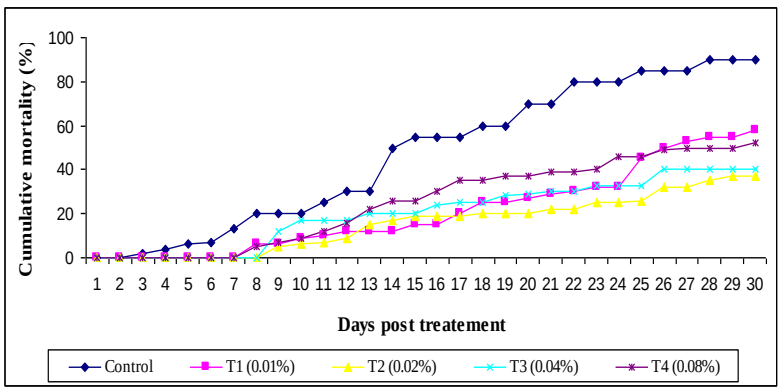

Figure 11. Effect of mixed algal blend after the *Aeromonas hydrophila* challenge on cumulative mortality of *L. rohita*.

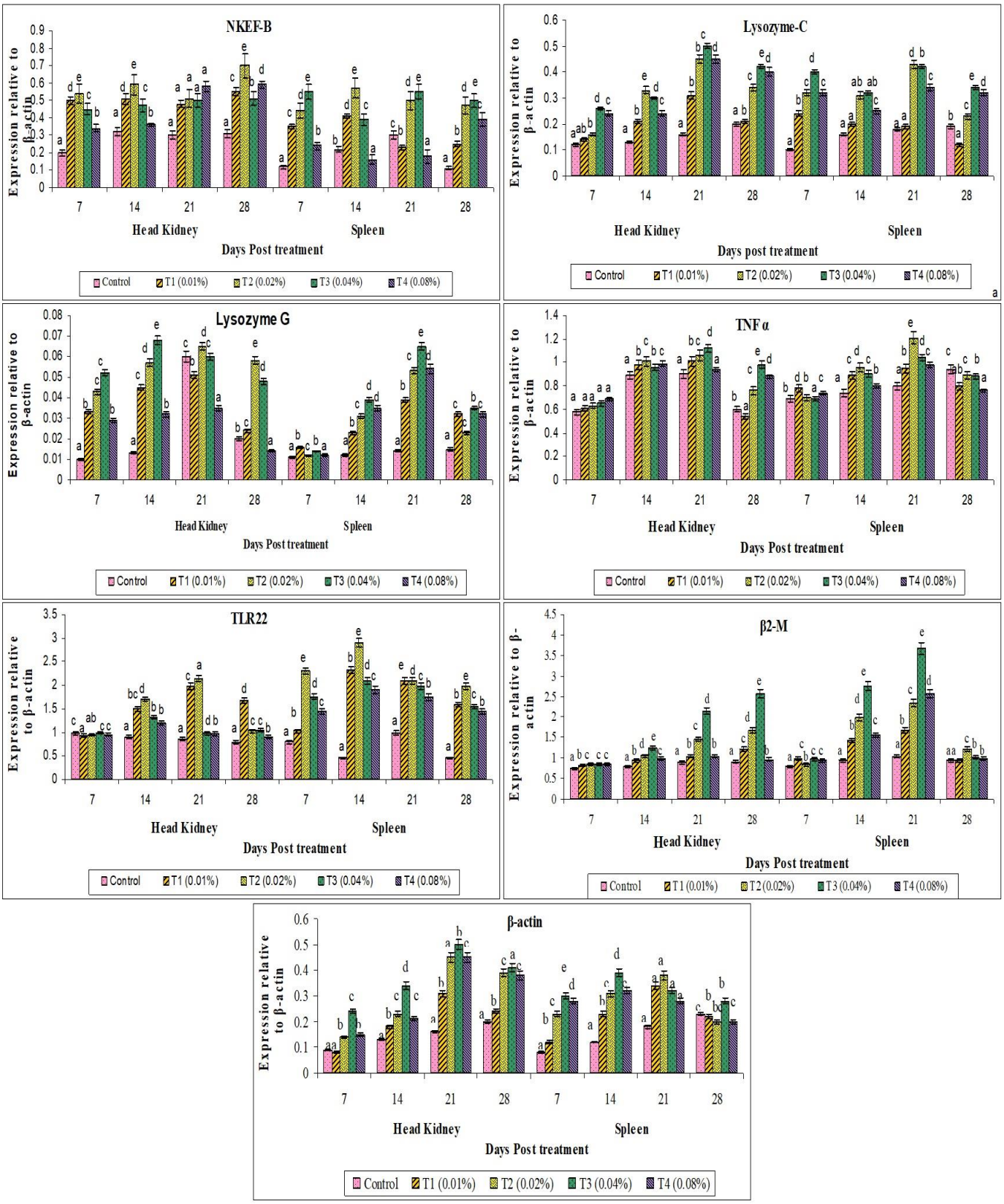

Figure 12. Expression in head kidney and spleen of *Aeromonas hdyrophila*-infected *Labeo rohita* at 7, 14, 21 and 28 days post-challenge of immune-related gens relative to the Beta actin gene. Mean values (+SE) for three samples are represented by bars. TNF alpha-tumor necrosis factor alpha; TLR 22-Toll-like receptor 22; β2M-β2 macroglobulin; Lysozyme C and G) NKEF-B, natural killer-cell enhancing factor B.

4. Discussion

Strengthening the defense system is crucial for sustaining the wellbeing of aquaculture species and halting the transmission of diseases across populations of farmed fish, which reduces disease-related economic losses and deaths. One strategy for improving the immune system, particularly non-specific defense mechanisms, is the use of immunostimulants for disease prevention and management in aquaculture. Because of their abundant nutrient and mineral content, as well as some of their useful qualities, seaweeds have been grown and eaten as food by humans and animals for a long time in some regions [42]. The function of this portion of the immune system and its elements is more important in fish since non-specific defense mechanisms are less complex processes in terms of particular immunological capabilities in fish than in higher vertebrates [43]. The effect of oral administration of this algal blend on the growth, biochemical, hematological, and immune responses of rohu is demonstrated in the current study by substantial differences in the immunological indices of fish fed with mixed (*Spirulina*, *Euglena*, and *Chlorella*) algal blend compared to the control group.

Dietary manipulation for growth and health management should be a prioritized topic in aquaculture research [44]. Growth promotion and better feed efficiency was reported in fish fed with various green algae by several researchers [45]. With a mixed algae blend supplementation of 0.02%, fish growth increased, and significant differences ($p < 0.05$) in WGR and SGR were observed between treatment groups and control. The fish improved their growth performance, which was like previous studies in *Huso husosturgeon* [46] and *Cyprinus carpio* [47,48]. *S. platensis* and *Chlorella* algae are good sources of protein for animal feed because they contain high amounts of essential fatty acids, vitamins, and minerals [48]. By supplementing with *S. platensis*, algae can also improve intestinal flora, digestive enzyme activity, and the breakdown of indigestible components, all of which may contribute to growth enhancement [48]. The serum total protein, albumin after 30 days fed with mixed algal blend increased in comparison to the control diet. Siwicki [49] observed an increase in total serum protein content after including beta-glucan (0.2%) and chitosan (0.5%) in the diet. Serum albumin and globulin values in fish fed with a mixed algal blend were higher than the control. Increase in serum protein, albumin levels are thought to be associated with a stronger innate immune response of fish [50]. According to Bai et al. [51], adding *Chlorella* to the diet had a good impact on Korean rock fish's body composition, growth, and feed utilization with no adverse effects. According to the findings in [52], the blood total protein, globulin, AST, and ALT levels have an impact on the supplementing effect of dietary *Chlorella*.

Incorporating hematological measures, the physiological condition of the L. rohita used in the experiment was evaluated. According to Svobodová et al. [53], these characteristics are also used to evaluate the nutritional status and feed composition in relation to the fish's habitat. When evaluating the toxicity of feed and the health of fish, white blood cells, red blood cells, and haematocrit (HCT) are used [54]. The RBC, WBC, and Hb levels all increased in a dose-dependent manner, with the highest increases seen in fish fed 0.02 to 0.08% of the mixed algae blend. The finding that the values of Hb, RBC, and WBC significantly changed throughout the supplementation levels suggests that substituting none of the three freshwater algae for fishmeal did not have a negative impact on the health of the fish. The results from every experimental diet are consistent with the haematological parameters of healthy catfish [55,56]. All of the experimental diets have considerably different levels of WBC and RBC, which show the presence of physiological characteristics and rising levels of antigens in the circulatory system [57]. An increase in RBC facilitates oxygen transmission, which enhances fish health. An increase in WBC suggests that *S. platensis* and *C. vulgaris* have anti-infection and immunostimulatory actions [58]. In rainbow trout [58] and Koi carp [59], the phycocyanin in spirulina and the -1,3-glucan in *chlorella* have both been connected to the aforementioned qualities [59]. The non-specific immune responses in aquatic species develop sooner and are crucial to the immune defense, whereas the specific immunological mechanisms are diverse and may be underdeveloped [60]. Marine

macroalgae have been shown to enhance stress tolerance, immunological responses, and survival in fish. For example, *Enteromorpha* sp., *Ulva rigida*, and *Chondrus crispus* have been shown to increase the respiratory activity of turbot phagocytes [61]. In this study, fish fed a diet containing 0.02% of a mixed algal blend showed significantly higher levels of all non-specific immunological measures, indicating that the fish had improved non-specific immunoregulation [62].

Lysozyme, immunoglobulin, and complement are examples of humoral substances that play significant roles in both specific and non-specific immunity in fish [63]. Enormous sturgeon's serum lysozyme, serum protease, and myleoperoxidase activities were increased in the current study by a dietary supplementation of mixed blend algae, with fish in the 0.02 and 0.04% supplementation groups displaying the highest levels of activity. Wan et al. [64] found that supplementing 10% of the algae boosted serum lysozyme and complement activity in Atlantic salmon, which is consistent with the findings of the current investigation. According to a study conducted by researchers, incorporating *A. platensis* in the diet boosted the serum lysozyme and complement activity in juvenile great sturgeon [65]. Seaweeds' rich polysaccharides (such agar and alginates) are thought to boost the immune system [64]. The utilization of diets, including seaweeds, was used in earlier studies to confirm positive stimulations of fish immunological parameters. For instance, 5% of a wet meal containing alginate from the brown alga *Ascophyllum nodosum* boosted plasma lysozyme activity in Atlantic salmon (*Salmo salar*) [66]. In a different study, *Pangasianodon hypophthalmus'* immunological characteristics, including antiprotease, serum bactericidal, respiratory burst activity, and lysozyme activity, were improved by the administration of a fucoidan-rich extract from the alga *Sargassum wightii* [67]. Similar studies found that consumption of *Spirulina platensis* increased immunological parameters in coral trout *Plectropomus leopardus* [68], *Ctenopharyngodon idella* [7], *Padina gymnospora* in *Cyprinus carpio* diet [69], and zebrafish (*Danio rerio*) Hoseinifar (neutrophil activity, lysozyme, immunoglobulin, and complement).

The MPO is a special kind of hemeprotein made by neutrophils. It is released and functions during neutrophil activation and is essential to the body's defensive system. It was shown that primary neutrophil azurophilic granules contain large amounts of accumulated MPO. It produces hydrochloric acid using hydrogen peroxide during respiratory bursts [16]. In this study, the MPO activity was higher in the mixed algal dietary groups fed 0.02 and 0.04% than in the control group. Other fish species displayed comparable patterns, with MPO content of oral administrations of algal glucan, lactoferrin, and levamisole significantly rising [45,70]. Alpha-1 antiprotease, alpha-2 antiplasmine, and alpha-2 macroglobulin are the three main protease inhibitors found in fish plasma, and they may help to prevent bacterial invasion and in vivo growth [18]. Ceruloplasmin, another acute phase protein, plays a role in superoxide dismutase activity, as well as anti-inflammation, copper transport from hepatocytes to other tissues, ferrous iron conservation to ferric state, and mobilization from cell to blood [34]. Ceruloplasmin levels were downregulated in this study compared to the control. This is in line with Rao and Chakrabarti's [34] observation that giving rohu an algal feed-incorporated diet for four weeks increased the serum level of ceruloplasmin may operate as a defense against invasive bacterial infections.

Almost as much as the nutrients present in feeds, such as fish meal and soy powder, seaweeds can contribute the nutritional requirements in aquatic diets. Seaweeds contain a variety of bioactive substances that can significantly help fish perform better [2]. In comparison to the control treatment (0.02 and 0.04%), the greatest serum anti-bactericidal and antiprotease activity was recorded in the algae supplement groups. The group that consumed a mixed algal mixture showed noticeably enhanced resistance to *A. hydrophila* infection. This might be as a result of the mixed algal diet stimulating and strengthening the experimental fish's non-specific immune system. Positive findings included an increase in antiprotease activity in *L. rohita* by *Chaetomorpha aerea* extract, as well as a significant rise in serum anti-bactericidal activity in animals fed E. viridis-containing diets following an *A. hydrophila* challenge [4,19]. Our results show that supplementation with a 0.02 and

0.04% mixed algal blend can improve fish non-specific immunity; therefore, more research is required to understand how it affects serum bactericidal activity.

However, there are other influencing elements as well, including fish species, age, physiological status, and various environmental conditions [71]. Additionally, the relative percent survival rate of *L. rohita* challenged with *A. hydrophila* was greatly improved with the addition of *L. rohita* to the diet. These findings support [72], which showed that spirulina improved the general health status of *Nile tilapia* juveniles. Additionally, *chlorella* contains a variety of immunostimulants in the form of polysaccharides, which are chemicals that stimulate immunological function in several animal species [59,73]. The serum globulin levels in the microalgae in the current study were elevated, which may promote resistance to infection. This finding was supported by [74], as globulins are produced by the mononuclear phagocyte system and are crucial components of the immune system. Increases in resistance to *A. hydrophila* were observed in tilapia fed a diet containing 5.0–10.0 g of *spirulina*/kg [75]. For its immune-boosting properties, the mixed algal blend supplementation in the current study may be helpful as a dietary supplement. The tumor necrosis factor (TNF) as a cytokine molecule plays an important role in stimulating the immune system secretion from white blood cells in the immune system of fish with the incidence of inflammations [74]. The IL-1β also plays an important role in the host response to microbial invasion, tissue damage, and impaired immune response [76]. Mollusks, invertebrates, mammals, birds, reptiles, fish, plants, and other species all readily express lysozyme, while some have been shown to have unique forms or tissue-specific expression patterns [77]. All vertebrates release the protein component macroglobulin, which is one of the most prevalent mammalian serum proteins. According to some experts, different cytokines can cause changes in the amount of β2M-β2 macroglobulin protein. For instance, in response to IL-1, the expression level of the β2M-β2 coding mRNA increased up to 40-fold [78]. The results of the current investigation demonstrated that after bacterial challenge, fish fed a diet containing 0.02% of algal extract expressed significantly higher levels of TNF and IL-1 cytokine genes, as well as lysozyme C, G and β2M-β2 protein compared to other treatments and the control.

Numerous studies found that certain fish species expressed higher levels of cytokine genes. Increased TNF and lower IL-10 gene expression levels were observed in a study looking at the impact of *S. platensis* on immune genes (TNF and IL-10) [79]. In fact, in infectious disorders, IL-1 modulates the expression of a number of genes [1]. According to Yuan et al. [80], common carp's diet containing astragalus plants boosted the expression of TNF and IL-1. The use of an algae extract (*Capparis spinosa*) in the diet of rainbow trout increased the transcription of cytokine genes (TNF and IL-1), which enhanced the immune response, according to Bilen et al. [12]. One of the most crucial elements in inflammatory events is TNF, which is produced from different cells when they are stimulated by endotoxins, inflammatory mediators, cytokines (IL-1), or after being stimulated alone by TNF [80,81].

Fish fed high amounts of horsemint extract showed increased post-challenge expression of TNF than the control group, according to Heydari et al. [82]. Aloe barbadensis food supplementation led to increased production of cytokine genes ((IL-1β, IL-6, IL-8, and TNFα) in rainbow trout, according to a study by Mehrabi et al. [83]. The expression of the IL-1 and lysozyme-C genes was correlated with the feeding of common carp with polysaccharides obtained from the marine macroalgae *Padina gymnospora* [69]. A green microalgae (*E. viridis*) supplement was also found to boost the expression of IL-1β, TNFα, and C3 genes in *Catla catla* [84]. The current study is the first to document elevated immune-related gene expression levels because of a diet supplementation with a mixed algal blend. Therefore, this algae species can be added to the group of immunostimulants that can promote the expression of critical immune genes. The results of our study on rainbow trout survival rates following *A. hydrophila* exposure showed increased fish resistance in treatments with mixed blend supplements, proving that the mixed algal blend (*S. platensis*, *E. viridis*, and *C. vulgaris*) supplement stimulates fish immune systems. Low molecular

weight polysaccharides from seaweeds were fermented by gut bacteria and revealed that the polysaccharides could be as potential source of prebiotics [85]. Pathogen inhibition, immune system bolstering, and improved digestibility are all benefits of these polysaccharides when combined with healthy intestinal bacteria such as *Bifidobacteria* and *Lactobacilli* [86]. For instance, adding low-molecular weight agar made from red algae to the diet of Basa fish (*Pangasius bocourti*) may strengthen their immune systems and increase their chances of surviving the *A. hydrophila* challenge [10]. Similarly, *Ctenopharyngodon idella* polysaccharide fed with *P. yezoensis* [7] and *C. carpio* fed with *P. gymnospora* polysaccharides [69] showed a higher relative percentage of survival after challenged with *A. hydrophila*.

5. Conclusions

In conclusion, the present study reported the effect of mixed algal blend (*Spirulina platensis, Euglena viridis*, and *Chlorella vulgaris*) on growth, biochemical, hematological, specific and non-specific immune parameters and expression of immune-related genes of *L. rohita*. The results of this study indicated that the dietary mixed algal extract blend in different concentrations significantly upregulates the growth performance, immune response, immune genes expressions and disease resistance in *L. rohita* challenged with *Aeromonas hydrophila*. Mixed algal blend diet exhibited the highest ($p < 0.05$) immunostimulatory activity. Therefore, multispecies of green algae should be taken into account while formulating a nutritionally balanced diet of carp for its better growth and immune performances. These findings provided a novel insight into the development of new feed additive in aquaculture sectors.

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Conflicts of Interest: The authors hereby declare that they have no conflict of interest and have no known competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.

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