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Lotus Elixir: A melody of gene expression variation in *Oncorhynchus mykiss* tissues.

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Highlights

- *Nelumbo nucifera* leaf extract (NNLE) plays an important role in enhancing the physiological status of the fish by significantly reducing the gill and liver SOD
- NNLE showed advantageous effects on the haematological profile of rainbow trout.
- NNLE fed groups showed upregulation of gene transcripts of defensin and hepcidin genes thereby highlighting its immunomodulatory effect.
- Both pro-inflammatory and regulatory cytokine gene expression was differentially modulated by incorporation dietary NNLE.
- The study underlines health benefits of NNLE incorporation in rainbow trout diets.

Abstract

The present study evaluated the survival and immune response of Rainbow trout (*Oncorhynchus mykiss*) fingerlings fed with *Nelumbo nucifera* leaf extract (NNLE) for 90-day time period. Two hundred and fifty fingerlings were randomly distributed into five treatment groups in triplicates. Each group was fed isonitrogenous and isoenergetic regimes containing 0% NNLE (control), 0.25% NNLE (T1), 0.75% NNLE (T2), 1.25% NNLE (T3), and 1.75% NNLE (T4) to satiation. NNLE was found to significantly ($P < 0.05$) reduce malate dehydrogenase and aspartate amino-transferase activity in the liver tissue. Alanine amino-transferase activity in the muscle tissue of NNLE fed groups was significantly higher than in the control. The liver and gill superoxide dismutase activity were significantly reduced in the NNLE fed groups compared to the control. Also, the catalase activity in the liver and gill got significantly lowered in the T4 group. NNLE fed groups showed elevated RBC, TLC, respiratory burst activity, lysozyme activity and phagocytic activity compared to control group. Also, mRNA expression of *defensin* and *hepcidin* genes showed significant upregulation in NNLE fed groups thereby highlighting its role in modulation of innate immune response. During pre-challenge phase, pro inflammatory cytokines were significantly ($p < 0.05$) down-regulated compared to control. Whereas, post-challenge period, T4 (1.75% NNLE) caused substantial ($p < 0.05$) up-regulation of *IL-1* and *TNF- α* expression. Regulatory cytokines were slightly up-regulated during pre-challenge and down-regulated post-challenge periods. Treatment groups T3 and T4 showed significantly ($p < 0.05$) higher survival rate after challenged with pathogenic bacteria *Aeromonas hydrophila*. The study highlights that supplementation of Indian lotus leaf extract at the rate of 1.75% in diet enhances the disease resistance in rainbow trout.

Keywords: *Nelumbo nucifera* leaf extract; Antimicrobial peptides; Nutraceutical; Immunomodulation, Aquaculture; Rainbow trout; *Aeromonas hydrophila*.

1. Introduction

Increasing aquaculture production can help address global hunger and malnutrition by closing the food demand gap (Villarino et al., 2022). However, with limited aquatic resources and the challenges of climate change, expanding fish production at low stocking densities is a complex task. Current efforts are shifting from extensive to intensive aquaculture practices, but this intensification often leads to stress, making fish more susceptible to diseases (Conte, 2004). The use of antibiotics and chemotherapeutics as alternative treatments has drawbacks that outweigh their benefits (Jang et al., 2018). While vaccines present a reliable solution, their limited commercialization and licensing hinder widespread availability for disease management in the global farming community (Ben Hamed et al., 2021).

An alternative to antibiotics and vaccines is the use of phytochemicals to stimulate the fish's innate immune system. These immunostimulants enhance nonspecific immune responses, offering a cost-effective and environmentally friendly approach to improving fish health (Yousefi et al., 2021). Research on plant-derived substances boosting fish immune responses has surged in the past decade (Jahazi et al., 2020; Hoseinifar et al., 2020; Abdel Rahman et al., 2019). Understanding the connection between nutrition, immune response, and disease resistance has become crucial, especially in farmed fish (Gabriel, 2019). Nutritional approaches play a vital role in preventing diseases among aquatic animals, with components like immunostimulants, antioxidants, pre- and probiotics considered to enhance fish growth, feed efficiency, stress tolerance, and disease resistance (Harikrishnan et al., 2021). Rainbow trout aquaculture faces challenges in nutrition and disease management, with the cost of feed and early-stage mortality causing significant economic losses.

Indian lotus (*Nelumbo nucifera* Gaertn.), a perennial rhizomatous plant with a history in traditional therapies, has been utilized in human and veterinary medicine (Sheikh, 2014; Sharma et al., 2017; Mehta, Patani, & Shah, 2013; Bhat & Sridhar, 2007; Abdel Rahman et al., 2019; Kim & Shin, 2012; Mukherjee, et al., 2010). While its applications in aquaculture for nutrition and health are relatively new, there are limited reports on its use as an immunostimulant in fish (Munglue, 2014, 2015, 2016; Sivagurunathan et al., 2012; Abdel Rehman et al., 2018). Utilizing Indian lotus in aquaculture can be a sustainable and environmentally friendly choice to mitigate disease-related economic losses. The current study aims to explore the impact of *Nelumbo nucifera* leaf extract on the growth and immune responses of rainbow trout, representing a novel investigation into its immunomodulatory properties in the trout diet.

2. Material and methods

2.1 Experimental Site and Fish

The feeding trial was conducted for 90 days at the trout hatchery of the Fisheries Department, Kokernag, Jammu & Kashmir. Fingerlings of rainbow trout, with an average weight of 20 ± 0.5 g were procured from trout hatchery, Kokernag and were carefully transferred to circular tanks of 350L capacity connected with running water at a 9L/min water exchange rate. The fish were left undisturbed overnight, to ameliorate the handling stress. The fish were given 0.2% salt treatment for 2 min in the next morning. The stock was acclimatized under continuous supply of oxygen rich cold-water and fed with a 45.32% crude protein diet.

2.2 Experimental Design

The feeding trial was conducted in 15 FRP tanks (2×3×2 feet, 350L capacity). A total of four hundred and fifty fingerlings were randomly distributed in the five distinct experimental groups with 90 fish in each group. Further, each group was divided into three replicates with 30 fish in each replicate. It was ensured that fish of uniform size were kept in each tank and covered with a net. No attempts were made to stimulate or control the environmental condition. The experimental conditions were kept the same throughout the experimental period. The body weight was measured at 15 days interval to assess the growth. The fishes were starved overnight before taking the bodyweight.

2.3 Experimental diet

Diets were formulated using purified ingredients following standard guidelines (NRC, 2011) and sourced from HiMedia Chemical Ltd (India). In the Fish Nutrition and Biochemistry Laboratory at SKUAST-K, five diets with consistent compositions, differing only in cellulose and *Nelumbo nucifera* leaf extract (NNLE) levels, were prepared. Cellulose powder served as a filler to maintain equal volume across all diets. The diets comprised 0% NNLE as control (C), 0.25% NNLE (T1), 0.75% NNLE (T2), 1.25% NNLE (T3), and 1.75% NNLE (T4). Ingredients were weighed accordingly (Table – 1). The preparation involved mixing gelatin with other components, followed by steaming in an autoclave for 30 minutes. Lipid, vitamin-mineral premix, and NNLE, extracted as per a previous study (Mudasir et al., 2017), were added and thoroughly mixed. The feed was then pelletized using a hand pelletizer. Samples from all treatment groups were stored in labeled, airtight plastic bags at -

20°C for subsequent proximate analysis (AOAC, 1995) in the Division of Animal Nutrition laboratory at FVSc & AH Shuhama, SKUAST-K.

2.4 Physico-chemical parameters of water

The water quality parameters were manually recorded on a daily basis. Temperature and dissolved oxygen were recorded with a dissolved oxygen meter (MERCK, Germany), pH with a digital pH meter (LABINDIA), free carbon dioxide (titrimetric method, APHA, 1998), total hardness (test kit, MERCK, Germany), and ammonia spectrophotometrically at 635 nm wavelength using the phenate method (APHA, 1998), nitrite, and nitrate spectrophotometrically at a wavelength of 543 nm (APHA, 1998) and compared with the standard graph.

2.5 Growth indicators

Fishes were starved overnight before taking the weight. Percentage of weight gain, specific growth rate (SGR), hepatosomatic index (HSI), viscerosomatic index (VSI) were determined according to Lugert et al.,2016.

2.6 Survival rate

At the end of the experiment of 75 days, all the experimental tubs were dewatered and the number of the experimental animals in each tub was counted and the survival rate (%) was calculated by the following formula

$$Survivalrate = \frac{Totalnumberofharvested}{Totalnumberofstocked} \times 100$$

2.7 Enzyme assays

The muscle, liver, gill, and intestine were meticulously extracted, weighed, and homogenized in a glass tube using a tissue homogenizer with a chilled sucrose solution (0.25M). To prevent heating, the tube was kept on ice during the process. The resulting homogenate underwent centrifugation at 5000rpm for 10 minutes at 4°C, and the supernatant was stored at 4°C for future use. A 5% homogenate was prepared for each tissue type. Lactate Dehydrogenase (LDH) activity was assessed using the method outlined by Wroblewski and Ladue (1955). Malate Dehydrogenase (MDH) activity was determined with the procedure described by Ochoa (1955). Alkaline Phosphatase (ALP) activity was determined using the Garen and Levinthal (1960) method. Acid phosphatase (ACP) activity was determined similarly to ALP but with acetate buffer (0.2M, pH 5) instead of bicarbonate

buffer. Aspartate Transaminase (AST) activity in tissue homogenates was assayed as per Wooten's method (1964). Total protein content in each tissue sample for enzyme assays was estimated using the Bradford method (Bradford, 1976). Superoxide dismutase activity was determined based on the Mishra and Fridovich (1972) method, involving the oxidation of the epinephrine–adrenochrome transition by the enzyme. Catalase activity was assayed according to the procedure outlined by Takahara et al., (1960).

2.8 Serum Immunological analysis

2.8.1 Collection of blood

Each fish was anesthetized with clove oil (50 µl of clove oil per litre of water) before collecting blood from the caudal vein. The collected blood was then transferred immediately to purple-cap tubes which were coated with EDTA to prevent haemolysis and clotting. After blood collection, fishes were allowed to recover from anaesthesia in well-aerated water before returning to respective treatment groups.

2.8.2 Collection of serum

For serum collection, the collected blood was immediately transferred to a dry Eppendorf tube. The tubes were allowed to stand in a tilted position at room temperature for an hour which allows the blood to clot. After clotting, yellow straw-colored serum was carefully collected after centrifugation at 5000g for 5 min and transferred to another Eppendorf tube.

2.8.3 Blood glucose

Blood glucose levels were determined using a digital glucometer (Accu-Chek Active Blood Glucose Meter Kit) (Sacks, 2008; Bartoňková and Hyršl, 2017). Reading was performed in triplicate for each treatment group.

2.8.4 Albumin- globulin

Plasma protein was estimated by the biuret method (Reinhold, 1953) using a kit. The absorbance of the standard (S) and test (T) was measured against the blank (B) using a spectrophotometer at 630 nm. The calculation was done as follows:

$$Total\ proteins(g\%) = \frac{Absorbance\ of\ test(T) \times 6}{Absorbance\ of\ standard(S)}$$

(a) Albumin

Albumin was estimated using the bromocresol green-binding method (Doumas et al., 1971). The absorbance of the standard (S) and test (T) was measured immediately against a blank (B) in a spectrophotometer at 630 nm. The calculation was done as follows:

$$Albumin(g\%) = \frac{Absorbance\ of\ Test}{Absorbance\ of\ Standard}$$

(b) Globulin

Globulin was calculated by subtracting albumin values from total plasma protein.

$$Globulins\ in\ (g\%) = Total\ protein\ in\ (g\%) - Albumin\ in\ (g\%)$$

(c) Albumin- globulin ratio

A/G ratio was calculated by dividing albumin values by globulin values

2.8.4 Haemoglobin content

The haemoglobin level of blood was analyzed following the Cyanmethemoglobin method using Drabkins Fluid (Qualigens). Blood (20 µl) was mixed with 5 ml of Drabkin's working solution. The absorbance was measured using a spectrophotometer at a wavelength of 540 nm. The final concentration was calculated by comparing it with the standard cyanmethemoglobin (Qualigens Diagnostics). The haemoglobin concentration was then calculated by using the following formula:

$$Haemoglobin(g\%) = \frac{OD\ of\ Test \times 251 \times 60}{OD\ of\ Standard \times 1000}$$

Where OD is Absorbance

2.8.5 Total erythrocyte count (TEC)

Blood (20 µl) was mixed with 3980 µl of RBC diluting fluid in a clean test tube. The mixture was shaken well to suspend the cells uniformly in the solution. A small drop of this mixture was charged to Neubauer's counting chamber of a haemocytometer. Care was taken that there were no air bubbles trapped. The red blood cells were counted in five groups of squares. All the cells lying inside the five small squares are counted under the high power (40X) of a light microscope. The following formula is used to calculate the number of RBC per mm³ of the blood sample:

$$Number\ of\ RBC\ mm^3 = \frac{N \times Dillution}{Area\ counted \times Depth\ of\ fluid} = \frac{N \times 200}{0.2 \times 0.1} = N \times 1000$$

Where N is the total number of red blood cells counted in 5 squares of the hemocytometer.

2.8.6 Total leucocyte count (TLC)

Twenty microlitres of blood were mixed with 3980µl of WBC diluting fluid in a clean glass vial. The mixture was shaken well to suspend the cells uniformly in the solution. Care was taken that there were no air bubbles trapped. The numbers of cells were counted in four big squares under high power (40x) magnification of the light microscope. The number of WBC per mm³ of the blood sample is calculated using the following formula:

$$\text{Number of WBC mm}^3 = \frac{N \times \text{Dilution}}{\text{Area counted} \times \text{Depth of fluid}} = \frac{N \times 200}{4 \times 0.1} = N \times 500$$

Where N denotes the total number of white blood cells counted in 4 squares of the hemacytometer.

2.8.7 Packed cell volume (PCV) / Haematocrit value (HCT)

PCV was determined by drawing non-dotted blood by capillary action into micro haematocrit tubes. One end of the tubes was sealed with synthetic sealant. The sealed tube was centrifuged in a microhematocrit centrifuge for 5 min at 10500 rpm. The PCV was measured using a microhematocrit reader and expressed as a percentage.

2.9 Phagocytosis assays

Phagocytic activity was determined by following the method of Anderson & Siwiki (1995). and was results were interpreted using the formula

$$\text{Phagocytic activity} = \frac{\text{Number of phagocytizing cells}}{\text{Total number of phagocytes counted}} \times 100$$

2.10 Nitro Blue Tetrazolium (NBT) assay

The method modified by Zanuzzo, et al., 2015, was used to perform the Nitro Blue Tetrazolium Assay. Heparinized blood and 0.2% NBT (in phosphate-buffered saline solution) were co-incubated in 0.1 mL for 45 min at room temperature. After adding 50 L of the resulting suspension to 1 mL of N,N-dimethylformamide, the mixture was centrifuged at 2,000 rpm for 10 minutes. On a UV-Visible spectrophotometer, the optical density (OD) of the supernatant was determined at 540 nm. Similar procedures and components were used in the blank, but the blood was substituted with distilled water.

2.11 Serum lysozyme activity

The lysozyme activity level was measured using the turbidimetric assay as described by Sankaran and Gurnani, (1972) and activity was expressed as U/min/mg protein.

2.12 LD₅₀ and Challenge study

At the end of the experiment, the fish in each group were challenged with the pathogenic isolates of *A. hydrophila* procured from Microbial Type Culture Collection (MTCC) 7966 and maintained at AAHM Laboratory, Faculty of Fisheries, SKUAST-K. Before challenging the fish, LD₅₀ value was determined using twenty-five fishes from five experimental groups. The glycerol stock of bacterial culture was revived in Mueller–Hinton Broth (MHB) to obtain single colony which was again regrown in MHB at 30°C for 24 h. The culture broth was centrifuged at 1006×g for 10 min. The supernatant was discarded and the pellets were resuspended in sterile phosphate buffer saline (PBS, pH 7.4) and the OD of the solution was adjusted to 0.50–0.48 nm. This corresponded to a count of 1.1×10^7 , confirmed by total plate count. Then the bacterial cells were diluted to get different concentrations required for the experiment.

Eighty fish were distributed randomly into eight groups. The fish of seven groups were injected intraperitoneally with 0.1 ml/g of *A. hydrophila* bacterial cell suspension at concentrations of 1.1×10^1 , 1.1×10^2 , 1.1×10^3 , 1.1×10^4 , 1.1×10^5 , 1.1×10^6 and 1.1×10^7 cfu/mL, respectively. The eighth group (control) was injected PBS at the concentration of 0.1 ml/g of fish weight. The fish were maintained for another 96 h and were observed regularly for any overt signs of disease including behavioral abnormalities and mortality. In case of mortality, the causative agent was confirmed by re-isolating *A. hydrophila* from the moribund fish. LD₅₀ value was calculated as per the trimmed Spearman–Karber method (Hamilton *et al.*, 1977).

At the end of 15 days of post-challenge, serum and blood samples of different challenged groups were collected to study the immuno-haematological parameters of challenged fishes. Liver and gill tissue of representative sample fish (moribund/dead) from control and each test group were removed and examined for oxidative stress enzyme activity.

2.13 Expression profiling of immune genes

2.13.1 Tissue sampling

The expression profiling of immune genes (β -*Defensin*, *Hepcidin* and Cytokines) was done for liver, head-kidney, intestine, and spleen of the fish. The sampling was done at 0 days, 45th, and 75th days intervals during the feeding trial. The organs were collected carefully using sterile scissors and forceps. Each specimen was preserved in RNALater for further use.

Tissue samples were carried to the Genomics laboratory of Division of Animal Biotechnology, F.V.Sc. & A.H. in liquid nitrogen at -194°C for molecular study.

2.13.2 Total RNA extraction

Total RNA was extracted using *Trizol*TM reagent (Sigma-Aldrich, USA) following the manufacturer's instructions. The quality of RNA was confirmed using a Nanodrop spectrophotometer (ThermoFisher, USA). The integrity of the isolated RNA was checked by running it in a 1% agarose gel.

2.13.3 Reverse transcription

The mRNA transcript of the gene was converted into its complementary DNA strand using the first-strand cDNA synthesis Kit (Fermentas USA) as per the protocol provided by the manufacturer. The extracted RNA was treated with DNase I (RNase free) to remove the genomic DNA contamination.

2.13.45 Real-Time PCR analysis analysis of immune genes of rainbow trout

Using the software Gene Runner 3.01, the primers for real-time PCR were created by choosing the conserved gene areas of closely related fish. To prevent non-specific products during PCR amplification, the primer sequences (Table – 2) were blasted with the *O. mykiss* contigs in GenBank. Rainbow trout immune genes were analysed using RT-PCR at different time points: 0 day, 45 day, 75 day, and post-challenge. Maxima R SYBR green PCR master mix (2x) (Fermentas, USA) was used to run real-time PCR using a LightCycler® 480II machine (Roche) real-time PCR machine. Each reaction was created in triplicates with a total volume of 20 ul. The reaction mix included 8.9 ul nuclease-free water, 0.3 ul forward and reverse primers (10 micromolar each), 10 ul 2X SYBR Green, and 0.5 ul cDNA (70ng/ul) to measure mRNA levels of selected genes. Components were pipetted into 96-well plates with 0.2 ml capacity per well. Plates were sealed with clear adhesive film and quickly mixed by spinning at 4000 rpm. Gene-specific beta-actin primers were used to normalize each sample. The qPCR amplification consisted of 40 cycles with denaturation at 95 °C for 5 minutes, annealing at gene-specific temperatures for 15 seconds, and extension at 72 °C for 15 seconds. The relative expression was calculated as $2^{-\Delta\Delta C_T}$ (Livak and Schmittgen, 2001), where $\Delta\Delta C_T$ corresponds to the difference between C_T value of the mRNA levels of each sample and the C_T values for the mRNA levels of the reference sample.

2.14 Statistical analysis

Data was analyzed using SPSS version 21. One-way ANOVA and Duncan's multiple range tests were used to find significant differences between means. Student t-test was used to compare pre- and post-challenge conditions. Comparisons were made at the 5% significance level.

Results

3.1 Growth and survival

The mean bodyweight of the fish was recorded at the beginning and the end of the experiment and also at every 15 days interval. There was a slight but significant ($p<0.05$) difference in the weight gain among the different treatments. The percentage weight gain, specific growth rate, protein efficiency ratio, and feed conversion ratio were also observed to be similar, among the different treatments (Table – 3). The survival rate at the end of the 75-day feeding trial was 100% in the T2, T3, and T4 treatment groups.

3.2 Serum and tissue immunological indices

3.2.1 Phagocytic oxidase activity

There was a significant ($p<0.05$) difference in the phagocytic activity of the different treatment groups in both the pre-and post-challenge periods. In addition, in the post-challenge period, there was a significant increase in the phagocytic activity and phagocytic index of the different treatment groups, except for the control group. In the pre-challenge period, the highest phagocytic activity and index were found in the T4 group, whereas the lowest were found in the control group. The same trend was observed during the post-challenge period. (Fig. 1a).

3.2.2 Lysozyme Activity

There was a significant ($p<0.05$) difference in serum lysozyme activity among the different treatments at both periods. In the pre-challenge period, the T4 group showed significantly higher serum lysozyme activity, and the lowest activity was observed in the control group. The same trend was followed in the post-challenge period. As expected, after infection, lysozyme activity in all treatment groups increased compared to their pre-challenge counterparts, with the highest activity observed again in T4 (Fig. 1b).

3.2.3 Respiratory Burst Activity (Nitro Blue Tetrazolium Assay)

The pre- and post-challenge respiratory burst activity of *Oncorhynchus mykiss* fingerling neutrophils was estimated using the NBT method. The control group exhibited the lowest NBT activity, which was similar to that of the T1 group. The highest activity was

observed in the T4 group. In the post-challenge study, the lowest NBT activity was observed in the control group, whereas the highest was observed in the T4 group (Fig. 1c).

3.2.4 Total serum protein

Pre- and post-challenge serum protein levels in different experimental groups differed significantly ($p < 0.05$). The T4 group had the highest levels of serum protein before and after the challenge, while the control group had the lowest. The T4 group also had the highest albumin levels throughout. Before the challenge, the T4 group showed the highest serum globulin levels, but after the challenge, the T3 group exhibited the highest levels. The control group had the lowest serum globulin levels. After the challenge, the 1.75% NNLE group had the highest serum globulin levels, while the control and T1 group had the lowest (Fig. 1d).

3.3 Metabolic Enzymes

In the liver, T2 showed lower AST and ALT values compared to the control group, except for ALT, which had insignificant differences. Muscle tissues had the lowest ALT and AST activity in the control group and the 1.75% NNLE-fed group (Fig 2a). MDH values in the liver were significantly different, with the lowest activity in the T2 group. LDH values in the liver and muscle, and MDH values in the muscle were not significantly different (Fig 2b). ALP and ACP activities in the intestine were not significantly different among treatments. G-6-PDH activity in the liver was significantly different ($p < 0.05$) in the 1.75% NNLE-fed group compared to other treatments, with the highest activity in this group. No significant difference was observed between T2 and T3 ($p < 0.05$). The control group had the lowest G6PDH activity among treatment groups (Fig 2c).

3.4 Enzymes of oxidative stress

Before the challenge, gill SOD activity did not differ significantly among groups ($p > 0.05$). However, liver SOD activity showed significant differences both before and after the challenge. The T4 group had the lowest catalase activity in the liver, while the control group had the highest. This trend was also observed in the gill (Fig. 3). After the challenge, all treatment groups showed increased catalase activity compared to their pre-challenge levels. In the post-challenge period, the 1.75% NNLE treatment group had the lowest catalase activity in both gill and liver compared to other treatments.

3.5 Haematological indices

T4 had significantly higher blood hemoglobin values than other groups, both before and after the challenge (Fig 4a). Total erythrocyte count (TEC) differed significantly ($p < 0.05$) among treatment groups in both pre-challenge and post-challenge periods, with 1.75%

NNLE-fed group showing the highest TEC (Fig 4b). Blood TLC was significantly affected by NNLE in both pre-challenge and post-challenge periods, with the 1.75% NNLE group having the highest TLC (Fig 4c). T4 had the highest hematocrit values in both pre-challenge and post-challenge periods, while the control group had the lowest (Fig 4d).

3.6 Relative expression of immune related genes

The mRNA expression of β -Defensin, hepcidin, and cytokine genes (IL-1 β , TNF- α 1, IL-10, TGF- β 6, IL-12p40, and INF- γ) was evaluated in different groups. There was no significant difference in β -Defensin expression throughout the experimental period, except for a significant increase in the intestine on the 75th day compared to the control group. In the liver, there was a non-significant increase in some NNLE-fed groups compared to the control. Significant changes in defensin expression were observed in the kidney and spleen in T2, T3 & T4 (Fig. 5a).

On the 75th day, significant changes in defensin expression were observed in the intestine, head-kidney, and spleen, but not in the liver among the treatment groups. During the post-infection period, defensin expression in the intestine and liver increased significantly in T4 compared to the control group. In the head-kidney and spleen, there was a slight but significant increase in defensin expression in T4.

3.6.1 Relative expression of Hepcidin

On day 0, hepcidin expression in the intestine remained unchanged, while in the liver, kidney, and spleen, it was significantly upregulated in T3 & T4 groups (Fig. 5b). Over the experimental period, mRNA levels increased linearly on the 45th and 75th days across all treatment groups compared to the control. On the 75th day, hepcidin expression in the liver was 7.9-fold higher in T4 than the control group. In the spleen, a slight non-significant increase of 0.36-fold was observed on the 75th day between T3 & T4. After the challenge, T4 showed significant up-regulation of hepcidin in the kidney, spleen, and liver compared to other treatments. The intestine showed no significant change in gene expression across treatments, but all treatment groups had a significant fold change of 3.43 ± 0.28 compared to the control group.

3.6.2 Relative expression of Cytokines

Cytokine expression in the kidney and liver decreased significantly in the treatment groups compared to the control. Overall, the pro-inflammatory cytokine gene expression was down-regulated during the pre-challenge study and slightly up-regulated during the post-challenge study period. However, there was no linear trend in fold change among the treatment groups. Regulatory cytokines showed a linear and significant ($p<0.05$) up-or down-regulation across treatment groups in the selected tissue of rainbow trout to keep the levels of pro-inflammatory cytokines in check. *TNF- α 1* expression was significantly decreased in all the treatments compared to the control group. The lowest expression of *TNF- α 1* (spleen: 0.07 ± 0.01 & kidney: 0.11 ± 0.03) was found on the 75th day in both T3 and T4, respectively. mRNA expression of *IL-1 β* was found to be significantly down-regulated towards the 45th up to 75th day (pre-challenge period) and then slightly increased towards the end of the experiment 90th day (post-challenge period). During the post-challenge period, among NNLE treatment groups highest expression (5.11 ± 0.58) of the *IL-1 β* was observed in the spleen in T4. *TGF- β* mRNA expression levels in the intestine were significantly ($p<0.05$) increased in all the treatment groups compared to the control (Fig – 6). Significantly lower mRNA levels (0.07 ± 0.03 to 1.73 ± 0.02) of *TGF- β* were found in the liver across treatment groups throughout the experimental period of 90 days. *IL-10* mRNA expression levels showed different but not significant expression in the head kidney compared to *TGF- β* . In the head-kidney, spleen, liver, and intestine, the *IL-10* mRNA expression in all treatment groups were down-regulated compared to control during the pre-challenge period. Significantly ($p<0.05$) higher mRNA levels of *IL-10* were found in the control group compared to treatments during the post-challenge period. In spleen and head-kidney tissues, *IFN- γ* gene expression was increased in T3 and T4 treatment groups compared to the control group on the 45th and 75th days. Similar trends were observed in fold change post-infection in head-kidney. In the liver, there was no significant change in fold change during the post-infection period. The highest fold change of 10.03 ± 2.71 in *IFN- γ* gene expression was noted in the spleen of the fish fed with the highest percentage of NNLE (1.75%) on the 75th day. The expression of the *IFN- γ* gene significantly ($p<0.05$) varied in the pre-and post-challenge study period.

3.7 Challenge Study

3.7.1 Percentage Survival Rate

The viable *A. hydrophila* (MTCC 1739) cells injected into rainbow trout fingerlings were found to have an LD50 of 1.1×10^7 cfu/mL after 5 days of observation. When 0.1 mL/g

of the pathogenic bacterium *A. hydrophila* was injected into each treatment group after the 75th day, the first death was noticed 15 hours later. The T4 group, which was given 1.75% NNLE, had the highest relative survival percentage at the end of the 15-day challenge period. The lowest relative percentage survival was seen in the groups fed NNLE at 0.25 and 0.75%, T1, and T2. The highest survival rate was observed in the T4 group whereas the lowest values were observed in the control group. The morbid fish exhibited typical symptoms (bacterial hemorrhagic septicemia, hyperemia of fin base mouth ulcers, gill necrosis). The cause of the mortality was ascertained by re-isolation of *A. hydrophila* on Rimler-Shots (RS) agar.

Discussion

Plant based ingredients are known to significantly modulate the innate immune parameters and thereby confer protection against harmful bacteria (Harikrishnan et al., 2011; Chakraborty et al., 2013; Van Hai, 2015; Bilen and Elbeshti, 2019; Dias et al., 2019; Rashidian et al., 2020). Herein, we investigated the effect of lotus leaf extract in rainbow trout diets for its potential to boost immunity and survival. The diets for *O. mykiss* fingerlings contained 44.9-45.9% crude protein and 17.2-18.9% crude lipid. Whereas, the gross energy content of the experimental diet was within the range of 14.98 KJ/g, which aligns with the 14-17 KJ/g digestible energy requirement for rainbow trout fingerlings as reported by Kim & Kaushik (1992). In the current study, there was no statistically significant impact ($p < 0.05$) of *Nelumbo nucifera* leaf extract on weight gain, growth rate, feed conversion, and protein efficiency in the experimental fish. It is likely that the extract may contain bioactive agents that promote growth, but this depends on the part of the plant used. Sivagurunathan et al., (2012) and Munglue (2015) in their separate studies reported the dietary effect of *Nelumbo nucifera* on growth in *Cirrhinus mrigala* and *Clarias gariepinus*. The fish diet was supplemented with crude *Nelumbo nucifera* leaf extract and this suggests that the extract may contain essential bioactive and growth-promoting agents as well, which depends on the part of the plant used for the extraction.

Interestingly, the T4 group showed a slight increase in weight gain compared to the control group, which was not statistically significant. This could be due to the immune-modulating effects of lotus leaf extract in rainbow trout, which may have improved the digestibility and absorption of nutrients. The HSI values of the experimental groups were not significantly different ($p < 0.05$). There is no existing data on the impact of *Nelumbo nucifera* leaf extract on HSI values in fish. Similarly, there was no significant difference in the VSI

values of the experimental groups. There is limited information available on the effect of *Nelumbo nucifera* leaf extract on VSI in fish.

The experimental groups fed with crude *Nelumbo nucifera* leaf extract had a 100% survival rate, indicating that the extract had no harmful impact on the survival of the fish. Our findings are supported by the previous studies by Sivagurunathan et al. (2012) and Munglue (2015) wherein the authors reported that lotus leaf extract had no negative effects on fish behaviour or survival.

In our study, the activity of SOD and Catalase was significantly reduced with increasing levels of *Nelumbo nucifera* leaf extract in the diet, which suggests that the extract effectively scavenges free radicals. The presence of phenolic compounds and sulfur-containing groups makes *Nelumbo nucifera* leaf extract a potent antioxidant, capable of protecting lipids, membranes, and other compounds from oxidative damage (Sharma et al., 2017). Additionally, Liu et al. (2014) reported that the bioactive compounds in *Nelumbo nucifera* leaves exhibit strong antioxidant activity. Further, the control group had the highest serum glucose content, while the treatment groups had lower levels, indicating improved glucose utilization. The results suggest that *Nelumbo nucifera* leaf extract plays a role in glucose utilization in fish. The groups provided *Nelumbo nucifera* leaf extract exhibited lower post-challenge serum glucose readings than the control group. This is consistent with earlier research by Talpur (2014), who fed peppermint to Asian seabass as a feed additive. The outcomes may be related to the hypoglycemic activity of NNLE in *O. mykiss*, which increased plasma insulin levels and enhanced peripheral glucose metabolism (Awad, 2010).

Fish phagocytes are known to produce superoxide anions and other reactive oxygen species during a process called the respiratory burst. These substances are toxic to bacterial pathogens in fish and are generated by phagocytes after stimulation, leading to increased oxygen consumption (Itou et al., 1996). In this study, the respiratory burst activity of phagocytes was measured through the reduction of nitroblue tetrazolium (NBT) by intracellular superoxide radicals produced by leucocytes. The results showed that *Nelumbo nucifera* leaf extract significantly ($p < 0.05$) increased the NBT values, suggesting its immune-protective role in fish. The group T4 fed with 1.75% *Nelumbo nucifera* leaf extract had the highest phagocytic activity (26%) and phagocytic index (1.81), while the control group had the lowest (12.66% and 1.37, respectively). After being challenged, the phagocytic activity in all treatment groups was consistently higher than before. The group fed with 1.75% *Nelumbo*

nucifera leaf extract again showed the highest values (29.33% and 1.9). The phagocytic index mirrored the trend of phagocytic activity. These results align with Rahman et al. (2018), who found an increase in phagocytic activity in Nile tilapia due to incorporation of *Nelumbo nucifera* leaf powder in their diet. Mukherjee et al. (2010) also supports our findings, with their study on Swiss albino mice revealing the benefits of *Nelumbo nucifera* seed and rhizome extract consumption. Whereas, the highest red blood cell count was found in the T4 group that was given 1.75% *Nelumbo nucifera* leaf extract. The same trend was observed after a bacterial challenge, with a significant ($p < 0.05$) decrease in red blood cell count and haemoglobin values. This is in agreement with findings of previous studies on infected fish and presents a sign of anaemia (Ranzani-Pavia et al., 2004; Miyazaki et al., 1985). The decrease in haematocrit value may also be due to the decrease in red blood cell count (Foda, 1973). White blood cells, or leucocytes, play a crucial role in the non-specific immunity of fish (Secombes and Wang, 2012). In this study, the highest number of white blood cells was observed in the T4 group that was given 1.75% *Nelumbo nucifera* leaf extract. The white blood cell count is a reflection of the health status of fish, although the normal range for healthy fish is not well established as reported by Fazio 2019 and Witeska et al., 2022. In the post-challenge period, there was a significant increase in white blood cells in all groups, which is an indication of fish defence to fight off infections.

Also, the serum proteins, albumins and globulins, play a crucial role in the immune response. The present study analysed the serum proteins, albumin and globulin, both before and after challenge, and found a significant difference between the treatment groups. The highest levels of plasma protein were found in the T4 group fed a diet with 1.75% *Nelumbo nucifera* leaf extract. The lowest levels were found in the control group. The results suggest that *Nelumbo nucifera* leaf extract can enhance the immunity of *O. mykiss* fingerlings. The non-specific immune parameters were stimulated by the extract in a dose-dependent manner, leading to an increase in immunostimulation.

Apart from phagocytes, lysozyme is a component of the innate immune system that inhibits the growth of harmful microorganisms by breaking down the bacterial cell wall (Alexander & Ingram, 1992). In this study, *Nelumbo nucifera* leaf extract was found to significantly increase serum lysozyme activity in fish (from 18.14 to 67.4 U/mg protein in the control and T4 groups, respectively) during the pre-challenge period. This is in line with previous reports suggesting that immunostimulants can increase serum lysozyme activity (Chen et al., 2022). During the post-challenge period, serum lysozyme activity continued to

show an increased trend, similar to the findings of Sahu et al., 2007, in which *Labeo rohita* displayed increased serum lysozyme activity after being challenged with *Aeromonas hydrophila*. The increased serum lysozyme activity in this study indicates activation of the non-specific immune system. Sivagurunathan et al. (2012) also observed substantial increases in total leucocyte count, neutrophils, and monocytes after feeding Indian lotus to Mrigal fish, which could explain the increased lysozyme activity seen in this study.

Transaminases are enzymes that transfer the amino nitrogen from one amino acid to another, creating new amino acids using the amino group from the pre-existing ones. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are the most crucial transaminases and are also referred to as glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT), respectively. In the present study, the AST and ALT activity was studied in the muscle and liver tissues. In the muscle tissues, the highest AST activity (12.31) was observed in the T4 group whereas the least (6.49) was observed in the T2 group. The higher activity of AST and ALT enzymes in the muscle indicates the synthesis of pyruvate and oxaloacetate which are involved in the synthesis of non-essential amino acids required for growth. Amino acid catabolism occurs mostly in the liver, resulting in the formation of 50–99 percent ammonia (Esmaeili et al., 2022). The use of lotus leaf extract resulted in a substantial decrease in liver AST and ALT activity. This indicates that the metabolism is preparing to save the protein for usage as an energy source (Kultz and Jurss 1993; Gopan et al., 2021). In the fish fed with NNLE, a rise in the level of muscle ALT was observed, which was in contrast to the liver trend. This is because muscle tissue metabolism differs from that of the liver in terms of the function of transaminases, which are involved in protein synthesis and breakdown (Lin and Luo, 2011). As a result of the enhanced transaminase activity in the muscle, lotus leaf extract may steer the metabolic balance of the fish toward growth.

Lactate dehydrogenase is the terminal enzyme of the glycolysis pathway and helps in maintaining the glycolysis cycle by supplying NAD^+ . Herein, the lactate dehydrogenase enzyme was not affected by dietary *Nelumbo nucifera* leaf extract, however, the liver MDH activity was lowered in the lotus leaf extract fed groups. The Krebs's cycle enzyme malate dehydrogenase is crucial. With the release of NADH^+ , it reversibly transforms malate to oxaloacetate. The reduced activity of MDH might be due to decreased liver AST activity, which would have resulted in a decrease in the substrate oxaloacetate (Verma et al., 2007).

As a result, nutritional supplementation with *Nelumbo nucifera* leaf extract did not affect fish metabolism.

The activity of acid phosphatase (ACP) and alkaline phosphatase (ALP) is critical for phosphorus metabolism in the body.). However, the effects of lotus leaf extract on the intestinal acid phosphatase and alkaline phosphatase enzymes in fish were not significant ($p < 0.05$), indicating no influence on phosphorus metabolism. Also, the enzyme Glucose-6-phosphate dehydrogenase is a part of the pentose phosphate pathway and converts glucose-6-phosphate into 6-phosphogluconolactone using NADP⁺ as a cofactor. This process generates NADPH, which is then utilized by NADPH oxidase to produce superoxide anions for the purpose of destroying phagocytized material. As a result, an increase in phagocytosis is expected to lead to an increase in the enzyme's activity (Das et al., 2002). In the present study, the activity of the G6PDH enzyme was found to be significantly higher (30.18 , $p < 0.05$) in the T4 group. The results are in line with the findings of the phagocytic activity observed by Das et al. (2002). The highest *Nelumbo nucifera* leaf extract fed groups had increased G6PDH activity compared to the control, which impacted the phagocytic activity by producing more superoxide anions (Chotigeat et al., 2004).

Aeromonas hydrophila is a major bacterial pathogen that causes infectious diseases in fish, such as haemorrhagic septicaemia, infectious dropsy, tropical ulcerative disease, fin rot, and leads to high mortality in the aquaculture industry (Kumar & Dey, 1988; Pridegon & Klesius, 2012). The present challenge study with *A. hydrophila* showed increased survival in fish fed with *Nelumbo nucifera* leaf extract compared to the control group, demonstrating the positive impact of the extract in resisting bacterial infection and promoting survival of *O. mykiss* fingerlings. The results showed a significant, dose-dependent increase in relative percentage survival ($p < 0.05$). Previous studies have also shown that the administration of various herbal plants can increase resistance to *A. hydrophila* in Nile tilapia and common carp (Kirubakaran et al., 2016; Sunitha et al., 2017; Yeasmin et al., 2015).

The present study also did mRNA expression profiling of the β -Defensin, hepcidin, and cytokine (IL-1 β , TNF- α 1, IL-10, TGF- β 6, IL-12p40, and INF- γ) in the different experimental groups. It was observed that the expression of defensins varied among different tissues. The highest increase was seen in the intestine (7.21 ± 0.99), followed by the liver (6.21 ± 0.78) and spleen (3.33 ± 0.54). This result is in line with a previous study by Marel et al. (2012) which showed an upregulation of defensin expression in the intestine of *Cyprinus*

carpio when fed with β -glucan. However, no significant upregulation was observed in the head-kidney, which aligns with the findings of Liang et al. (2013) who reported low expression of defensins in the kidney of *Megalobrama amblycephala*. The spleen showed a low but significant ($p < 0.05$) increase in defensin expression on the 75th day. Whereas, the upregulated expression of hepcidin was observed in the liver, head kidney, and spleen of the NNLE fed groups compared to the control group. This is consistent with Liu et al.'s (2010) earlier research, which showed that hepcidin was significantly upregulated in the liver of *Ctenopharyngodon idella*. The results of the study showed that integration of lotus leaf extract changed the expression of defensin and hepcidin in a dose-dependent manner, with 1.75% *Nelumbo nucifera* leaf extract having a bigger impact than 0.25% NNLE. The findings in this study demonstrate the significance of antimicrobial peptides in innate immunity by demonstrating that both defensin and hepcidin mRNA levels increased considerably in the T3 and T4 therapy groups during the post-challenge period. According to the findings, NNLE may have an impact on rainbow trout's acute phase protein and antibacterial activities. Na-Phatthalung et al. (2018) also demonstrated the benefits of *Rhodymyrtus tomentosa* leaf extract injection in rainbow trout (*Oncorhynchus mykiss*), which may indicate better fish resistance to infection, stress, or inflammation.

Cytokines are proteins made by immune cells that play a part in cell growth, differentiation, and the body's response to infections (Ellis, 2001; Wei, et al., 2009). IL-1 β and TNF- α are key cytokines involved in inflammation and the immune response (Aoki, Takano, Santos, Kondo, & Hirano, 2008; Corripio Miyar, Bird, Tsamopoulos, & Secombes, 2007). The current study showed that a 1.75% lotus leaf extract increased IL-1 β and TNF- α levels in rainbow trout after exposure to a bacterial challenge. Similar findings have been reported in other studies of fish fed plant extracts (Adel et al., 2020; Hosenif, 2018; Giri, Sukumaran, and Park 2019; Nanji et al., 2003; Sharma, Steward, & Gesche, 2007; Larmonier et al., 2008; Gutierrez et al., 2012). Our study also found that feeding rainbow trout a diet containing NNLE reduced TNF- α expression in all studied tissues. However, the expression of IL-1 β decreased in a non-linear way as the amount of NNLE in the diet increased. The study results were in line with previous research conducted by Yin et al. (2018) who found that curcumin reduces IL-1 β secretion and prevents inflammation in mice. Similarly, Giri et al. (2019) showed that curcumin decreases the expression of IL-1 β in *C. carpio*. Ming et al. (2020) also noted that a diet supplemented with curcumin decreases both TNF- α and IL-1 β expression.

IL-10 is a cytokine with immunosuppressive properties that controls the inflammatory response and prevents harm to the host. The mRNA expression of IL-10 in all studied tissue sample was down-regulated in comparison to the control group after exposure to a challenge. Lower expression levels of IL-10 has also been found following the administration of different stimulants, including Spirulina (Attiya, 2022; Ibrahim et al., 2022; Awad et al., 2022) . This study revealed that cytokines with regulatory or immune-modulating effects, such as IL-10 and TGF- β , got activated. The major role of IL-10 appears to be the control of the inflammatory response, preventing harm to the host from an excessive reaction (Raida & Buchmann 2008). Thus, IL-10 suppresses the activation of macrophages/monocytes by blocking chemokine receptors and inhibiting the action of pro-inflammatory cytokines, hence controlling cytokine synthesis and nitric oxide (NO) generation. In the head-kidney, spleen, liver, and intestine, the mRNA expression in all treatment groups was down-regulated compared to control in the post-challenge period. Lower expression levels of IL-10 were also found following the administration of different stimulants such as Spirulina (Watanuki et al., 2006). The present study showed that the regulating or immune-modulatory cytokines such as IL-10 and TGF- β got activated.

Regulating cytokines like IL-10 and TGF- β can suppress the production of pro-inflammatory cytokines, like IL-1 and TNF- α , while directing adaptive immune pathways that involve B lymphocytes (Abbas et al., 1996; D'Amico et al., 2000; Dinarello 2000; Zou & Secombes 2016). The increased expression of IL-10 in the NNLE fed groups may be due to the presence of anti-inflammatory bioactive compounds like neferine, nuciferine, and phenolics (Rahman et al., 2018). IL-12p40 helps regulate cell-mediated immunity and stimulates the production of IFN- γ from Th1 and NK cells for protection against parasites, viruses, and bacteria (Vergrd et al., 2012). The expression of IL-12p40 was up-regulated in T3 and T4 groups in various tissues. Caper extract has been shown to increase IL-12p40 expression in rainbow trout (Bilen et al., 2016). IFN- γ is a crucial cytokine for cell-mediated immunity against viral and bacterial agents (Biswas et al., 2013), and its expression increased in fish after 45 days and after a challenge period of feeding. Probiotics have also been shown to increase the expression of IFN- γ (Panigrahi et al. 2011; Biswas et al. 2013).

Conclusion:

Based on the current findings, it can be concluded that using *Nelumbo nucifera* leaf extract in the *Oncorhynchus mykiss* diet increases overall welfare of the fish to better deal

with anthropogenic stress and combat opportunistic pathogens in aquatic habitat. NNLE is a strong antioxidant and inhibits pathogenic bacteria as confirmed by *in vitro* and *in vivo* experiments. It enhances the non-specific/innate immunity of rainbow trout fingerlings challenged with *Aeromonas hydrophila*. The lotus extract significantly down-regulates the pro-inflammatory cytokines during the pre-challenge study. Overall, the NNLE-fed groups showed a better survival rate (RPS) compared to control and this suggests that NNLE incorporation in diet is a better prophylactic option to prevent fish disease outbreak.

Declaration of interest: none.

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Animal Ethics declaration: The study was approved by Institutional Animal Ethics Committee, Faculty of Veterinary Sciences & AH SKUAST-Kashmir (**Certificate No. AU/FVSc/PS-57/15198 Dated: 19/03/2018**).

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References

- Abdel Rahman, A. N., ElHady, M., & Hassanin, M. E. 2018. Effect of Indian lotus (*Nelumbo nucifera* Gaertn.) leaf powder on immune status and disease resistance of Nile tilapia. *Aquaculture Research*, **49**(10):3392-3399.
- Abdel Rahman, A., Hassanin, M. and ElHady, M., 2019. Growth performance, haematology and intestinal histo-morphology of Nile tilapia fed on Indian Lotus (*Nelumbo nucifera*

- Gaertn.) leaf powder at different concentrations. *Aquaculture Research*, 50(11), pp.3211-3222.
- Agina, O. A., Okoye, C. N., & Dan-Jumbo, S. O. (2020). Glucose levels are stable in stored blood and plasma samples of cultured female African catfish (*Clarias gariepinus*). *Comparative Clinical Pathology*, 29, 385-389.
- Alexander, J. B. and Ingram G. A., 1992. Noncellular nonspecific defense mechanism of fish. *Annu. Rev. Fish Dis.* 2: 249-279
- AOAC, 1995. Official methods of Analysis of AOAC International, Vol. 1, 16th edn. (ed.Cunnif, P. A.). *AOAC International*, Arlington, USA.
- APHA-AWWA-WEF, 1998. Standard methods for the estimation of water and waste water, 20th edn. (ed. Clesceri, L.S., Greenberg, A.E. and Eaton, A. D.). *American Public Health Association, American Waters Works Association, Water Environment Federation*, Washington DC.
- Attiya, H. (2022). Protective Effect of Spirulina against Aspartame Induced Oxidative Stress and Molecular Gene Brain damage. *Journal of the Hellenic Veterinary Medical Society*, 73(1), 3689-3698.
- Awad E.S. (2010). Studies on plant based dietary supplements for control of *Aeromonas hydrophila* infections in rainbow trout (*Oncorhynchus mykiss* Walbaum). A thesis of PhD. Scholl of Life Sciences, Heriot Watt University, Edinburgh, UK. 204 p.
- Awad, E. S. (2010). Studies on plant based dietary supplements for control of *Aeromonas hydrophila* infections in rainbow trout (*Oncorhynchus mykiss* Walbaum) (Doctoral dissertation, Heriot-Watt University).
- Awad, L. Z., El-Mahallawy, H. S., Abdelnaeim, N. S., Mahmoud, M. M., Dessouki, A. A., & ElBanna, N. I. (2022). Role of dietary Spirulina platensis and betaine supplementation on growth, hematological, serum biochemical parameters, antioxidant status, immune responses, and disease resistance in Nile tilapia. *Fish & Shellfish Immunology*, 126, 122-130.
- Bartoňková, J., Hyršl, P., & Vojtek, L. (2017). Glucose determination in fish plasma by two different moderate methods. *Acta Veterinaria Brno*, 85(4), 349-353.
- Ben Hamed, S., Tapia-Paniagua, S.T., Moriñigo, M.Á. and Ranzani-Paiva, M.J.T., 2021. Advances in vaccines developed for bacterial fish diseases, performance and limits. *Aquaculture Research*, 52(6), pp.2377-2390.

- Bilen, S., & Elbeshti, H. T. A. G. (2019). A new potential therapeutic remedy against *Aeromonas hydrophila* infection in rainbow trout (*Oncorhynchus mykiss*) using tetra, *Cotinus coggygia*. *Journal of fish diseases*, 42(10), 1369-1381.
- Chakraborty, S. B., Horn, P., and Hancz, C. (2013). Application of phytochemicals as growth-promoters and endocrine modulators in fish culture. *Rev. Aquacult* **5**:1–19.
- Chen, P., Jin, D., Yang, S., Yu, X., Yi, G., Hu, S., ... & Xia, L. (2022). *Aeromonas veronii* infection remarkably increases expression of lysozymes in grass carp (*Ctenopharyngodon idellus*) and injection of lysozyme expression cassette along with QCDC adjuvant significantly upregulates immune factors and decreases cumulative mortality. *Microbial Pathogenesis*, 169, 105646.
- Conte, F.S., 2004. Stress and the welfare of cultured fish. *Applied Animal Behaviour Science*, 86(3-4), pp.205-223.
- Dias, M. K. R., Yoshioka, E. T. O., Rodriguez, A. F. R., Ribeiro, R. A., Faria, F. S. E. D. V., Ozório, R. O. A., & Tavares-Dias, M. (2019). Growth, physiological and immune responses of *Arapaima gigas* (Arapaimidae) to *Aeromonas hydrophila* challenge and handling stress following feeding with immunostimulant supplemented diets. *Fish & shellfish immunology* **84**:843-847.
- Doumas, B. T., and Biggs, H. G., 1972. Determination of serum albumin. **In**: Stand. Method Clin. Chem., Acad. Press, New York **7**: 175
- Esmaceli, N., Carter, C. G., Wilson, R., Walker, S. P., Miller, M. R., Bridle, A. R., & Symonds, J. E. (2022). Protein metabolism in the liver and white muscle is associated with feed efficiency in Chinook salmon (*Oncorhynchus tshawytscha*) reared in seawater: Evidence from proteomic analysis. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, 42, 100994.
- Fazio, F. (2019). Fish hematology analysis as an important tool of aquaculture: a review. *Aquaculture*, 500, 237-242.
- Fazio, F. (2019). Fish hematology analysis as an important tool of aquaculture: a review. *Aquaculture*, 500, 237-242.
- Foda, A., 1973., Changes in hematocrit and haemoglobin in Atlantic Salmon (*Salmo salar*), as a result of furunculosis disease. *J. Fisheries. Res. Board. Canada* **30**:467-468.
- Gabriel, N.N., 2019. Review on the progress in the role of herbal extracts in tilapia culture. *Cogent Food & Agriculture*, 5(1), p.1619651.

- Garen, A., and Levinthal, C. A., 1960. Fine structure, genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*: Purification and characterization of alkaline phosphatase. *Biochem. Biophys. Acta.*, 38: 470
- Gopan, A., Maiti, M. K., Varghese, T., Lalappan, S., Sardar, P., Gupta, S., & Sahu, N. P. (2021). Nutritional evaluation of protein isolate from neem seed cake in *Labeo rohita* (Hamilton, 1822) fingerlings concomitant with oil extraction processing industry. *Animal Feed Science and Technology*, 274, 114858.
- Harikrishnan, R., Balasundaram, C., and Heo, M.-S. (2011). Impact of plant products on innate and adaptive immune system of cultured finfish and shellfish. *Aquaculture*.
- Harikrishnan, R., Devi, G., Van Doan, H., Balasundaram, C., Arockiaraj, J. and Jagruthi, C., 2021. Efficacy of ulvan on immune response and immuno-antioxidant gene modulation in *Labeo rohita* against columnaris disease. *Fish & Shellfish Immunology*, 117, pp.262-273.
- Hoseinifar, S.H., Jahazi, M.A., Mohseni, R., Raeisi, M., Bayani, M., Mazandarani, M., Yousefi, M., Van Doan, H. and Mozanzadeh, M.T., 2020. Effects of dietary fern (*Adiantum capillus-veneris*) leaves powder on serum and mucus antioxidant defence, immunological responses, antimicrobial activity and growth performance of common carp (*Cyprinus carpio*) juveniles. *Fish & Shellfish Immunology*, 106, pp.959-966.
- Ibrahim, D., Abd El-Hamid, M. I., Al-Zaban, M. I., ElHady, M., El-Azzouny, M. M., ElFeky, T. M., ... & Omar, A. E. (2022). Impacts of fortifying Nile tilapia (*Oreochromis niloticus*) diet with different strains of microalgae on its performance, fillet quality and disease resistance to *Aeromonas hydrophila* considering the interplay between antioxidant and inflammatory response. *Antioxidants*, 11(11), 2181.
- Jahazi, M.A., Hoseinifar, S.H., Jafari, V., Hajimoradloo, A., Van Doan, H. and Paolucci, M., 2020. Dietary supplementation of polyphenols positively affects the innate immune response, oxidative status, and growth performance of common carp, *Cyprinus carpio* L. *Aquaculture*, 517, p.734709.
- Jang, H.M., Kim, Y.B., Choi, S., Lee, Y., Shin, S.G., Unno, T. and Kim, Y.M., 2018. Prevalence of antibiotic resistance genes from effluent of coastal aquaculture, South Korea. *Environmental pollution*, 233, pp.1049-1057.
- Kim, J. D., & Kaushik, S. J. (1992). Contribution of digestible energy from carbohydrates and estimation of protein/energy requirements for growth of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 106(2):161-169.

- Kot, B., Kwiatek, K., Janiuk, J., Witeska, M. and Pękala-Safińska, A., 2019. Antibacterial activity of commercial phytochemicals against *Aeromonas* species isolated from fish. *Pathogens*, 8(3), p.142.
- Kultz D, Jurss K (1993) Biochemical characterization of isolated branchial mitochondria-rich cells of *Oreochromis mossambicus* acclimated to fresh water or hypersaline sea water. *J Comp Physiol* 163B:406–412
- Liu CM, Kao CL, Wu HM, et al. 2014. Antioxidant and anticancer aporphine alkaloids from the leaves of *Nelumbo nucifera* Gaertn. cv. Rosa-plena. *Molecules* **19**:17829–17838.
- Livak, K.J. and Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻ΔΔCT method. *methods*, 25(4), pp.402-408.
- Lugert, V., Thaller, G., Tetens, J., Schulz, C., & Krieter, J. (2016). A review on fish growth calculation: multiple functions in fish production and their specific application. *Reviews in aquaculture*, 8(1), 30-42.
- Mehta, N.R., Patel, E.P., Patani, P.V. and Shah, B., 2013. *Nelumbo nucifera* (Lotus): a review on ethanobotany, phytochemistry and pharmacology. *Indian Journal of Pharmaceutical and Biological Research*, 1(04), pp.152-167.
- Mishra, H. P., and Frodovich, I., 1972. The role of superoxide anion in the autooxidation of epinephrine and a simple assay for SOD. *J. Biol. Chem.*, 247: 3179-3175 118
- Miyazaki T., and Kiege, N., 1985. A histopathological study on motile aeromonad disease in Crucian Carp. *Fish Pathol.* **21**:181-185.
- Mukherjee, D., Khatua, T.N., Venkatesh, P., Saha, B.P. and Mukherjee, P.K., 2010. Immunomodulatory potential of rhizome and seed extracts of *Nelumbo nucifera* Gaertn. *Journal of ethnopharmacology*, **128**(2), pp.490-494.
- Munglue, P., 2014, November. Effects of dietary *Nelumbo nucifera* (lotus) peduncle extract on growth performance of Nile tilapia (*Oreochromis niloticus*). In *Proceedings of the 1st Environmental and Natural Resources International Conference (ENRIC 2014)*, Bangkok, Thailand (pp. 6-7).
- Munglue, P., 2015. Effect of *Nelumbo nucifera* Gaertn. leaf extract on growth performance of Nile tilapia (*Oreochromis niloticus*). *Journal of Food Health and Bioenvironmental Science*, **8**(1), pp.45-56.
- Na-Phatthalung, P., Teles, M., Voravuthikunchai, S. P., Tort, L., & Fierro-Castro, C. (2018). Immune-related gene expression and physiological responses in rainbow trout (*Oncorhynchus mykiss*) after intraperitoneal administration of *Rhodomyrtus*

- tomentosa leaf extract: A potent phytoimmunostimulant. *Fish & shellfish immunology*, 77, 429-437.
- Ranzani-Pavia, M.J.T., Ishikawa, C.M., Eiras, A.C. and Silveira, V.R., 2004. Effects of an experimental challenge with *Mycobacterium marinum* on the blood parameters of Nile Tilapia. *Braz. Arch. Biol. Tech.* **47**:945-953.
- Rashidian, G., Bahrami Gorji, S., Farsani, M. N., Prokić, M. D., & Faggio, C. (2020). The oak (*Quercus brantii*) acorn as a growth promotor for rainbow trout (*Oncorhynchus mykiss*): growth performance, body composition, liver enzymes activity and blood biochemical parameters. *Natural Product Research*, 34(17), 2413-2423.
- Renhold, J. G., 1953, Manual determination of serum total protein, albumin and globulin fraction by Biuret method. **In**: Standard method of clinical chemistry (ed. Reiner, M.). Academic Press, New York, pp 88.
- Sacks DB (2008) Carbohydrates. In: Burtis CA, Ashwood ER, Brunis DE (eds) Tietz fundamentals of clinical chemistry, 6th edn. St. Louis, Saunders Elsevier, p 373
- Sahu, S., Das, B. K., Mishra, B. K., Pradhan, J., & Sarangi, N. (2007). Effect of *Allium sativum* on the immunity and survival of *Labeo rohita* infected with *Aeromonas hydrophila*. *Journal of Applied Ichthyology*, 23(1), 80-86.
- Sheikh, S.A., 2014. Ethno-medicinal uses and pharmacological activities of lotus (*Nelumbo nucifera*). *Journal of Medicinal Plants Studies*, 2(6), pp.42-46.
- Sankaran, K. and Gurnani, S.H.A.N.T.O.O., 1972. On the variation in the catalytic activity of lysozyme in fishes. *Indian journal of biochemistry & biophysics*, 9(2), pp.162-165.
- Secombes, C. J., & Wang, T. (2012). The innate and adaptive immune system of fish. In *Infectious disease in aquaculture* (pp. 3-68). Woodhead Publishing.
- Sharma, B.R., Gautam, L.N.S., Adhikari, D. and Karki, R., 2017. A comprehensive review on chemical profiling of *Nelumbo nucifera*: potential for drug development. *Phytotherapy Research*, 31(1), pp.3-26.
- Sivagurunathan, A., Innocent, B.X. and Lakshmi, S.M., 2012. Immunomodulatory effect of dietary *Nelumbo nucifera* (Lotus) in growth and haematology of *Cirrhinus mrigala* challenged with *Pseudomonas aeruginosa*. *Journal of Applied Pharmaceutical Science*, 2(7), pp.191-195.
- Stratev, D., Zhelyazkov, G., Noundou, X.S. and Krause, R.W., 2018. Beneficial effects of medicinal plants in fish diseases. *Aquaculture International*, 26(1), pp.289-308.

- Sutuli, F.J., Gatlin III, D.M., Heinzmann, B.M. and Baldisserotto, B., 2018. Plant essential oils as fish diet additives: benefits on fish health and stability in feed. *Reviews in Aquaculture*, 10(3), pp.716-726.
- Takhara, S., Hamiton, H.B., Neel, J. V., Kobara, T.Y., Ogura, Y and Nishimura, E. T., 1960. Hypoctalasemia, a new genetic carrier state. *J. Clin. Invest.* **39**:610-619.
- Talpur, A. D. (2014). *Mentha piperita* (Peppermint) as feed additive enhanced growth performance, survival, immune response and disease resistance of Asian seabass, *Lates calcarifer* (Bloch) against *Vibrio harveyi* infection. *Aquaculture*, 420, 71-78.
- Villarino, Ma Eliza J., Millicent Gay Buenaseda Tejada, and Sala Elise Patterson. "From agricultural statistics to zero hunger: How the 50x2030 Initiative is closing data gaps for SDG2 and beyond." *Statistical Journal of the IAOS* 38.1 (2022): 63-73.)
- Witeska, M., Kondera, E., Ługowska, K., & Bojarski, B. (2022). Hematological methods in fish—Not only for beginners. *Aquaculture*, 547, 737498.
- Witeska, M., Kondera, E., Ługowska, K., & Bojarski, B. (2022). Hematological methods in fish—Not only for beginners. *Aquaculture*, 547, 737498.
- Wooten i. D. P. 1964. Microanalysis. **In:** Medical Biochemistry 4th ed (ed. Churchill, J. and Churchill, A.) London. pp 101-107
- Wrobleiuski, F. and Laude, J.S.,1955. LDH activity in blood *Proc. Soc. Exp. Biol. Med.* **90**:210-213.
- Yousefi, M., Zahedi, S., Reverter, M., Adineh, H., Hoseini, S.M., Van Doan, H., El-Haroun, E.R. and Hoseinifar, S.H., 2021. Enhanced growth performance, oxidative capacity and immune responses of common carp, *Cyprinus carpio* fed with *Artemisia absinthium* extract-supplemented diet. *Aquaculture*, 545, p.737167.
- Zanuzzo, F. S., Biller-Takahashi, J. D., & Urbinati, E. C. (2012). Effect of Aloe vera extract on the improvement of the respiratory activity of leukocytes of *matrinxa* during the transport stress. *Revista Brasileira de Zootecnia*, 41, 2299-2302.

Table 1. Composition and proximate composition analysis of the experimental diets (g/100 g).

Ingredients	Control	T1	T2	T3	T4
Casein	29.7	29.7	29.7	29.7	29.7
Gelatin	10	10	10	10	10
Dextrin	18.3	18.3	18.3	18.3	18.3
Starch	21.5	21.5	21.5	21.5	21.5
Cellulose	10	10	10	10	10
Cod oil	3	3	3	3	3
Sunflower oil	3	3	3	3	3
Vitamin-mineral mix	2	2	2	2	2
CMC	1.5	1.5	1.5	1.5	1.5
Choline	0.9	0.9	0.9	0.9	0.9
BHT	0.1	0.1	0.1	0.1	0.1
Lotus extract	0	0.25	0.75	1.25	1.75
Total	100	100	100	100	100
Proximate composition (g/100 g)					
Moisture	10 ± 0.98	10.7 ± 0.98	10 ± 0.98	9.75 ± 0.98	10.5± 0.98
In dry matter					
Crude protein	45.3 ± 0.11	45.5 ± 0.21	45.5 ± 0.01	45.7 ± 0.01	44.95 ± 0.15
Crude fibre	11.2 ± 0.98	11.1 ± 1.1	11.8 ± 0.45	11.9 ± 0.2	11.9 ± 0.2
Ether extract	1.8 ± 0.23	1.4 ± 0.3	1.7 ± 0.3	1.7 ± 0.3	1.7 ± 0.3
Ash	10.75 ± 0.98	11.5 ± 0.98	12 ± 0.98	11.7 ± 0.98	11.75± 0.98

Note: Composition of vitamin mineral mix (PREEMIX PLUS) (quantity/2.5 kg): Vitamin A, 5 500 000 IU; Vitamin D3, 1 100 000 IU; Vitamin B2, 2000 mg; Vitamin E, 750 mg; Vitamin K, 1000 mg; Vitamin B6, 1000 mg; Vitamin B12, 6 mcg; Calcium Pantothenate, 2500 mg; Nicotinamide, 10 g; Choline Chloride, 150 g; Mn, 27 000 mg; I, 1000 mg; Fe, 7500 mg; Zn, 5000 mg; Cu, 2000 mg; Co, 450 L-lysine, 10 g; DL-Methionine, 10 g; Selenium, 50 ppm.

Table 2. Primer sequences used for expression study (qPCR) of immune genes in *Oncorhynchus mykiss*

S.No	Gene code	Forward Sequences (5'-3')	Reverse Sequences (5'-3')	Annealing (°C)	Amplicon Size (bp)
1.	<i>β-Actin</i>	TCTGGCATCACACCTTCTAC	ATCTTCTCCCTGTTGGCTTT	59	144
2.	<i>IL-10</i>	GCTGGACGAAGGGATTCTAC	CGTTGTTGTTCTGTGTTCT	58	160
3.	<i>IL-12p40</i>	ATGTGGAAGTGGTGGAGATG	CAGCGTGTGGTTGAGGTAG	60	111
4.	<i>IL-1β</i>	GTATCCCATCACCCCATCAC	TTCTTCCACAGCACTCTCCA	59	114
5.	<i>TNF-α</i>	GCTGAACGAAGGGGACAA	GCAAACACACCAAAGAAG	56	108
6.	<i>TGF-β</i>	CGTGGCTCTCTGTGGATGT	TTGGTGGAGGGAACGAAG	59	200
7.	<i>INF-γ</i>	CTGTTTTCCCAAGGACAC	TCCAGAACCACACTCATCAAC	59	118
8.	<i>β-Defensin</i>	GTGCTCCTCGCTTTCTTG	AGACCCCGCTGTAGTTTGAG	59	140
9.	<i>Hepcidin</i>	CGGAGCATTCAGGTTCA	AGAAGCCACAGCCCTTGTT	59	144

Table 3. Body weight gain (g) and survival of *Nelumbo nucifera* leaf extract fed Rainbow trout fingerlings at 15days intervals during the experimental period of 75 days.

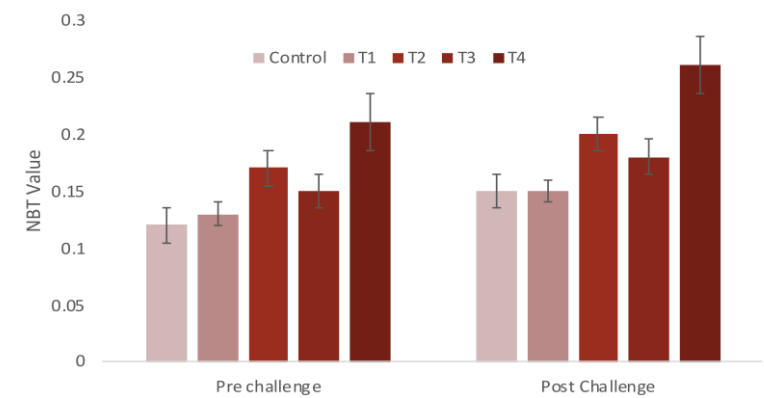
Treatments	Time (Days)						Survival (%)*
	0th	15th	30th	45th	60th	75th	

Control	20.98±0.09	28.24±00.14	35.00±0.34	44.90±0.05	55.90±0.05	72.90±0.07 ^c	98
T1	20.99±0.09	27.36±0.17	36.35±0.05	47.16±0.18	57.16±0.18	76.16±0.28 ^b	99
T2	19.93±0.07	28.13±0.08	35.40±0.1	46.67±0.17	56.67±0.17	79.67±0.11 ^a	100
T3	20.73±0.09	28.51±0.11	34.65±0.25	48.66±0.13	57.66±0.13	79.66±0.03 ^a	100
T4	20.63±0.14	27.70±0.15	35.48±0.23	49.53±0.31	59.53±0.31	81.53±0.01 ^a	100

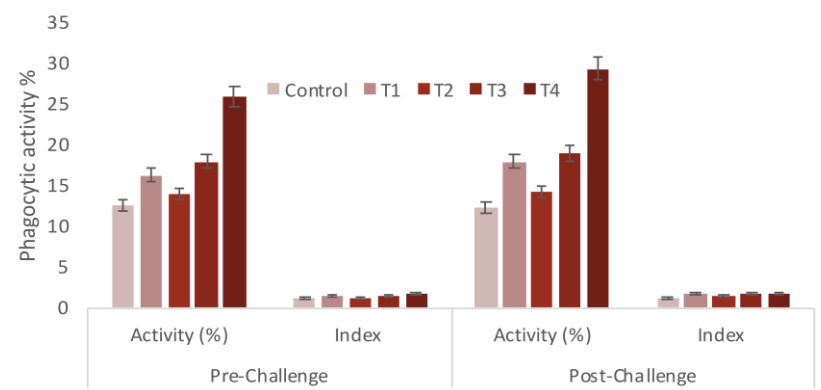
Data expressed as Mean ± SE, n=3, values with different superscripts differ significantly at p<0.05. LDH and MDH activity of liver and muscle tissues of different experimental groups.

**Data represented is Mean ± SE, n=3*

Figure 1. Serum and tissue immunological indices a) respiratory burst activity (NBT), b) phagocytic activity, c) lysozyme activity, and d) total serum protein



a
c



b
d

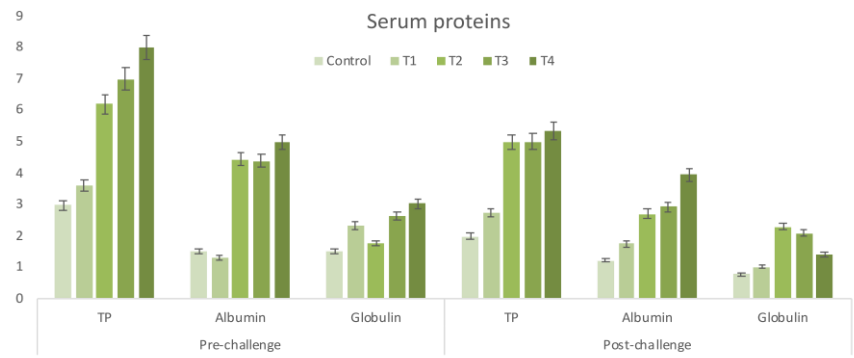
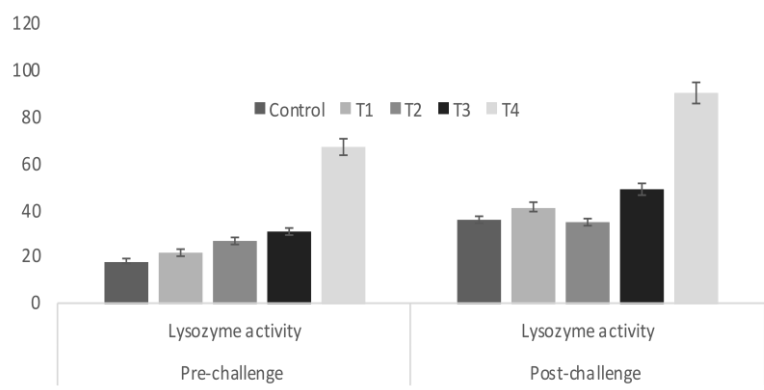
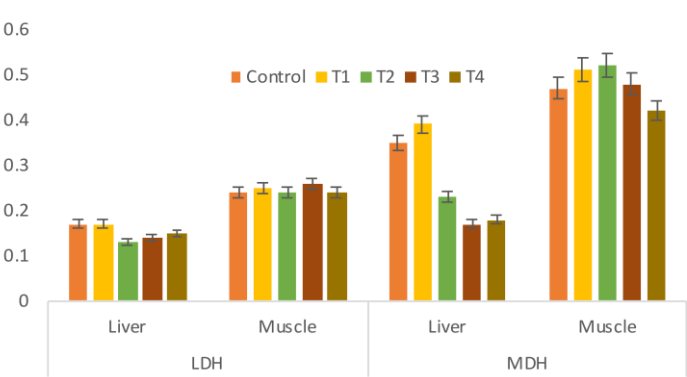
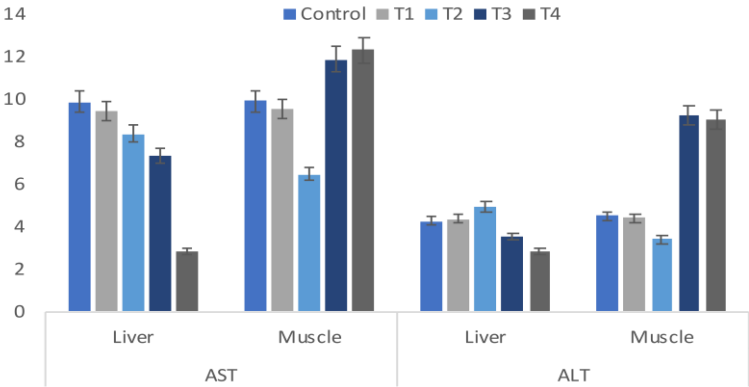


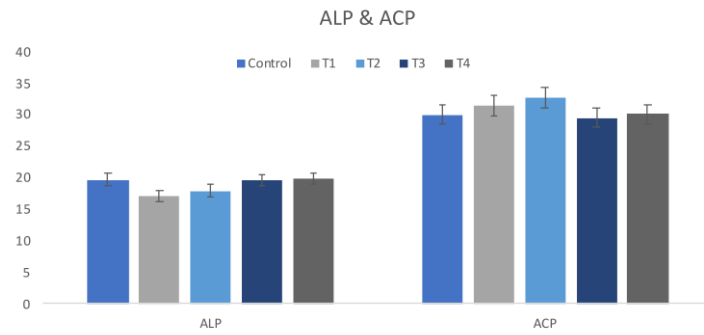
Figure 2. Metabolic enzymes (a) LDH and MDH (b) ALT, AST (c) ALP and ACP (d) blood glucose of Rainbow trout fed with *Nelumbo nucifera* leaf extract.



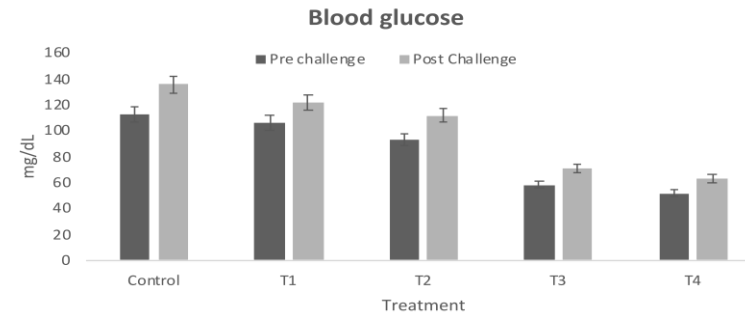
a



b



c



d

Figure 3. Enzymes of oxidative stress (a) SOD and (b) CAT of rainbow trout fed with *Nelumbo nucifera* leaf extract.

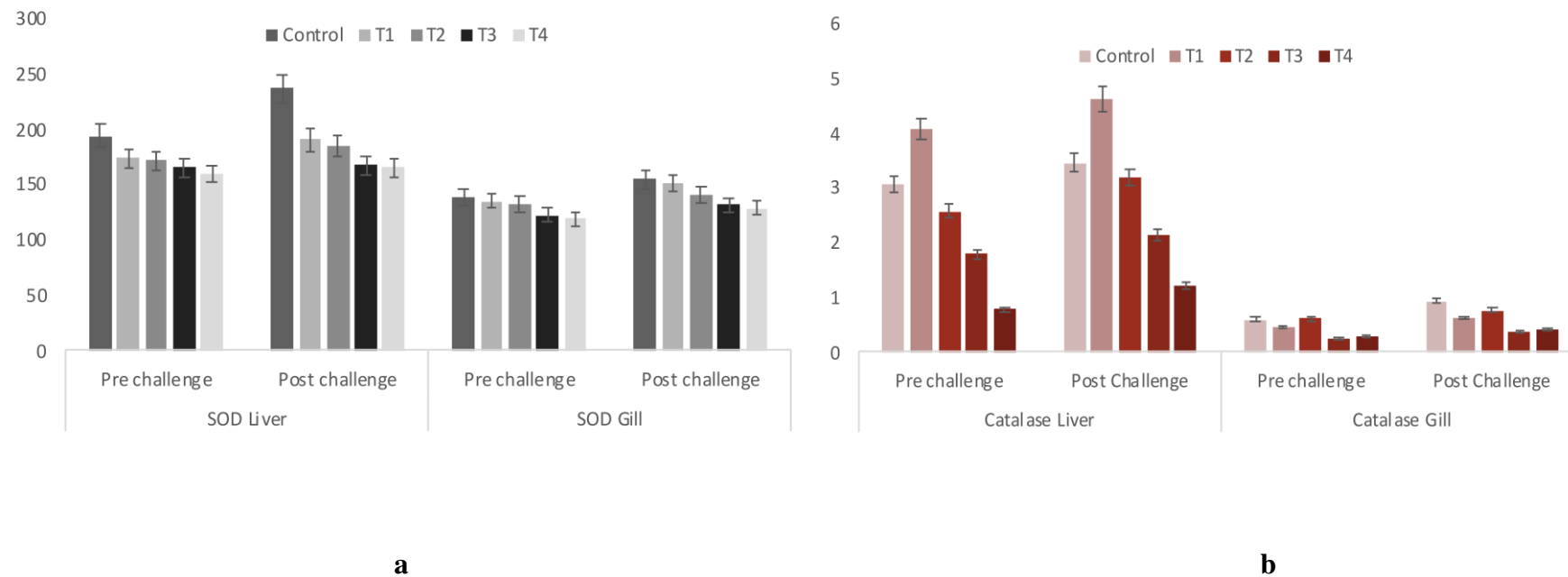


Figure 4. Haematological indices of *Nelumbo nucifera* leaf extract fed-Rainbow trout, Pre- and Post-challenge period.

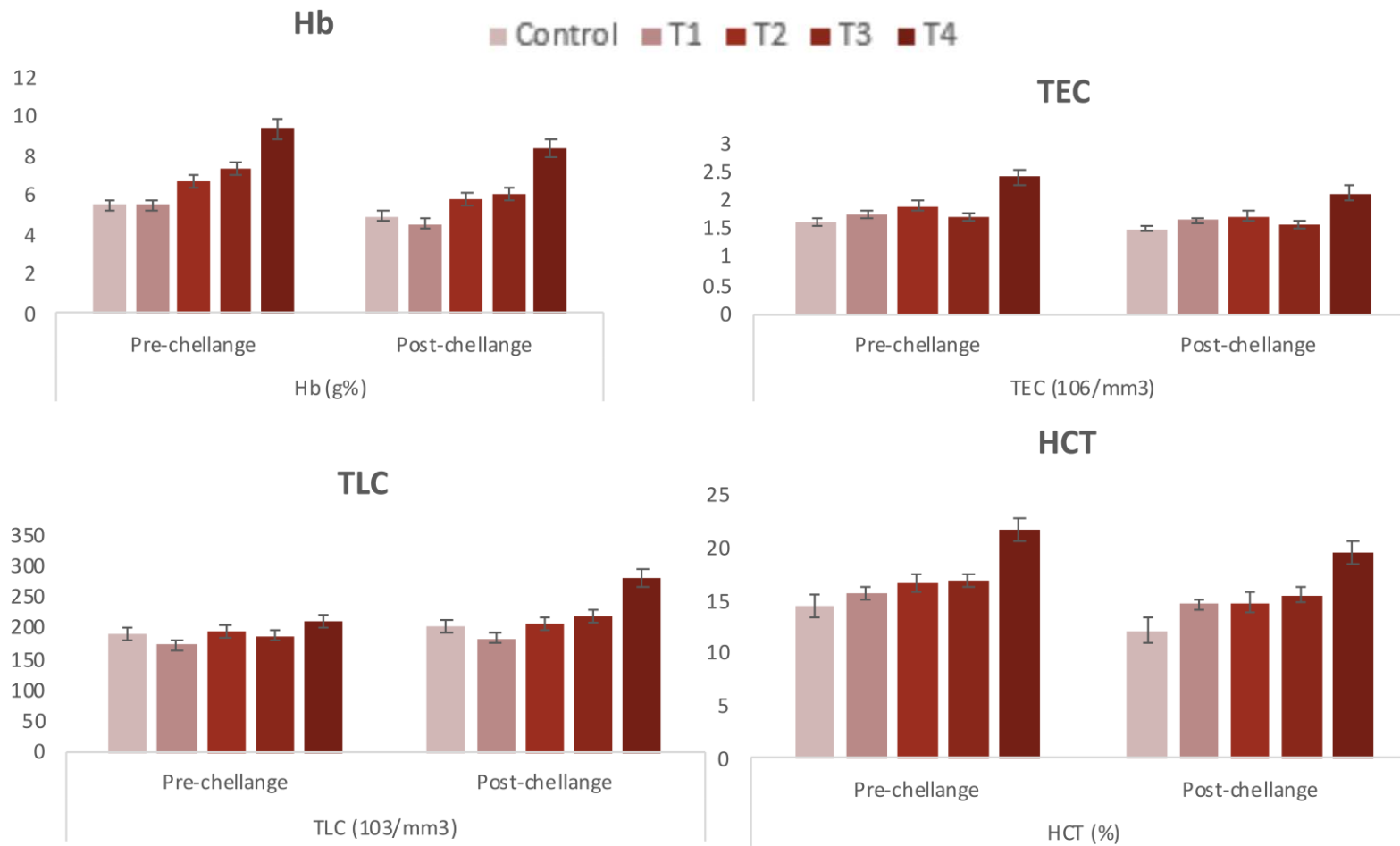
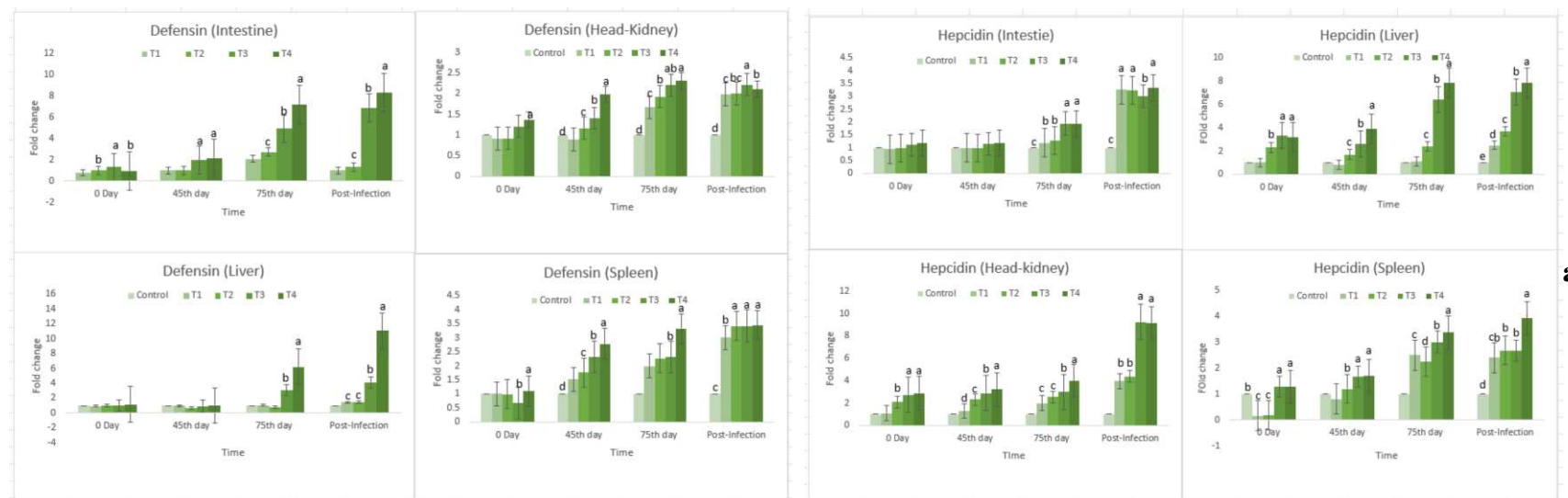


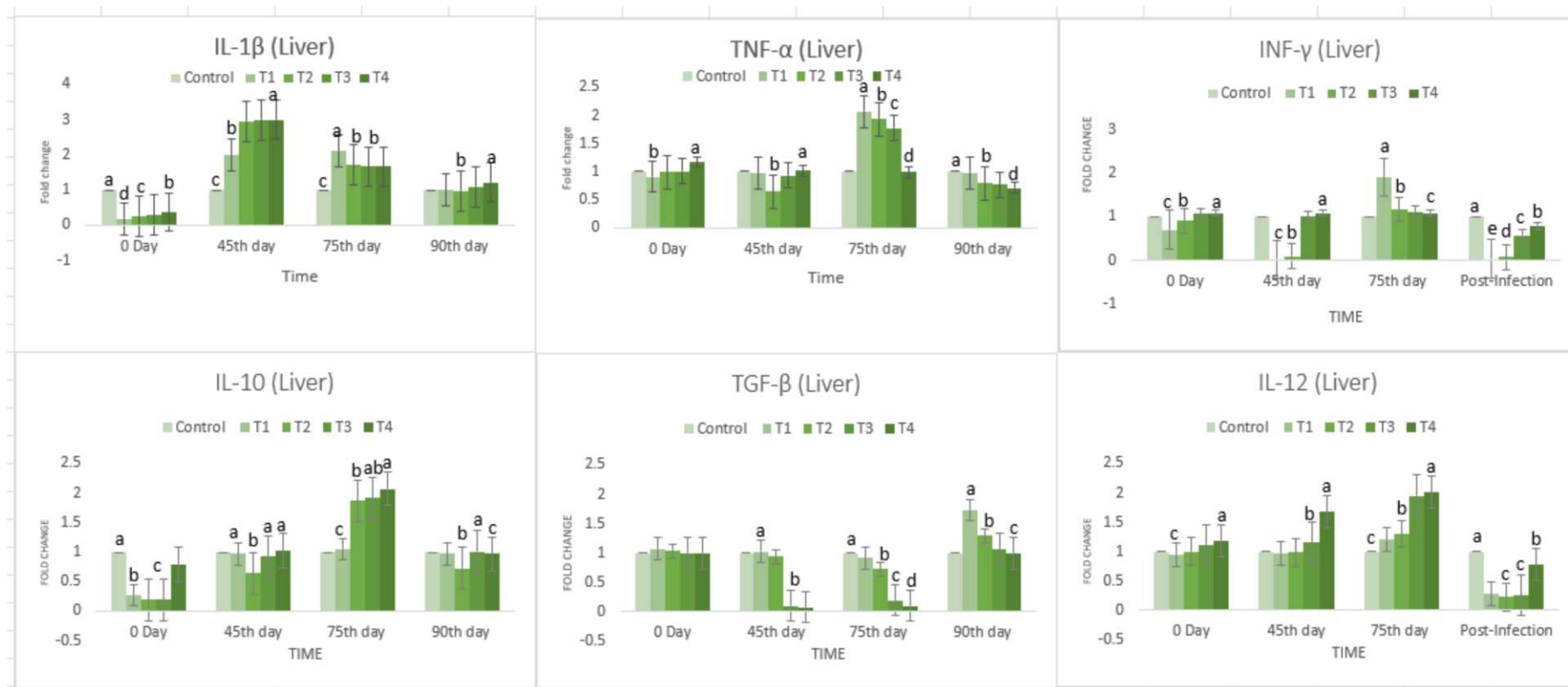
Figure 5. Relative gene expression of innate immune genes (a) Defensin and (b) Hepcidin, in liver, intestine, spleen and head-kidney of Rainbow trout fed with *Nelumbo nucifera* leaf extract on 0day, 45th day, 75th day and post-challenge.



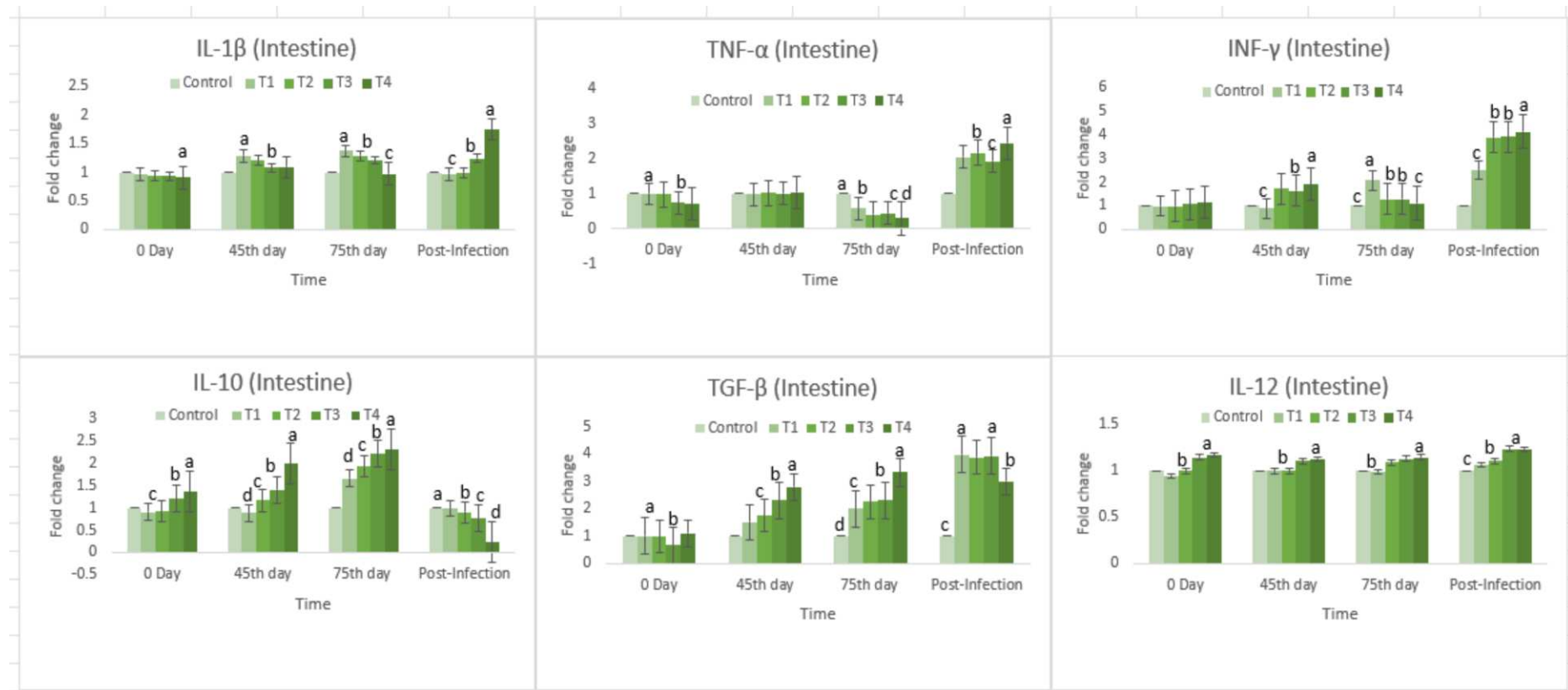
b

a

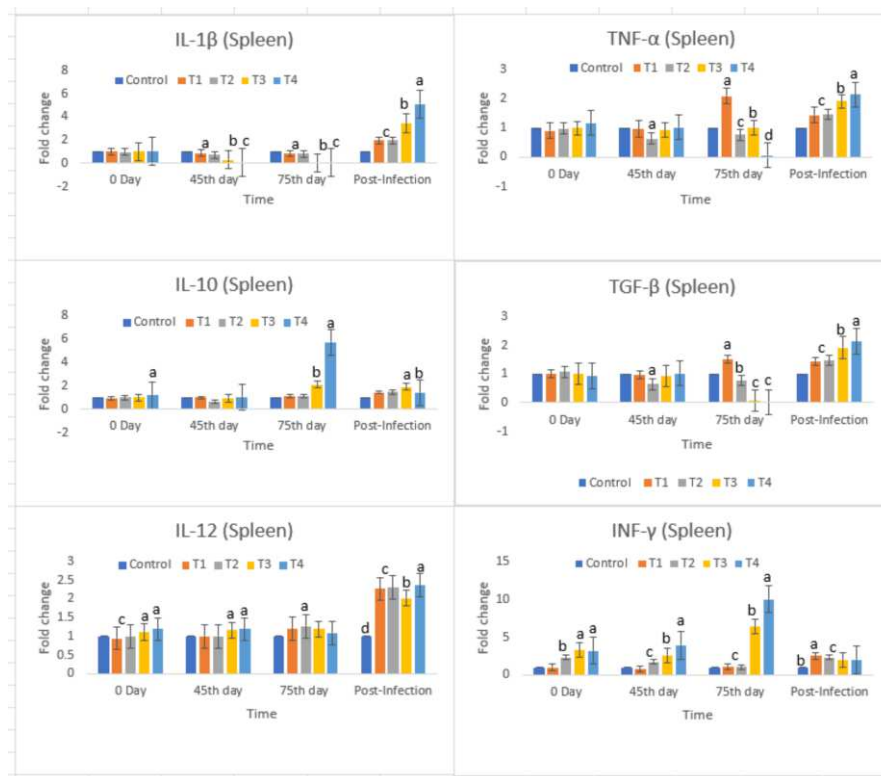
Figure 6. Relative gene expression of cytokines in (a) liver, (b) intestine, (c) spleen and (d) head-kidney of Rainbow trout fed with *Nelumbo nucifera* leaf extract on 0day, 45th day, 75th day and post-challenge.



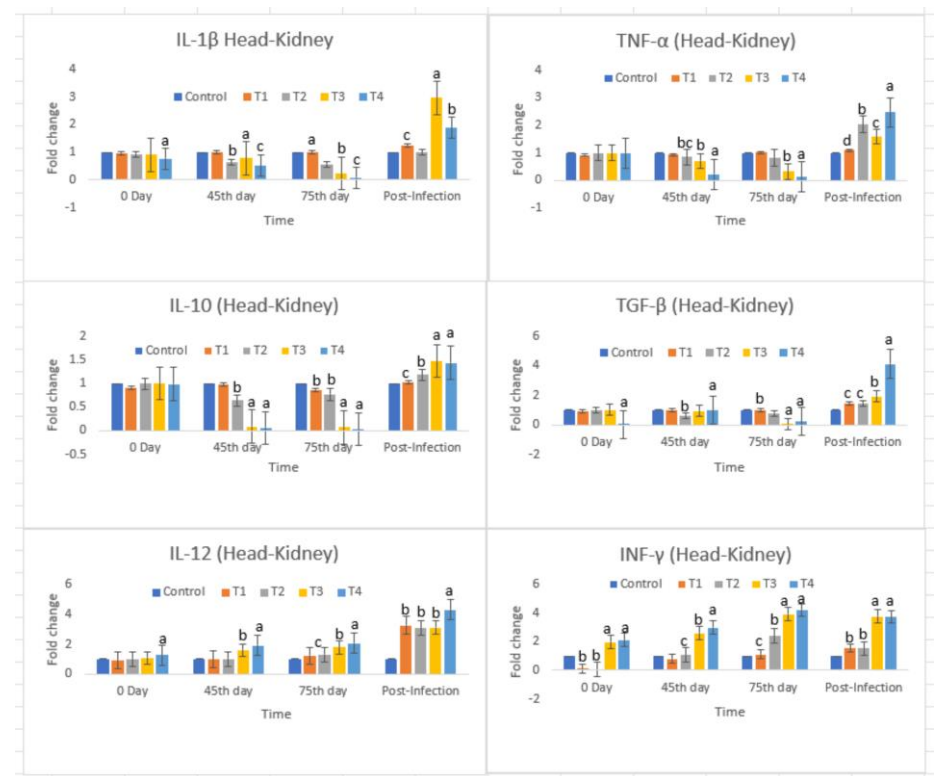
a



b



c



d