



# Potentials of *Aloe barbadensis* inclusion in fish feeds on resilience to *Aeromonas hydrophila* infection in freshwater fish *Labeo rohita*

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**Abstract** Aquatic bacterial pathogens can cause severe economic loss in aquaculture industry. An opportunistic pathogen, *Aeromonas hydrophila* is responsible for Motile Aeromonas Septicemia, leading to high mortality rates in fish. The present study was focused on the efficacy of *Aloe barbadensis* replacing fishmeal diets on hematological, serum biochemical, antioxidant, histopathological parameters, and disease resistance against *A. hydrophila* infection in *Labeo rohita*. Isonitrogenous fishmeal replaced diets (FMR) were prepared with varying levels of *A. barbadensis* at D1 (0%) (control), D2 (25%), D3 (50%), D4 (75%) and D5 (100%) then fed to *L.*

*rohita*. After 60 days of post-feeding, the experimental fish were challenged with *A. hydrophila*. Blood and organs were collected and examined at 1- and 15-days post infection (dpi). The results demonstrated that on 1 dpi, white blood cells (WBC), total protein, cholesterol and low-density lipoprotein (LDL) levels were significantly increased in D3 diet fed groups. The D2 and D3 diet fed group showed decreasing trends of serum glutamic pyruvic transaminase (SGPT) and antioxidant enzymes activity on 15 dpi. The histopathological architecture results clearly illustrated that the D3 diet fed group had given a higher protective effect by reducing the pathological changes associated with *A. hydrophila* infection in liver, intestine and muscle. Higher percentage of survival rate was also observed in D3 diet fed group. Therefore, the present study suggested that the dietary administration of *A. barbadensis* up to 50% fishmeal replacement (D3 diet) can elicit earlier antioxidant activity, innate immune response and improve survival rate in *L. rohita* against *A. hydrophila* infection.

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## Introduction

Aquaculture is one of the fastest growing food producing sectors that contributes to global food and nutritional security. Among the global aquatic

production, the aquaculture sector reached a record of 49.2% in 2020 (FAO 2022). The global aquaculture production is dominated by freshwater finfish culture due to the species demand, easy to breed, and high tolerance of low level dissolved oxygen (Zhang et al. 2022). India is the second largest country after China in cultivable fish production. India's total production of finfish was 7.1 million tonnes, and inland finfish species account for 6.3 million tonnes (89.2%) (FAO 2020). Indian major carps such as catla, rohu and mrigal fish are of paramount interest. The freshwater fish rohu (*Labeo rohita*) is the most popular among carp species, accounting for about 35% of total carp production in the last decade and it is the most economically affordable, nutritious, and delicious fish with high market value in India and neighbouring countries (Devi et al. 2019 Sridhar et al. 2021b). The major drawback of the aquaculture industry is disease outbreaks, which affect productivity. Fishes are susceptible to pathogenic or non-pathogenic diseases. Non-pathogenic diseases are related to poor water quality, malnutrition, etc., and are not contagious. On the other hand, pathogenic diseases are more threatening because they are contagious and cause huge mortalities (Nasr-Eldahan et al. 2021).

Infectious diseases are caused by pathogens such as opportunistic bacteria (Haldar et al. 2010; Manchanayake et al. 2023), viruses (Pan et al. 2023), fungi (Dananjaya et al. 2017), and parasites (Zeng et al. 2023). Significant losses in aquaculture are attributed to outbreaks of bacterial diseases including vibriosis caused by *Vibrio anguillarum* (Kapetanović et al. 2022), redmouth disease caused by *Yersinia ruckeri* (Fajardo et al. 2022), enteric septicemia caused by *Edwardsiella ictaluri* (Yang et al. 2023), and septicemia caused by *Aeromonas sp.* (Zhao et al. 2023), and other more bacterial species (Chong et al. 2023). Recently, many reported fish diseases are related to *Aeromonas* species such as *A. caviae* (Xue et al. 2022), *A. veronii* (Reda et al. 2022), *A. salmonicida* (Moreau et al. 2023), *A. hydrophila* (Raissy et al. 2022), *A. sobria* (Almarri et al. 2023), and *A. bestiarum* (Fuentes-Valencia et al. 2022). *Aeromonas* are rod-shaped, gram-negative bacteria that are facultative anaerobes belong to the family *Aeromonadaceae*. These bacteria are abundant in nature and have high level of environmental adaptation, including brackish, estuarine and freshwater (Li et al. 2020). They have turned into prominent disease causing agents

in fish and leads to 80% of mortality (Abd El Latif et al. 2019; Zhou et al. 2021). Among the species, *A. hydrophila* has been considered as the most harmful bacteria, causing hemorrhagic septicemia and epizootic ulcerative syndrome in cultured fishes (Marinho-Neto et al. 2019; Wang et al. 2023). The pathogenesis of infection was due to various virulent factors derived from *A. hydrophila* (El-Barbary 2010). The clinical symptoms include fin/tail rot, abdominal distension, and exophthalmia (Kumar et al. 2022a; Mursalim et al. 2022). It can also cause infections like endocarditis, gastroenteritis, peritonitis, and septicemia in humans (Chan et al. 2015; Chen et al. 2019; Matys et al. 2022).

In an intensive aquaculture farming, antibiotic treatments are essential to minimize bacterial infection related growth impairment and considerable economic losses. Antibiotics are administered to fish as part of their diet and also through baths and injections (Cabello et al. 2016; Nawaz et al. 2022). The constant presence of high levels of antibiotic residues in aquatic environments and sediments have the potential to affect normal flora and plankton in those niches, leading to shifts in microbiota diversity and causing deterioration of water quality (Cabello et al. 2016; Bhat et al. 2022). The longer use of antibiotics enable antimicrobial resistance gene exchange between bacteria (Muziasari et al. 2016; Watts et al. 2017; Okeke et al. 2022). The transfer of antibiotic residue and resistance genes from aquatic species and their habitats to terrestrial animals enhance the prevalence of drug-resistant bacteria (Santos and Ramos 2018). Vaccines could be a reasonable alternative for disease prevention in aquaculture. However, issues with inoculation and pathogen specificity limit their efficacy. Moreover, there are no commercial vaccines available for many bacterial infections (Wu et al. 2013; Ben Hamed et al. 2021; Pollard and Bijker 2021).

Another major obstacle in aquaculture is feed cost, fishmeal is considered as a prime feed ingredient for its high protein content, fatty acid and balanced amino acid profile. Nevertheless, researchers and feed manufacturers are looking for suitable dietary protein sources to replace fish meal because of its soaring price, higher demand and decline in supply (Ayiku et al. 2020). With increased accessibility, fewer hazards to aquatic habitats and lower cost, the use of plant-derived immunostimulants has become more popular in aquaculture over the last few years (Van Hai 2015; Mehrabi

et al. 2019). Natural plant-based herbal immunostimulants or supplementation of herbal medicine is one of the alternative approaches of fishmeal and antibiotics due to its richness in various compounds, including phenols, tannins, alkaloids, terpenoids, and polysaccharides (Hoseinifar et al. 2020; Hosseini et al. 2022). In addition, plant based diets have antibacterial properties and also provide considerable growth, antioxidant, and immuno-modulation in fish (Panigrahi et al. 2021). These herbal supplements boost the immune system of cultured fish, making them more resistant to stress and diseases (Kumar et al. 2022b).

*Aloe barbadensis* belonging to the family of Liliaceae has been recognized as one of the most well-known medicinal plants with various applications (Gupta et al. 2012; Giannakoudakis et al. 2018; Yazarlu et al. 2021). It is a drought-resistant succulent, stemless herb, native to tropical and subtropical countries (Saljooghianpour and Javaran 2013; Heş et al. 2019). *Aloe barbadensis* is characterized by its abundance in compounds with biological activity, namely mono-, di-polysaccharides, phenolic compounds, anthraquinones, minerals, water- and lipid- soluble vitamins, organic acids, and lipids (Folliero et al. 2022; Palaniyappan et al. 2023). Our previous study evaluating partial and complete fishmeal replacement by *Aloe barbadensis* with a concentration of 0% (D1), 25% (D2), 50% (D3), 75% (D4) and 100% (D5) experimental diets on freshwater fish *Labeo rohita* showed positive effects on growth, hematology, serum biochemistry, digestive and antioxidant enzyme activity (unpublished data). The previous literature suggests that, Aloe have the potential properties to act against aquatic pathogen in various fish species, such as *Piaractus mesopotamicus* (de Assis and Urbinati 2020), *Cyprinus carpio* (Khanal et al. 2021), *Danio rerio* (Mehrabi et al. 2020), *Oncorhynchus mykiss* (Mehrabi and Firouzbakhsh 2020). In light of this, the present study was aimed to investigate the protective effect of *Aloe barbadensis* diets replacing fishmeal against *A. hydrophila* infection in *L. rohita* fish.

## Materials and methods

### Experimental diets

Five experimental fishmeal replacement (FMR) diets were prepared to fulfil the nutrient requirement of

*L. rohita* using ingredients such as fishmeal (FM), *Aloe barbadensis* (Ab), soy bean meal, ground nut oil cake, wheat flour, tapioca flour, corn flour, vitamin and mineral mix. The isonitrogenous diets were formulated according to Kari et al. (2021) to replace fishmeal gradually with the concentrations 0% (D1), 25% (D2), 50% (D3), 75% (D4) and 100% (D5) of *A. barbadensis*. All the ingredients were powdered, mixed thoroughly, autoclaved and prepared in small pellets. The formulation of experimental diets were given in Table 1. The prepared experimental diets were dried and stored at  $-20\text{ }^{\circ}\text{C}$  before being used.

### Preparation of *A. hydrophila*

In order to determine the disease resistance rate of *L. rohita* after fed with fishmeal replaced *A. barbadensis* diets, the fish were challenged with *A. hydrophila*. The pathogenic bacteria *A. hydrophila* was cultured in Luria Bertani broth for 12 h at  $37\text{ }^{\circ}\text{C}$ . The overnight bacterial culture was then centrifuged at 7500 rpm for 10 min in a cooling centrifuge. The supernatant was discarded and the bacterial pellet was resuspended in PBS (Phosphate Buffer saline, pH 7.4). The bacterial suspension was adjusted to 0.5 absorbance or optical density (O.D.) at 456 nm corresponding to  $1 \times 10^7$  colony forming unit (CFU/mL) using Synergy HT

**Table 1** Formulation of experimental diets for *Labeo rohita*. D1-0% *A. barbadensis* (positive control); D2-25% *A. barbadensis*; D3-50% *A. barbadensis*; D4-75% *A. barbadensis*; D5-100% *A. barbadensis* replacing fishmeal

Ingredients (g/100 g)	D1	D2	D3	D4	D5
Fish meal	25	18.75	12.5	6.25	0
<i>Aloe barbadensis</i>	0	6.25	12.5	18.75	25
Soybean meal	25	28	31	35.5	45
Groundnut oil cake	13	14.5	18	18	19
Wheat bran	12	11	14.5	9.5	4.5
Tapioca flour/ Cassava	12	9.5	11.5	6	2.45
Corn flour	11	10	8.5	6	2.05
Palm oil	1	1	1	1	1
Vitamin and mineral mix*	1	1	1	1	1
Total	100	100	100	100	100

\*Vitamin and mineral mix (Each serving of soft gel capsule): Saturated fatty acid- 120 mg; Eicosatetraenoic acid-90 mg; Docosahexaenoic acid-60 mg; Alpha lipoic acid-30 mg; Vitamin C-25 mg; Vitamin E acetate-8 mg; Beta-carotene-2.4 mg; Vitamin B12-1.5 mg; Vitamin B1-0.8 mg; Folic acid-50 mg; Zinc-9 mg; Selenium-30 mg; Chromium-30mcg

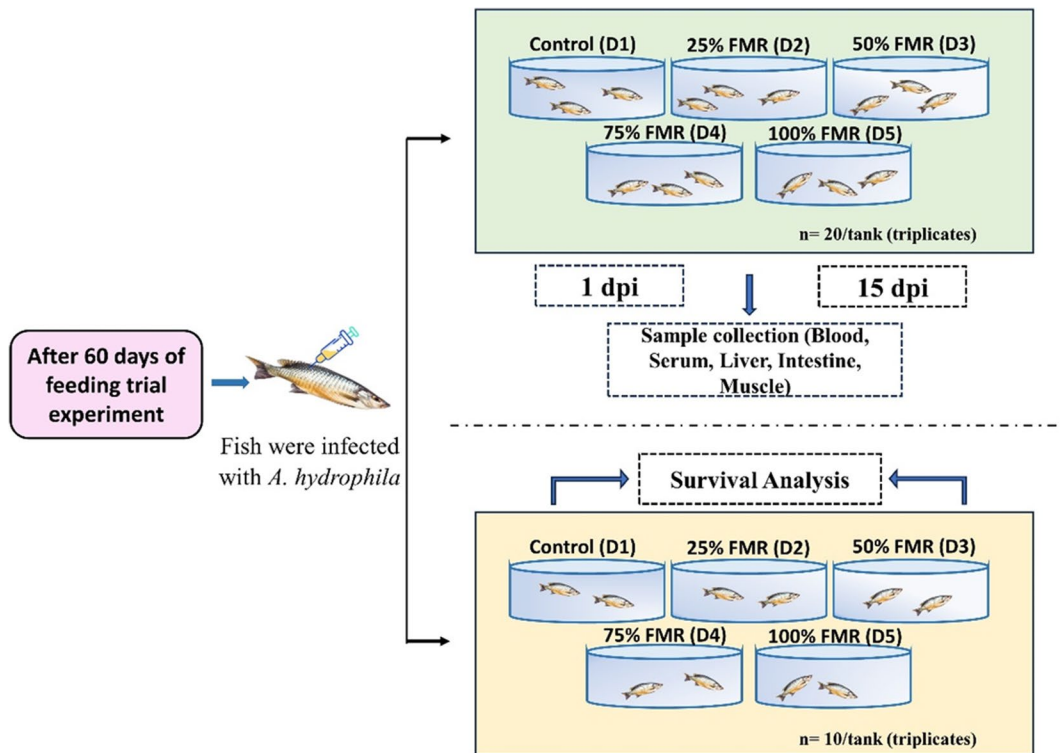
Multimode Reader (Bio Tek Instruments, Inc., Winooski, USA) (Devi et al. 2019).

### Experimental design and challenging study

The *L. rohita* fingerlings were obtained from Guna fish farm, Rapoosal, Pudukkottai, Tamil Nadu, India, and transported to laboratory in aerated polyethylene bags. They were acclimatized to laboratory conditions for 10 days with commercial feed. After acclimation to the experimental conditions, fish ( $7.22 \pm 0.48$  g) were randomly distributed into the experimental tanks in five groups with each group in triplicates. Fish were fed with experimental diets (D1, D2, D3, D4 and D5) at a rate of 5% total fish biomass twice a day (06:00 and 18:00 h) for 60 days. The water temperature was maintained at  $27 \pm 2$  °C and pH at  $7.1 \pm 0.4$  for the whole experimental period and the tanks were well aerated. The water was exchanged at a rate of 50% once a day. After 60 days of feeding, challenging study was carried out. Thirty healthy fish from each treatment tank, fed with different experimental diets (D1, D2, D3, D4 and

D5) were randomly collected and subjected to artificial bacterial infection. The bacterial infection was induced by injecting 0.2 mL of  $1 \times 10^7$  CFU/mL of *A. hydrophila* suspended in PBS intramuscularly (IM) above the caudal fin. The experimental fish from the control group (D1) was IM injected with 0.2 mL of PBS and *A. hydrophila* considered as negative control (NC) and positive control (PC) respectively. The respective experimental feed was given twice a day to the infected fish during the period of 15 days post infection (dpi). The schematic representation of experimental design was given in Fig. 1. The challenged fishes were monitored frequently for abnormal behaviour and clinical signs. External modifications were assessed, daily mortality was recorded and at the end of the challenging experiment, relative percentage survival (RPS) was calculated by using the following formula (Sattanathan et al. 2023).

$$\text{RPS} = \left( \frac{\text{No. of surviving fish after challenge}}{\text{No. of fish injected with bacteria}} \right) \times 100$$



**Fig. 1** Schematic representation of experimental design

## Sample collection

After challenging with *A. hydrophila*, 5 fish were randomly collected from each tank on 1- & 15-dpi and euthanised using benzocaine (Yonar et al. 2019). The blood was collected from the fish branchial arch using 1 mL syringe and stored in heparinized and non-heparinized tubes separately (Carbonara et al. 2019). The heparinized blood samples were used for the analysis of hematological parameters. The non-heparinized blood samples were kept under room temperature for 2 h and centrifuged at 5000 rpm for 10 min; the serum was separated and stored at  $-20^{\circ}\text{C}$  for serum biochemical studies. Further, the fish were dissected and organs (intestine, muscle and liver) were collected. For histopathological examination, some of the excised organs were stored in 10% formalin. The remaining organs were homogenized separately and stored at  $-20^{\circ}\text{C}$  until used for analysing antioxidant activities.

## Hematological parameters

Hematological indices such as white blood cell (WBC), red blood cell (RBC), hemoglobin (HB), mean corpuscular hemoglobin (MCH), packed cell volume (PCV), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) were estimated by using 200  $\mu\text{L}$  of blood sample through Fully Automatic Medonic M51, 5 part-Haematology Analyzer using Medonic M51-D diluent, L1 and L2 lyse reagents.

## Serum biochemistry

Serum of 400  $\mu\text{L}$  was used to analyse biochemical indices such as serum glutamic pyruvic transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT), alkaline phosphatase (ALP), total proteins, glucose, triglycerides, cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), and creatinine levels by Erba XL 640 fully automated biochemistry analyzer (Erba Trans, India) using Erba's biochemical kit procedures.

## Immunological assay

### *Lysozyme activity*

Lysozyme activity was determined by turbidimetric method as described by Ellis (1990) with slight

modifications. Briefly, the bacterial suspension, containing sodium phosphate buffer (0.05 mM, 6.2 pH) and lyophilized *Micrococcus lysodeikticus* (0.03%), was prepared. Then, 250  $\mu\text{L}$  of bacterial suspension was added to 20  $\mu\text{L}$  of fish serum in a 96 well plate. The absorbance was read at 490 nm using Synergy HT Multimode Reader (Bio Tek Instruments, Inc.). Decrease in the absorbance of 0.001/min was considered as one unit of lysozyme activity.

### *Immunoglobulin M (IgM) assay*

Total IgM activity of serum was measured based on the method of Sharma et al (2010). Briefly, after measuring the protein concentration of serum by Bradford (1976) method, 100  $\mu\text{L}$  of polyethylene glycol (12% w/v) was added to 100  $\mu\text{L}$  of serum and incubated under continuous shaking for 2 h. After incubation, the reaction mixture was centrifuged at 5000 rpm for 10 min. Then, the protein concentration of supernatant was determined. The total IgM level was calculated by subtracting the protein concentration of supernatant from the serum protein concentration and it was expressed as unit mg/mL.

## Antioxidant assays

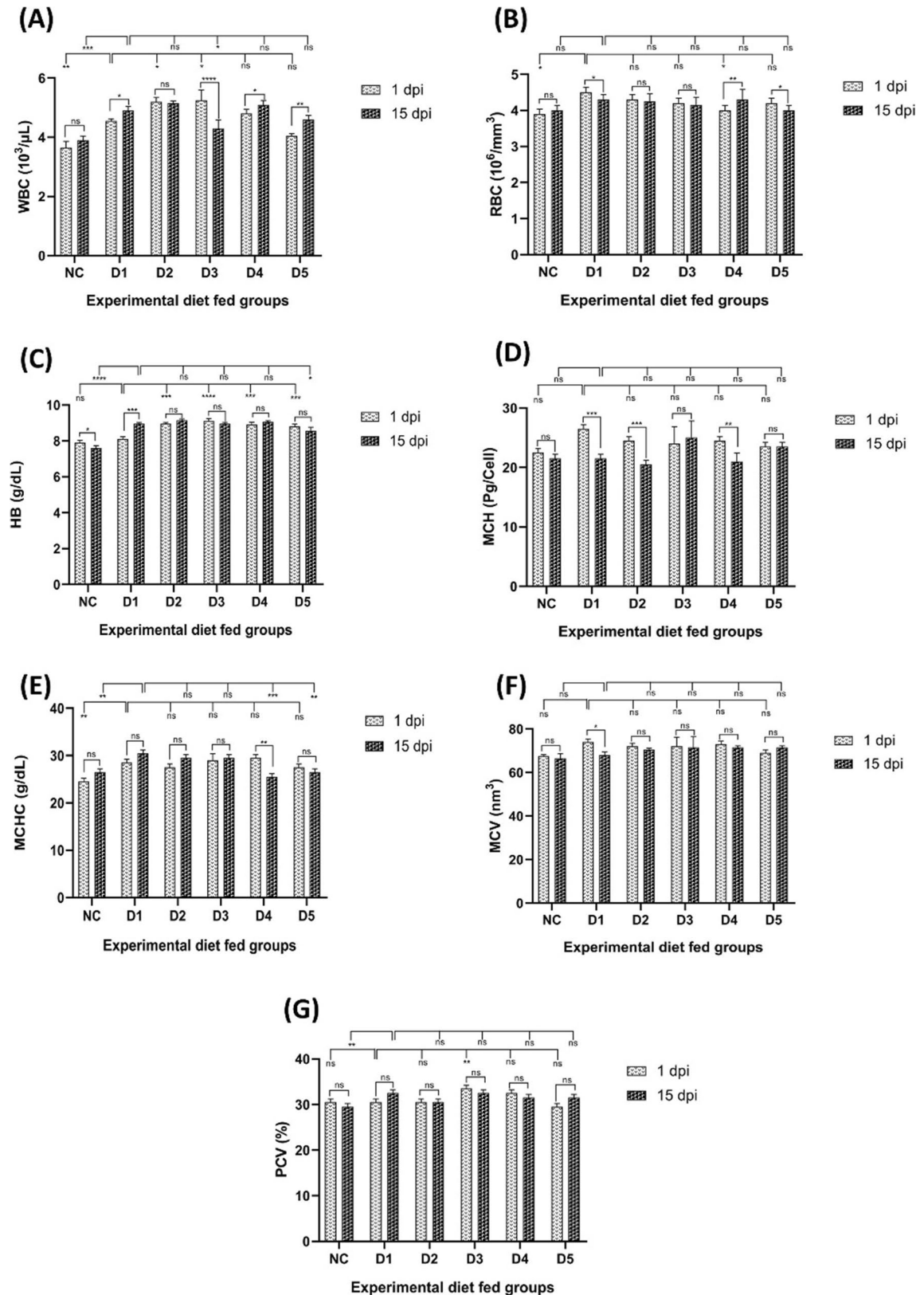
### *Superoxide dismutase (SOD) activity*

The superoxide dismutase activity was determined using the method of Marklund and Marklund, (1974) with minor modifications. In brief, 50  $\mu\text{L}$  of the sample was added with 15  $\mu\text{L}$  of chloroform. The mixture was mixed well in a shaker for 15 min. The reaction mixture was then centrifuged at 13000 rpm for 15 min at  $4^{\circ}\text{C}$ . Then, 15  $\mu\text{L}$  supernatant was collected and 2 mL of 0.01 M EDTA buffer was added to it followed by 0.5 mL of pyrogallol. Finally, 1 mL of distilled water was added to the mixture. The absorbance was read at 420 nm using Synergy HT Multimode Reader (Bio Tek Instruments, Inc.). The enzyme activity was expressed in terms of units/mg protein in which one unit corresponds to the amount of enzyme that inhibited the auto-oxidation reaction by 50%.

### *Catalase (CAT) activity*

The catalase activity was determined by the method of Sinha, (1972) with slight modifications. The reaction





**Fig. 2** Effect of replacing fishmeal with *Aloe barbadensis* in *Labeo rohita* blood parameters on 1 and 15 days post infection (dpi) with *Aeromonas hydrophila*. (A) White blood cell (WBC); (B) Red blood cell (RBC); (C) Hemoglobin (HB); (D) Mean corpuscular hemoglobin (MCH); (E) Mean corpuscular hemoglobin concentration (MCHC); (F) Mean corpuscular volume (MCV); (G) Packed cell volume (PCV). \*, \*\*, \*\*\*, \*\*\*\* indicates significant difference to positive control (D1) at  $\leq 0.05$ ,  $\leq 0.01$ ,  $\leq 0.001$  and  $\leq 0.0001$  respectively. NC, negative control; D1-0% *A. barbadensis* (positive control); D2-25% *A. barbadensis*; D3-50% *A. barbadensis*; D4-75% *A. barbadensis*; D5-100% *A. barbadensis* replacing fishmeal; ns, non-significant

mixture contained 0.5 mL of hydrogen peroxide, 1 mL of phosphate buffer, 0.4 mL of distilled water, 0.1 mL of diluted homogenate (1:10). This mixture was incubated for 1 min at 37 °C. Then, 2 mL of potassium dichromate acetic acid was added. The mixture was kept in boiling water bath for 10 min and the colour appeared was read at 570 nm using Synergy HT Multimode Reader (Bio Tek Instruments, Inc.). The activity of catalase was determined from the amount of hydrogen peroxide consumed and expressed as  $\mu\text{moles}$  of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) consumed/minute/mg protein.

#### Glutathione peroxidase (GPx) activity

The glutathione peroxidase activity was measured using the standard method described by Rotruck et al., (1973). In brief, the reaction mixture contains 0.5 mL of sodium phosphate buffer (0.3 M, pH 7), 0.1 mL of 10 mM sodium azide, 0.2 mL of 4 mM reduced glutathione, 0.1 mL of 2.5 mM hydrogen peroxide and 0.1  $\mu\text{L}$  tissue homogenate. The reaction mixture was made up to 2 mL with distilled water and incubated at 37 °C for 3 min. Then, 0.5 mL of 10% trichloroacetic acid (TCA) was added and centrifuged at 13000 rpm for 15 min in a cooling centrifuge. The supernatant was collected and 3 mL of disodium hydrogen phosphate (0.3 mM) was added. Finally, 0.5 mL of DTNB (5,5'-dithiobis-2-nitrobenzoic acid) was added into it. The colour developed was read against the blank at 412 nm in the Synergy HT Multimode Reader (Bio Tek Instruments, Inc.). The activity was expressed in terms of  $\mu\text{g}$  of glutathione utilized /minute/mg protein.

#### Histopathology

Histopathological analysis was carried out according to the method of (Krishnasamy Sekar et al. 2023). In

order to prevent the tissue degradation and to maintain the intact structures, the tissues were fixed with 10% formalin initially. After 12 h, the fixatives were removed and washed with distilled water thoroughly to remove the fixative. Dehydration was done with various concentrations of ethanol (50, 70, 90 and 100%) for an hour. Chloroform was used to remove the alcohol residues. After infiltration, the tissue was embedded into the paraffin wax to give an external support while cutting. Then, the sections (5  $\mu\text{m}$ ) were made by Leica microtome (Leica Microsystems GmbH, Wetzlar, Germany), and stained with Hematoxylin and Eosin (H & E) solution. Finally, the histological sections were analyzed using microscope (Leica, DM750, Germany).

#### Statistical analysis

Experiments were performed in triplicates and the results were presented as mean  $\pm$  standard deviation. The data were analysed by applying two-way ANOVA with Dunnett's and Sidak's multiple comparisons test using GraphPad Prism version 8 (GraphPad Software, Inc., San Diego, CA). The data were presented in the form of descriptive statistics through tables and graphs. \*, \*\*, \*\*\*, and \*\*\*\* indicate P-values of respectively  $\leq 0.05$ ,  $\leq 0.01$ ,  $\leq 0.001$  and  $\leq 0.0001$ .

## Results and discussion

### Hematological parameters

Hematological parameters are an important diagnostic tool for examining the health status of fishes. Periodic hematological analysis provides simple way of evaluating chronic stress, diseases, reproductive dysfunction, and metabolic disorder, by environmental conditions both in the field and captive populations of fish species (Ahmed and Sheikh 2019). The results of hematological indices of the present study were given in Fig. 2. In this study, increased WBC count was observed in D3 diet fed group fish on 1 dpi (Fig. 2A), the count was significantly decreased on 15 dpi. In addition, the D3 diet fed group showed significant variations on both 1 & 15 dpi when comparing with D1. The groups with higher WBC count will fight infection more efficiently than other groups and these results are in line with Harikrishnan et al.,

(2020) in *L. rohita* after being fed with 2.5 and 5 g/kg of dried lemon peel diets. No significant variations were observed in MCH and MCV count between D1 and other diet fed groups on both 1 & 15 dpi except RBC which showed significant difference in D4 diet fed group on 1 dpi when comparing to D1 fed group (Fig. 2B, D, F). Nevertheless, significant variations in the count were observed between 1 & 15 dpi in some diet fed groups.

D3 diet fed fish showed significantly higher hemoglobin content on 1 dpi among all experimental diet fed groups compared to D1 (Fig. 2C). Increased level of hemoglobin suggests that iron consisting protein level was also increased. Hemoglobin prohibits anemia and favour for respiration (Das et al. 2022). On 15 dpi, no significant reduction was observed in all experimental groups except D5 diet fed group. It might be due to the swelling of cells (Vignesh et al. 2022) and our results were in line with that of Rathore et al., (2021) and Talpur et al., (2014). No significant variations were observed in MCHC except D4 & D5 diet fed groups which showed significantly decreased level compared to D1 on 15 dpi (Fig. 2E). The present study results were supported by the findings of Mbokane and Moyo, (2018), MCV represents the volume of the red blood corpuscle. On 1 & 15 dpi, the MCV values were comparatively similar in all groups and no significant variations were observed except D1 (Fig. 2F), whereas, a significant decrease in values were noted in D1 group on 15 dpi when compared to 1 dpi. The declining level of MCV may be imputed to the erythrocyte shrinking, which results microcytic anemia. Shrinking in erythrocytes occur due to insufficient synthesis of hemoglobin (Kumar et al. 2013). Similarly, no significant variations were observed in the levels of PCV except D3 diet fed group, which showed significantly increased value on 1 dpi comparing to D1 and other experimental diet fed groups (Fig. 2G).

### Serum biochemistry

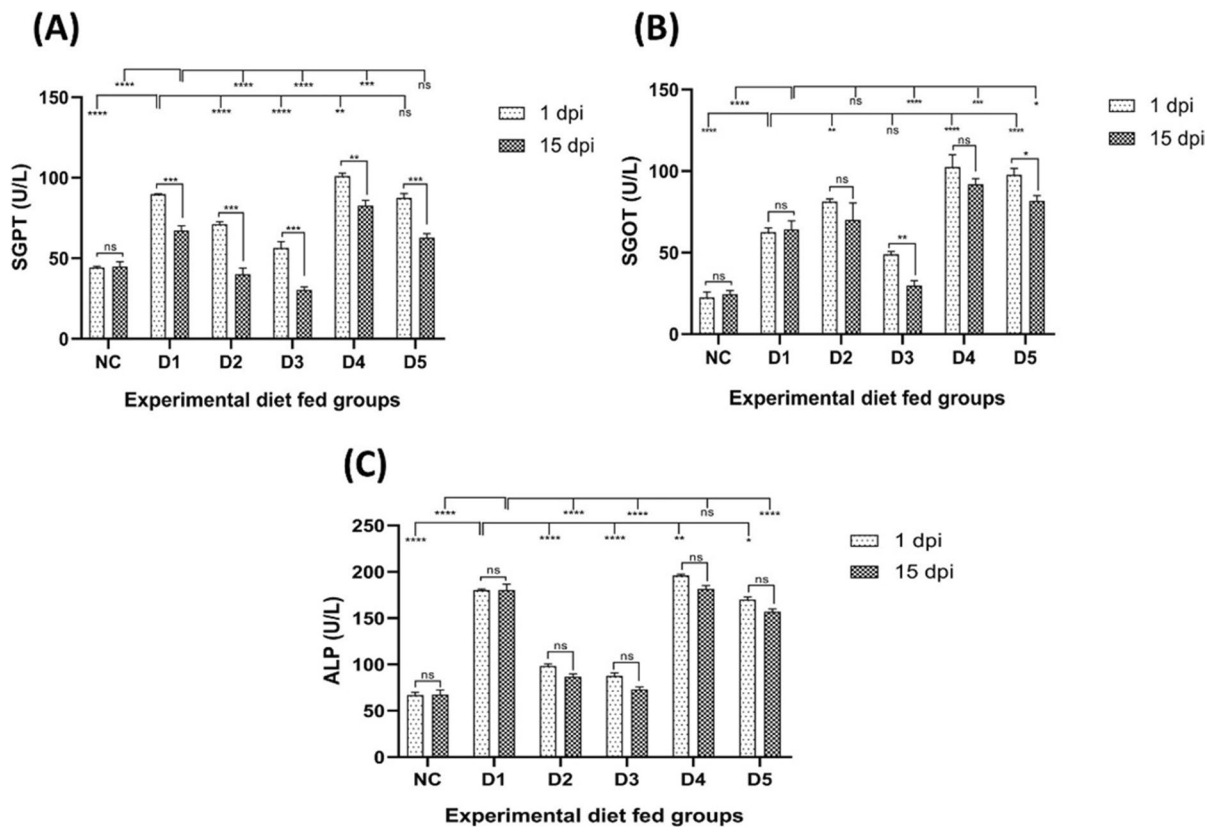
Serum biochemical study is also a crucial component in disease diagnosis. It is one of the ancillary diagnostic methods, many pathological abnormalities are reflected in serum well before clinical illnesses. Serum biochemical studies will give an insight information of internal environment of animals including fish (Ahmed and Sheikh 2019). Fish have a complex

combination of serum proteins which are involved in a wide range of physiological functions in both healthy and diseased state and have an important role in understanding physiological parameters of fish (Fazio et al. 2013).

Serum glutamic pyruvic transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) enzymes are found in hepatocyte cytosol. Due to the linkage of SGPT with liver parenchymal cells (Veerabhadran et al. 2023), it is highly present in liver and to a lesser amount in skeletal muscle and kidney (Saha and Bandyopadhyay 2020). The SGPT is a specific indicator of liver inflammation and damage (Nair et al. 2021). On the other hand, SGOT is commonly found in liver, skeletal muscle and heart. It also indicates the status of liver health. In this study, the concentration of SGPT is higher in liver. The optimum range of SGPT level in fish is usually between 5 and 45 U/L (Kumar et al. 2013). The current study results showed a significant increase in all the experimental diet fed fish groups on 1 dpi (Fig. 3A). The increasing level of these enzymes probably due to the liver damage caused by the pathogenic bacteria or oxidative stress (Hore et al. 2023; Kumar et al. 2022a). A significant SGPT decline was observed on 15 dpi in all the experimental diet fed groups when compared to 1 dpi. It indicates that the SGPT enzyme level increased during early stages of infection and reduced gradually. Moreover, significantly decreased values were observed in D2 & D3 diet fed group when compared to D1. Especially, the D3 diet fed group showed lower SGPT and SGOT enzymes level among the experimental diet fed groups. Significant differences in the SGOT enzyme levels were observed in D3 & D5 groups between 1 & 15 dpi. These values suggest that the diet D3 fed fish showed less infection than other diet fed groups (Fig. 3B). The decreasing trend of SGPT and SGOT was also documented in azadirachtin administered goldfish (*Carassius auratus*) against *A. hydrophila* (Kumar et al. 2013). In contrast, Haque et al., (2021) reported significantly higher SGPT and SGOT activity in *Anabas testudineus* (climbing perch) infected with *Pseudomonas aeruginosa*.

The alkaline phosphatase (ALP) is a multifunctional enzyme that can serve as transphosphorylase in the alkaline pH and plays a major role in membrane transport and skeletal mineralization in aquatic animals (Bernet et al. 2001; Rahimikia 2017). ALP





**Fig. 3** (A) Serum glutamic pyruvic transaminase (SGPT); (B) Serum glutamate oxaloacetate transaminase (SGOT); (C) Alkaline phosphatase (ALP) activity of *Labeo rohita* fed with *Aloe barbadensis* on 1 and 15-days post infection (dpi) with *Aeromonas hydrophila*. \*, \*\*, \*\*\*, \*\*\*\* indicates significant

difference to positive control (D1) at  $\leq 0.05$ ,  $\leq 0.01$ ,  $\leq 0.001$  and  $\leq 0.0001$  respectively. NC, negative control; D1-0% *A. barbadensis* (positive control); D2-25% *A. barbadensis*; D3-50% *A. barbadensis*; D4-75% *A. barbadensis*; D5-100% *A. barbadensis* replacing fishmeal; ns, non-significant

is one of the lysosomal enzymes, acts as an excellent antibacterial agent and protects fish during infection (Sridhar et al. 2022). Increased production of alkaline phosphatase activity is associated with the elevating enzyme production by macrophage cells (Zhu and Su 2022). In this study, between 1 & 15 dpi, no significant changes in ALP level were observed in all the experimental diet fed groups (Fig. 3C). The higher level of ALP enzyme was detected in D1, D4 and D5 diet fed groups. It shows that the ALP enzyme was produced in higher level in these particular groups to evade the severity of *A. hydrophila* infection. In contrast, D2 and D3 diet fed groups showed ALP enzyme levels similar to the negative control (NC) and significantly reduced when compared to D1. It suggests that the level of *A. hydrophila* infection was controlled in these groups. The similar increased trend was observed by Liang

et al. 2020, at 48 h post infection in *Onchidium reevesii* against *Vibrio harveyi*. Gobi et al. 2018 also reported an increased level of ALP in dietary supplementation of probiotic *Bacillus licheniformis* Dahb1 in tilapia (*Oreochromis mossambicus*) challenged with *A. hydrophila*.

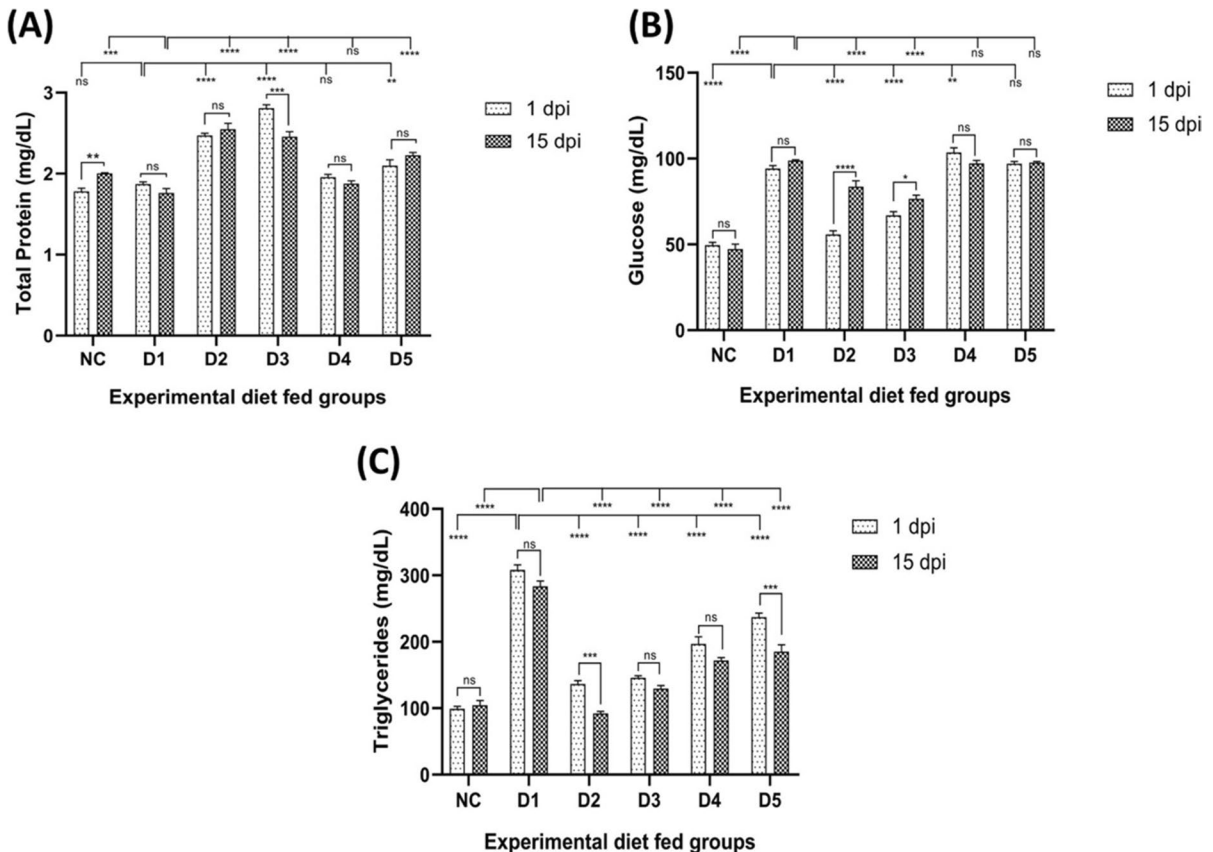
One of the important components in serum is proteins. It consists of globulins and albumins (Tan et al. 2017). In fish, stronger innate immunity response is based on the level of serum/plasma total protein (Zhou et al. 2015). The increase in the level of serum total protein indicates enhanced antibody development, and several humoral constituents related to innate immune response (Schieber and Chandel 2014). In this study, the diets D2 and D3 fed fish showed higher protein level than the other experimental diet fed groups (Fig. 4A). It indicates that the *A. barbadensis* supplementation in these groups

elevated the protein level and escalated the innate immune response in experimental fish. Medicinal herbs can directly strengthen the fish immunity by generating antibodies with response to the pathogenic organisms (Li et al. 2022). Reduced level of protein was observed by Vignesh et al., (2022) on *Channa striata* after infecting with *A. hydrophila*.

Blood glucose level is a major factor to identify the second level stress conditions in fish (Ahmed and Sheikh 2019). Glucose acts as an indicator of hypoxic and environmental stress (Malini et al. 2018). The increased level of blood glucose indicates escalated levels of stress/infection (Citarasu et al. 2006). According to Patriche 2009, 40–90 mg/dL is the normal level of fish blood glucose. In our study there was a significant decrease in the level of serum

glucose in D2 and D3 diet fed groups when compared to D1 (Fig. 4B) on 1 dpi. It might be the reason of slow pathogenesis caused by *A. hydrophila* due to the immunoprotective effect of *A. barbadensis* in these diet groups. Talpur and Ikhwanuddin 2013 stated that the blood glucose level was increased in the *V. harveyi* infected fish Asian seabass (*Lates calcarifer*) fed with 2 g/kg of neem leaf supplemented diet.

Glucose can also be formed from glycerol in triglycerides (Wen et al. 2021). Consequently, elevated levels of blood glucose likely due to the increase of triglycerides. The present results demonstrated the declined levels of glucose and triglycerides in the D2 and D3 diet fed groups and higher level of triglycerides were observed in D1 group, which acts as positive control (Fig. 4C). Our results were in agreement



**Fig. 4** (A) Total protein; (B) Glucose; (C) Triglycerides level of *Labeo rohita* fed with *Aloe barbadensis* on 1 and 15-days post infection (dpi) with *Aeromonas hydrophila*. \*, \*\*, \*\*\*, \*\*\*\* indicates significant difference to positive control (D1) at  $\leq 0.05$ ,  $\leq 0.01$ ,  $\leq 0.001$  and  $\leq 0.0001$  respectively. NC, nega-

tive control; D1-0% *A. barbadensis* (positive control); D2-25% *A. barbadensis*; D3-50% *A. barbadensis*; D4-75% *A. barbadensis*; D5-100% *A. barbadensis* replacing fishmeal; ns, non-significant

with the reports of Banihashemi et al. 2022 indicating increased levels of blood glucose and triglycerides in the *Yersinia ruckeri* exposed fishes.

Cholesterol is a lipid that is mainly produced by liver. The increased levels of cholesterol in serum is utilized by fish to assuage the excess energy demand during stress conditions. On 1 dpi, the cholesterol levels were increased in D1 diet fed group when compared to the other groups. Increasing cholesterol level was reported in *Cyprinus carpio* under stressful conditions (Abdel-Tawwab et al. 2022). During 15 dpi, the cholesterol levels were increased in all groups except D1 and D3 diet fed groups when compared to 1 dpi but it was vice versa in other experimental diet fed groups. The values were statistically significant comparing to D1 except 15 dpi on D5 group which showed no significant alterations. (Fig. 5A). In fish, cholesterol levels could be emblematic of common and traditional response to stress (Saha and Bandyopadhyay 2020).

Chen et al. 2020 stated that the serum HDL mainly collect excess phospholipids and cholesterol and reverse them to liver. The elevated levels of HDL activity nullify the ROS (reactive oxygen species) produced from oxidative stress (Zahran et al. 2020). In our study, lower level of HDL was observed in D2 and D3 diet fed groups (Fig. 5B) which suggests that the lesser oxidative stress was generated in these particular groups post challenged with *A. hydrophila*. Contrarily, increasing level of HDL was reported by Gabriel et al., (2015a) on GIFT – tilapia (*Oreochromis niloticus*) fed with *A. barbadensis* against *Streptococcus iniae*. Adeli et al., (2021) reported the elevated level of HDL in the *Oncorhynchus mykiss* (rainbow trout) fed with *Aloysia thriphylla*. The LDL levels were similar in D3 and NC group on 1 dpi and decreased values were noted in other experimental diet fed groups (Fig. 5C). Gabriel et al., (2015a), mentioned that, LDL content is usually correlated with the levels of free radicals which can lead to damage cells, DNA and tissues. The present study demonstrated that the *Aloe barbadensis* supplementation diets positively altered the LDL levels of the experimental fish. The similar decreasing trend of LDL was also observed in *Labeo rohita* fed with *Geotrichum candidum* (Amir et al. 2019).

Creatinine is a kind of protein which is produced by the breakdown of creatinine phosphate in the muscle and discharged into blood for energy. Creatinine acts as a helpful marker for assessing the health of fish gill and kidney. It constitutes more than 50% of nitrogenous waste eliminated by fish kidney and

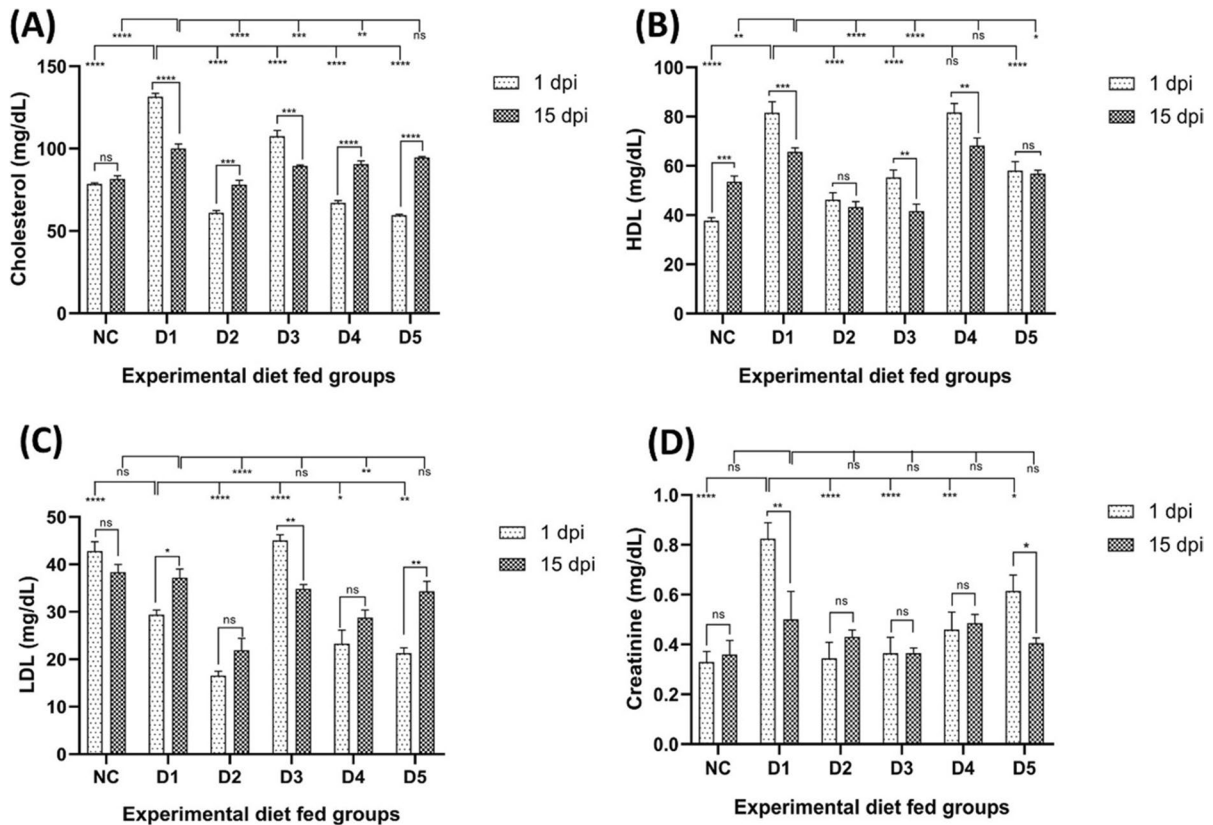
kidney filtration capability is measured by the level of creatinine (Pastorino et al. 2022). In this study, D1 diet fed group showed increased creatinine level than other experimental diet fed groups. The D5 diet fed group showed significant difference in creatinine level between 1 & 15 dpi (Fig. 5D). In addition, significantly decreased creatinine levels were observed on 1 dpi in all the experimental diet fed groups when compared with D1 diet fed group. Mahboub et al., (2022) reported increased level of creatinine in the Nile tilapia (*Oreochromis niloticus*) post infected with *Burkholderia cepacia*. Increased level of creatinine was recorded in *Aeromonas hydrophila* infected Nile tilapia (*O. niloticus*) fed with micro algae *Spirulina*, *Chlorella* diet (Fadl et al. 2017).

#### Immunological assay

According to previous studies, the medicinal plants and their extracts can ameliorate the specific and non-specific immune response of various fish species such as *Poecilia reticulata* (Ahmadniaye Motlagh et al. 2020), *Pelteobagrus fulvidraco* (Hou et al. 2022), *Achycentron canadum* (Lee et al. 2020) and *Oncorhynchus mykiss* (Mehrabi et al. 2020). In line with these previous reports, this current study investigated that whether the administration of *Aloe barbadensis* can enhance the humoral immunity of experimental fish *Labeo rohita* by analyzing the activity of immunological parameters such as lysozyme and IgM.

#### Lysozyme activity

Usually, neutrophils, submucosal glands and macrophages secrete lysozyme enzymes (Ganz 2006; Dotta et al. 2014). It serves a crucial role in innate immunity by protecting against pathogens (Sridhar et al. 2021a) and also used as a biomarker (Das et al. 2015; Sutthi et al. 2020). Bacterial lysis, stimulation of complement system and phagocytosis are accomplished by the enzyme lysozyme. The declining activity of this enzyme might be due to the reduction in the count or function of macrophages and neutrophils (Farag et al. 2021). In this present study, increased level of lysozyme activity was observed in the D3 diet fed fish group in both 1 & 15 dpi (Table 2). The results revealed that the supplementation of *A. barbadensis* positively modulates in D3 diet fed group. Plant based ingredients usually contain antinutritional



**Fig. 5** (A) Cholesterol; (B) High density lipoprotein (HDL); (C) Low density lipoprotein (LDL); (D) Creatinine levels of *Labeo rohita* fed with *Aloe barbadensis* on 1 and 15-days post infection (dpi) with *Aeromonas hydrophila*. \*, \*\*, \*\*\*, \*\*\*\* indicates significant difference to positive control (D1) at  $\leq 0.05$ ,  $\leq 0.01$ ,  $\leq 0.001$  and  $\leq 0.0001$  respectively. NC, negative control; D1-0% *A. barbadensis* (positive control); D2-25% *A. barbadensis*; D3-50% *A. barbadensis*; D4-75% *A. barbadensis*; D5-100% *A. barbadensis* replacing fishmeal; ns, non-significant

factors and it was reflected in the higher inclusion diets (D4 & D5). Identical results were obtained by Sutthi et al., (2020), in which 0.25 g/kg inclusion of ethanolic extract of *Apium graveolens* leaves diet fed fish *Labeo chrysophekadion* showed elevated levels of lysozyme activity and declined in higher inclusion diets against *Aeromonas hydrophila* infection. Teoh and Loo, (2022) mentioned that lysozyme enzyme protects the fish by rupturing the peptidoglycan layer of pathogenic bacterial cell wall. Gabriel et al., (2015b) reported that higher lysozyme activity was observed in the aloe vera inclusion diet fed GIFT tilapia post infected with *Streptococcus iniae*. Significant increase in the level of lysozyme was also noted after *A. hydrophila* infection on *Piaractus mesopotamicus* fed with 1 and 2% aloe vera inclusion diets (Zanuzzo et al. 2017). El-Kassas et al. (2022) observed elevated

levels of lysozyme activity on *A. hydrophila* infected *Oreochromis niloticus* fed with *Moringa oleifera* leaf powder at 48 h of post infection.

#### Immunoglobulin M (IgM) assay

IgM is a major fish immune component in serum (Zhou et al. 2021). IgM is a main constituent in humoral immune system of teleost and indicator of fish health (Yeganeh et al. 2021). IgM is believed to serve a crucial role in various immune processes including phagocytosis, toxins and pathogenic viruses, bacteria neutralization, and opsonization in the affected fish (Magnadottir 2010; Yonar et al. 2019). In our study, IgM level was increased in D3 diet fed group (Table 2). Higher *Aloe barbadensis* inclusion diets (D4 & D5) showed no significant

**Table 2** Immunological parameters of *Labeo rohita* fed with *Aloe barbadensis* on 1 and 15-days post infection (dpi) with *Aeromonas hydrophila*, \*, \*\*, \*\*\*, indicates significant difference to positive control (D1) at  $\leq 0.05$ ,  $\leq 0.01$ , and  $\leq 0.001$ 

Enzyme	Days post infection (dpi)	Negative control	D1	D2	D3	D4	D5
Lysozyme (U/mL)	1	13.5 ± 0.71 <sup>ns</sup>	16.0 ± 1.41	20.5 ± 2.12 <sup>ns</sup>	25.5 ± 2.12 <sup>**</sup>	12.25 ± 1.77 <sup>ns</sup>	13.0 ± 1.41 <sup>ns</sup>
	15	13.5 ± 2.12 <sup>ns</sup>	12.5 ± 0.71	21.0 ± 2.82 <sup>**</sup>	22.5 ± 2.12 <sup>***</sup>	11.5 ± 2.12 <sup>ns</sup>	13.5 ± 2.12 <sup>ns</sup>
IgM (unit mg/mL)	1	9.45 ± 1.63 <sup>ns</sup>	10.76 ± 0.36	16.50 ± 1.41 <sup>**</sup>	19.05 ± 1.20 <sup>***</sup>	10.50 ± 1.84 <sup>ns</sup>	11.35 ± 1.49 <sup>ns</sup>
	15	10.45 ± 1.48 <sup>ns</sup>	12.30 ± 1.27	17.40 ± 0.99 <sup>*</sup>	18.25 ± 0.92 <sup>**</sup>	9.40 ± 1.84 <sup>ns</sup>	9.20 ± 0.99 <sup>ns</sup>

respectively. D1-0% *A. barbadensis* (positive control); D2-25% *A. barbadensis*; D3-50% *A. barbadensis*; D4-75% *A. barbadensis*; D5-100% *A. barbadensis* replacing fishmeal; ns, non-significant

variations on 1 & 15 dpi when compared with D1. The increased IgM levels are related to augmented fish immunity. Das et al., (2013) reported the correlation between immunoglobulin levels and proteins. The present study revealed a notable higher IgM content in experimental diet D3 fed group fishes and suggesting that the fishmeal replaced with *A. barbadensis* at 50% may enhance the immunity of *Labeo rohita*. The inclusion of plant-derived compounds in fish feed increase the non-specific immune responses in fish (Zhang et al. 2020). Increased level of IgM level was reported by Adeli et al., (2021) on rainbow trout (*Oncorhynchus mykiss*) juveniles fed with Lemon verbena (*Aloysia triphylla*).

#### Antioxidant activity

Common oxidative stress could make fish susceptible to diseases by creating an imbalance between the generation and removal of ROS (Sheikhzadeh et al. 2012; Yuan et al. 2019). An antioxidant is a type of compound that stabilizes, scavenges, and suppresses the generation of oxidants and free radicals. External stress is directly responsible to stimulate the antioxidant system (Ding et al. 2022).

#### Superoxide dismutase (SOD)

SOD is an important antioxidant enzyme (metalloenzyme) that converts superoxide radicals into hydrogen peroxide and molecular oxygen (O<sub>2</sub>). It acts as the defence mechanism against free radicals (Weydert and Cullen 2010) and protects cells from oxidative damage due to external stimuli. It is also a crucial component in the first line of protection in fish against invading pathogens (Wang et al. 2016). Kaur et al. (2020) reported elevated level of SOD activity

in *Aeromonas veronii* challenged *Labeo rohita* supplemented with turmeric powder. In the current study, lesser SOD activity was observed in D2 & D3 groups compared to other diet fed groups though they did not show any significant alterations (Table 3). Similar level of SOD was recorded in liver, intestine and muscle. Dotta et al., (2018) also observed no significant variations in SOD and GPx activity in *A. hydrophila* infected Nile tilapia fed with the 1% extracts of propolis and *Aloe barbadensis*. In this study, no significant variations were observed between 1 & 15 dpi in all the analyzed organs. When comparing to the negative control, all the experimental diet fed fish showed increased SOD enzyme level which indicates that the treated fish have experienced some disruption in the antioxidant defense system. In contrast, no significant variation was observed between the infected and uninfected silver cat fish (*Rhamdia quelen*) with *A. caviae* (Baldissera et al. 2018).

#### Catalase activity

Catalase (CAT) is a primary antioxidant enzyme found in almost all living organisms. It catalyzes the decomposition of hydrogen peroxide into water and oxygen and helps to protect cells from oxidative stress caused by ROS (Weydert and Cullen 2010). All the experimental diet fed groups showed increased catalase activity than negative control and implies that *A. barbadensis* significantly modulates the immunity of *Labeo rohita*. Significant higher antioxidant activity was noted in D1 & D4 diet fed fish on both 1 & 15 dpi irrespective of tissues (Table 3). An increase in the catalase enzyme activity denotes an accumulation of H<sub>2</sub>O<sub>2</sub> (Garcia et al. 2011). The decreased catalase activity was observed in D2 and D3 diet fed fish on 1 & 15 dpi in all the tissues indicating



**Table 3** Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) activity of *Labeo rohita* fed with *Aloe barbadensis* on 1 and 15-days post infection (dpi) with *Aeromonas hydrophila*. \*, \*\*, \*\*\*, \*\*\*\* indicates significant difference to positive control (D1) at  $\leq 0.05$ ,  $\leq 0.01$ ,  $\leq 0.001$

and  $\leq 0.0001$  respectively. NC, negative control; D1-0% *A. barbadensis* (positive control); D2-25% *A. barbadensis*; D3-50% *A. barbadensis*; D4-75% *A. barbadensis*; D5-100% *A. barbadensis* replacing fishmeal; ns, non-significant

Enzymes	Days post infection (dpi)	Organs	Negative control	D1	D2	D3	D4	D5
SOD (units/mg protein)	1	Liver	7.71 ± 1.02****	10.21 ± 0.02	9.91 ± 0.05 <sup>ns</sup>	9.01 ± 0.72*	10.25 ± 0.02 <sup>ns</sup>	10.27 ± 0.01 <sup>ns</sup>
		Intestine	7.45 ± 0.75****	9.97 ± 0.01	9.49 ± 0.01 <sup>ns</sup>	9.52 ± 0.01 <sup>ns</sup>	9.91 ± 0.08 <sup>ns</sup>	9.67 ± 0.13 <sup>ns</sup>
		Muscle	7.44 ± 0.63****	9.95 ± 0.02	8.53 ± 0.02**	8.47 ± 0.21 <sup>ns</sup>	8.48 ± 0.06 <sup>ns</sup>	8.54 ± 0.05 <sup>ns</sup>
	15	Liver	7.58 ± 0.49***	10.78 ± 0.66	9.32 ± 0.06 <sup>ns</sup>	9.30 ± 0.03 <sup>ns</sup>	11.64 ± 1.02 <sup>ns</sup>	13.35 ± 1.39**
		Intestine	7.66 ± 0.48***	10.85 ± 1.40	9.88 ± 0.02 <sup>ns</sup>	9.87 ± 0.01 <sup>ns</sup>	13.39 ± 0.68**	10.38 ± 0.70 <sup>ns</sup>
		Muscle	8 ± 0.01*	10.24 ± 0.01	9.36 ± 0.7 <sup>ns</sup>	9.35 ± 0.7 <sup>ns</sup>	10.73 ± 0.7 <sup>ns</sup>	10.06 ± 0.13 <sup>ns</sup>
CAT (μmol H <sub>2</sub> O <sub>2</sub> consumed/min/mg protein)	1	Liver	6.46 ± 0.60****	16.33 ± 2.26	13.45 ± 2.47 <sup>ns</sup>	10.3 ± 0.64***	16.91 ± 1.27 <sup>ns</sup>	13.37 ± 0.64 <sup>ns</sup>
		Intestine	5.34 ± 0.89**	10.05 ± 0.37	7.36 ± 0.78 <sup>ns</sup>	7.91 ± 0.64 <sup>ns</sup>	9.06 ± 0.64 <sup>ns</sup>	8.64 ± 0.52 <sup>ns</sup>
		Muscle	4.63 ± 1.73**	9.08 ± 0.47	7.45 ± 0.64 <sup>ns</sup>	6.70 ± 0.85 <sup>ns</sup>	6.60 ± 0.57 <sup>ns</sup>	10.02 ± 1.00 <sup>ns</sup>
	15	Liver	5.29 ± 0.54****	15.37 ± 0.66	11.81 ± 2.53**	8.26 ± 0.50****	16.36 ± 0.92	14.25 ± 0.64
		Intestine	5.32 ± 0.43*	8.48 ± 0.76	7.05 ± 1.63 <sup>ns</sup>	6.26 ± 0.35 <sup>ns</sup>	8.15 ± 0.35 <sup>ns</sup>	7.35 ± 0.21 <sup>ns</sup>
		Muscle	4.89 ± 0.28**	8.31 ± 0.96	7.45 ± 0.64 <sup>ns</sup>	5.46 ± 0.62*	7.35 ± 0.64 <sup>ns</sup>	7.48 ± 0.60 <sup>ns</sup>
GPx (μg glutathione utilized/min/mg protein)	1	Liver	18.15 ± 0.27****	25.31 ± 0.95	19.13 ± 0.27****	19.91 ± 0.89****	24.30 ± 0.64 <sup>ns</sup>	21.89 ± 1.21***
		Intestine	16.25 ± 0.60****	23.99 ± 0.19	15.99 ± 0.56****	17.02 ± 0.65****	21.16 ± 0.47**	19.25 ± 0.31****
		Muscle	14.80 ± 0.77***	18.19 ± 0.22	14.99 ± 0.66****	17.34 ± 0.79 <sup>ns</sup>	20.03 ± 0.10 <sup>ns</sup>	19.03 ± 0.37 <sup>ns</sup>
	15	Liver	15.98 ± 0.01****	23.23 ± 1.26	15.81 ± 0.23****	16.37 ± 2.13****	21.16 ± 0.57 <sup>ns</sup>	22.61 ± 0.69 <sup>ns</sup>
		Intestine	15.38 ± 0.01****	22.88 ± 1.10	15.34 ± 0.45****	16.82 ± 0.08**	17.42 ± 0.63***	17.32 ± 0.78***
		Muscle	16.61 ± 0.33**	22.13 ± 2.36	16.68 ± 0.45****	17.19 ± 2.55**	23.81 ± 0.23 <sup>ns</sup>	21.80 ± 0.07 <sup>ns</sup>

lesser infection in these diets fed groups. Especially in the liver, the D3 diet fed fish showed significantly decreased activity on 1 & 15 dpi when compared with D1. This result suggests that the inclusion of *A. barbadensis* enhanced the *L. rohita* immunity. Elevated level of CAT activity was observed in licorice (Abdel-Tawwab and El-Araby 2021) and mulberry leaves (Neamat-Allah et al. 2021) fed *Oreochromis niloticus* against *A. hydrophila*.

#### Glutathione peroxidase activity

Another crucial antioxidant enzyme is GPx (Glutathione peroxidase) which converts hydrogen peroxide into water and oxygen and reduce peroxide radicals into alcohols and oxygen (Weydert and Cullen 2010). It acts as a strong free radical and lipid peroxidase scavenging enzyme. All the three tissues analyzed in this study showed higher GPx activity in D1, D4 & D5 diet fed groups similarly to the catalase activity (Table 3). There were no significant variations in the tissues between 1 & 15 dpi. Significantly

decreased GPx activity was observed in D2 and D3 group when compared with D1. Sahreen et al., (2021) reported the elevated levels of SOD, CAT and GPx activity in Indian catfish, *Wallago attu* against *Isoparorchis hypselobagri* infection and indicated that the host organism had adaptive changes to the pathogens or infections.

The results of the antioxidant assays conclude that the antioxidant enzymes (SOD, CAT, GPx) studied in the present work showed increased levels on 1 dpi than 15 dpi. When comparing all the experimental diet fed groups, D4 showed elevated levels of antioxidant enzymes. Whereas, decreased activity was observed in D3 followed by D2 diet fed group. The activation of these enzyme cause deleterious effects to ROS (Halliwell 2012). Antioxidant activity such as SOD, CAT, GPx was significantly influenced in the D1 due to the stress caused by the severe infection of *Aeromonas hydrophila*. The phytoconstituents like phenols, flavonoids, vitamins, etc., in the extracts of medicinal plants impede oxidation process (Rajasekaran et al. 2006; Hoseinifar et al. 2020).

Study by Vicente et al., (2019) also reported that the phytoconstituents of orange peel enriched diets influenced the antioxidant enzyme activity in Nile tilapia *Oreochromis niloticus*.

### Histopathology

Histopathology is a well-established method to determine the qualitative alterations in the organs caused by biological infectious agents/chemicals and the patterns of recovery (Forouhar Vajargah et al. 2018; Abraham et al. 2021). The histopathological lesions clearly demonstrate the interactions of bacterial virulence factors with the host immune system. The bacterium *Aeromonas* cause cellular membrane rupture by producing hemolysin and aerolysin, resulted in cell death (Hussain Bhat et al. 2021). In this study, severe infection was observed on 1 dpi in all the experimental diets fed fish groups (Fig. 6). D3 and D4 group fish showed lesser damage than other experimental diets fed fish groups.

In liver at 1 dpi, the D1 diet fed group showed severe necrosis of hepatocytes and extreme diffused hepatic vacuolation. The D2 diet fed group showed moderate necrosis of hepatocytes and vacuolation. The D3 diet fed group revealed slight necrosis of hepatocytes with severe diffused vacuolation. The D4 diet fed group showed very slight necrosis of hepatocytes with high diffused vacuolation. The D5 diet fed group exhibited diffused vacuolization and necrosis of hepatocytes and sinusoidal dilation. On 15 dpi (Fig. 7), the D3 and D5 diet fed fish liver showed mild vacuolation and necrosis of hepatocytes and blood sinusoid were clearly seen when compared to 1 dpi. The D4 diet fed group on 15 dpi showed severe diffused vacuolation and moderate necrosis of hepatocytes. Islam et al., (2013) demonstrated structural abnormalities like focal necrosis, massive atrophy, hepatic abscess, hemorrhagic area and vacuolation in the liver of *Heteropneustes fossilis* (Asian stinging catfish) infected with *Aeromonas hydrophila*. Vinosha et al., (2020) reported histopathological alterations like necrosis, degraded hepatocyte and vacuolations in liver, muscle and gill tissue of *A. hydrophila* infected Nile tilapia. Whereas, sulfated galactan from *Halymenia dilatata* treated fish groups showed positively upgraded tissue structures.

At 1 dpi, D1 group showed the infected intestine with complete degeneration along with severe necrosis, and sloughing of lamina mucosa. The D2 and D3 diet fed fish intestine showed partial sloughing

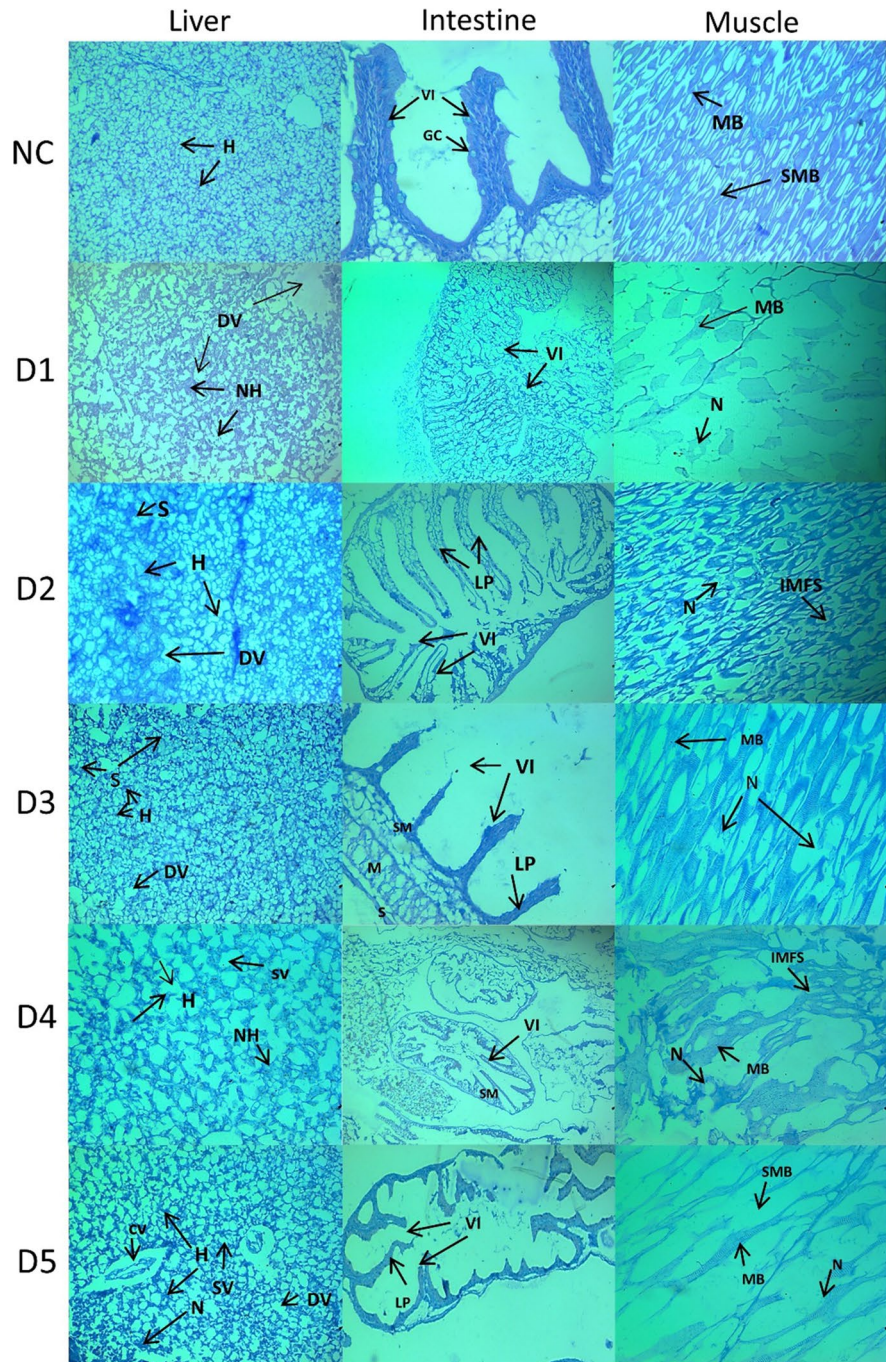
of intestinal villi and moderate necrosis. The same modifications were reported by Hoseini et al., (2022) in the intestine of *Cyprinus carpio* exposed to copper and polyvinyl chloride microparticles. Sloughing of intestinal villi was observed in the *Aeromonas* infected fishes such as *Cyprinus carpio* (Di et al. 2017), *Oreochromis niloticus* (Assane et al. 2022), *Ictalurus punctatus* (Abdelhamed et al. 2017), and *Labeo rohita* (Sidiq et al. 2023). Blood innervates the submucosa's connective tissue, causing submucosa congestion that eventually results in haemorrhage with leukocyte infiltration and sloughing of the epithelial submucosa (Hussain Bhat et al. 2021). D4 and D5 diet fed fish intestine on 1 dpi showed severe necrosis and degeneration along the length of intestinal villi. In the case of 15 dpi, degeneration and necrosis were observed in D4 and D5 diet fed fish intestine. Intact structure was observed in the D2 and D3 diet fed groups.

In the case of muscle histoarchitectural analysis, D4 and D5 diet fed group fish showed severe necrosis and atrophy of muscle bundles. Abdel-Latif et al. (2022) reported the slight edema, loss of muscle striation in *A. hydrophila* challenged Nile tilapia. Necrosis was reported by ELbially et al. (2023) in *Oreochromis niloticus* infected with *A. hydrophila*. Intact and clear structures were noted in the negative control, D2 and D3 diet fed fish on 1 dpi (Fig. 6). Necrosis level was also decreased in those group fish on 15 dpi (Fig. 7). It suggests the reduction in the infection rate on 15 dpi. In this current study, *Aloe barbadensis* inclusion diet D2 & D3 reduced the pathological changes associated with *A. hydrophila* infection in all examined tissues with an obvious effect in D3 diet fed group (50% FMR). In the same context, Ostaszewska et al., (2008) have showed an improvement in organ structures in silver bream (*Vimba vimba*) fed on natural plant feed additives.

### Survival rate

The survival percentage of fish challenged with *A. hydrophila* after fed with different concentrations of *A. barbadensis* diet was showed in Fig. 8. In this study, D3 diet fed group showed higher survival rate (80%) followed by D2 (75%), D5 (65%), D1 (50%) diet fed group and the least survival percentage was obtained in D4 (40%) diet fed group. Giri et al., (2023) reported higher survival percentage on *A. hydrophila* infected *Cyprinus carpio* fed with *Bougainvillea glabra* leaf inclusion diets and

**Fig. 6** The histopathological photomicrographs of liver, intestine and muscle of *Labeo rohita* fed with *Aloe barbadensis* on 1 day post infection (dpi) with *Aeromonas hydrophila*. H-hepatocytes, DV-diffused vacuolation, S-sinusoid, SV-sinusoidal vacuolation, N-necrosis, NH-necrotic hepatocyte, CV-central vein, LP-lamina propria, VI-villi, SM-submucosa, E-tunica mucosa, M-tunica muscularis, S-tunica serosa, GC-goblet cell, MB-muscle bundle, SMB-space between muscle bundle, IMFS-intra myofibril space. NC, negative control; D1-0% *A. barbadensis* (positive control); D2-25% *A. barbadensis*; D3-50% *A. barbadensis*; D4-75% *A. barbadensis*; D5-100% *A. barbadensis* replacing fishmeal

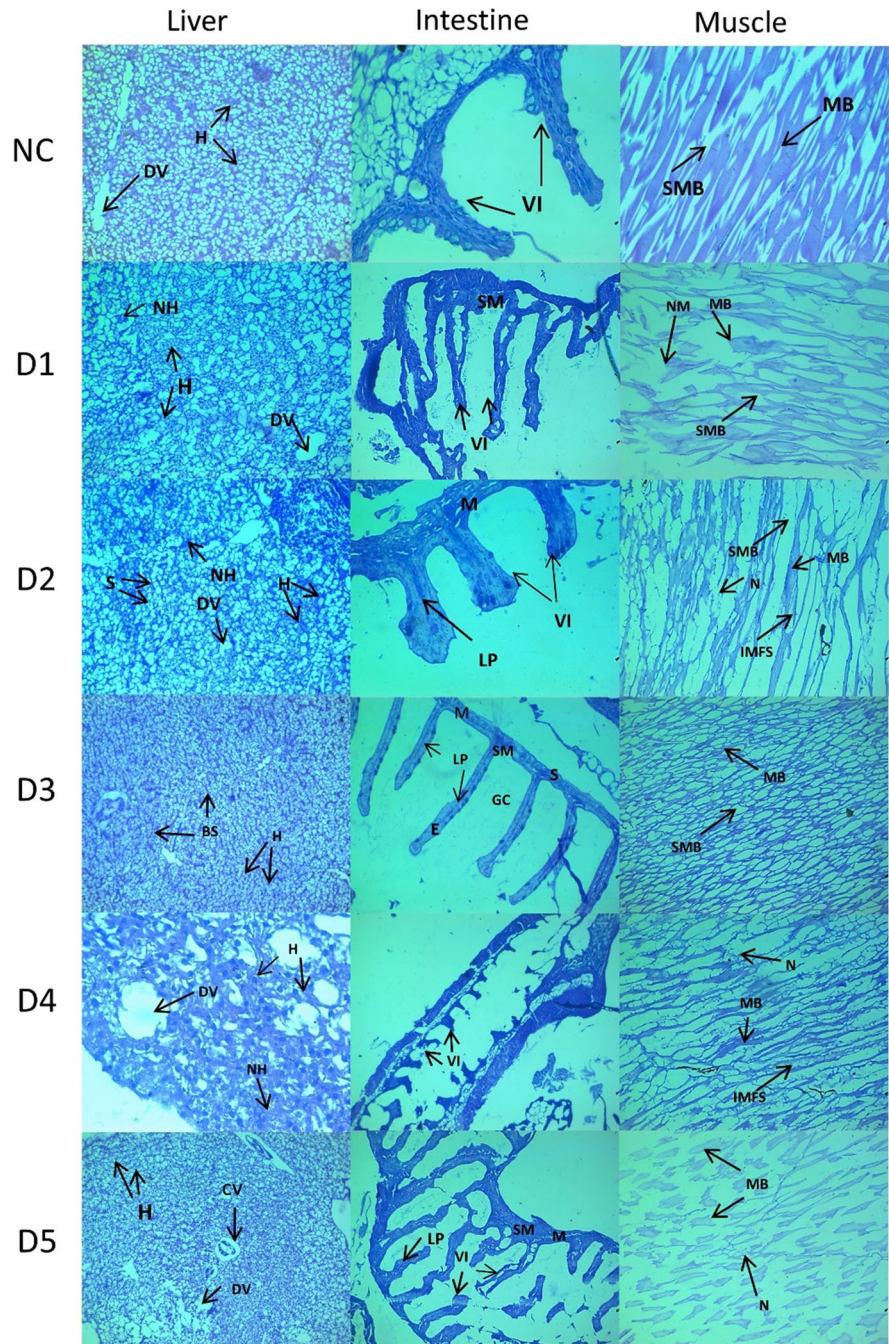


indicated that the dietary supplementation of herbal immunostimulants increase the disease resistance mechanism in fish. Administration of *Mentha piperita* enhanced the survival of *Labeo rohita* against *A. hydrophila* infection (Padala et al. 2021). Among the experimental diet fed groups challenged with

*A. hydrophila* infection, D3 diet fed group showed increased lysozyme and IgM activity, better antioxidant activity and higher survival rate which indicates that the *Aloe barbadensis* inclusion at the rate of 50% replacing fishmeal enhance the disease resistance by elevating the immune activity of *Labeo rohita*.



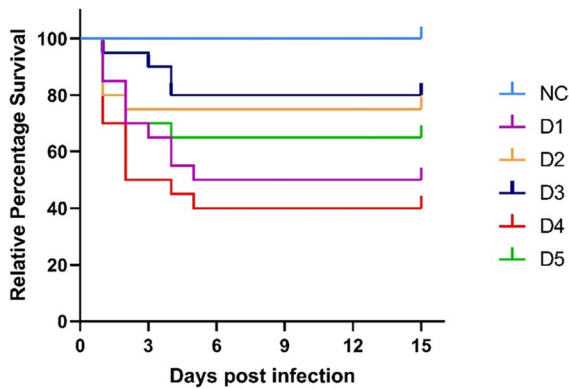
**Fig. 7** The histopathological photomicrographs of liver, intestine and muscle of *Labeo rohita* fed with *Aloe barbadensis* on 15 days post infection (dpi) with *Aeromonas hydrophila*. H-hepatocytes, DV- diffused vacuolation, S-sinusoid, SV-sinusoidal vacuolation, N-necrosis, NH-necrotic hepatocyte and CV-central vein, LP-lamina propria, VI-villi, SM-sub-mucosa, E-tunica mucosa, M-tunica muscularis, S-tunica serosa, GC-goblet cell, MB-muscle bundle, SMB-space between muscle bundle, N-necrosis, IMFS-intra myofibril space. NC, negative control; D1-0% *A. barbadensis* (positive control); D2-25% *A. barbadensis*; D3-50% *A. barbadensis*; D4-75% *A. barbadensis*; D5-100% *A. barbadensis* replacing fishmeal



## Conclusion

The findings of the current study demonstrated that *L. rohita* fed with *A. barbadensis* replacing 50% of fishmeal (D3 diet group) exhibited effective immune protection and increased disease resistance to *A. hydrophila* infection. However, higher level of *A.*

*barbadensis* replacing fishmeal (75 & 100%—D4 and D5 group respectively) diets had a potential risk of decreasing the disease resistance and survival rate of the fish *L. rohita*. Therefore, based on the results obtained in this study, Diet 3 (50% of *A. barbadensis* replacement in fishmeal) could be considered as strategy to increase the disease resistance against *A.*



**Fig. 8** Relative percentage survival of *Labeo rohita* fed with *Aloe barbadensis* replacing fishmeal post challenged with *Aeromonas hydrophila*. NC, negative control; D1-0% *A. barbadensis* (positive control); D2-25% *A. barbadensis*; D3-50% *A. barbadensis*; D4-75% *A. barbadensis*; D5-100% *A. barbadensis* replacing fishmeal

*hydrophila* associated disease occurrences in *L. rohita* farming. *A. barbadensis* could enhance the fish immunity against aquatic disease problems through its sustainable, non-drug-resistant, non-noxious, and non-negligible characteristics.

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**Author contributions** **S. P.:** Methodology, Investigation, Formal analysis, Data curation, Writing – original draft. **A. S.:** Resources, Formal analysis, Writing– review & editing. **Z. A. K. & G. T.:** review & editing. **T. R.:** conceptualization, project administration, supervision, validation, visualization, writing – review and editing.

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**Data availability** All data and materials are included in this published article.

## Declarations

**Ethical approval** The animal study was reviewed and approved by Institutional Animal Ethical Committee (IACE) Department of Animal Science, Bharathidasan University, Tiruchirappalli – 620 024. Ref. No: BDU/IAEC/P19/2021.

**Consent to participate** Not applicable.

**Consent for publication** Not applicable.

**Competing interests** The authors declare no competing interests.

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