Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v31i7.6957

EFFECT OF ROSELLE ANTHOCYANIN EXTRACT ON GUT MICROBIOTA AND IMMUNE RESPONSE IN *CYPRINUS CARPIO* AND *CTENOPHARYNGODON IDELLA*

Muhammad Ahmad[1], Shakeela Parveen[1*], Fayyaz Rasool[2], Amina Ayub[3], Shahid Mahmood[4], Zeeshan Ashraf[2], Shumaila Maryam[2], Tahreem Fatima[1], Kashif Manzoor[4]

[1*]Department of Zoology, Wildlife and Fisheries, University of Agriculture Faisalabad
[2]Department of Zoology, University of Education Lahore, Faisalabad Campus
[3]Department of Zoology, Wildlife and Fisheries, Depalpur Okara Campus, University of Agriculture Faisalabad, Pakistan
[4]Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences Lahore

***Corresponding Author**: Shakeela Parveen
*Email: drshakeela.fayyaz@uaf.edu.pk

ABSTRACT

Cyprinus carpio and *Ctenopharyngodon idella* are widely cultivated in polyculture where high stocking density and uncontrolled use of variety of antibiotics which cause severe disease outbreaks, posing risks to both fish and consumer health status. These risks can be overcome by using roselle anthocyanin extract from *Hibiscus sabdariffa*, known for its antibacterial, immunomodulator and antioxidant properties used to overcome these risks. The study aimed to investigate the effect of roselle anthocyanin extract on gut microbiota and immune response in *C. carpio* and *C. idella*. For this purpose, 40 samples of each of *C. carpio* and *C. idella*, were collected from fish farms and subsequently divided into control group fish fed commercial feed, while the experimental group fish fed roselle anthocyanin extract for 60 days. Survival rates, bacterial cultures, hematological parameters, and immunological assays were analyzed. The different treatments were compared using the Two-way factorial ANOVA. Results showed that roselle anthocyanin significantly (p<0.05) increased WBCs, while decreasing platelet counts and gut microbial counts in both fish species compared to controls. In the experimental group, WBCs, neutrophils, eosinophils, and basophils were non-significant (p>0.05) between the species, but lymphocytes, monocytes, platelets, and microbial counts showed significant differences (p<0.05). Roselle anthocyanin prevented thrombosis by stabilizing platelet activity due to its antioxidant properties, improved intestinal morphology, boosted the immune response by increasing white blood cell counts, and promoted beneficial microorganisms while inhibiting harmful bacteria. The study concluded that roselle anthocyanin enhances intestinal health, immunity, and mitigates thrombotic events in both *C. carpio* and *C. idella*.

Key words: Anthocyanin, Immunomodulator, Microbiota, Antioxidant, Thrombosis, Microbial counting

Introduction

The rapid increase in the human population has caused major challenges for food security. Aquaculture is playing a significant role in providing value sources of protein through fish and its products (Manzoor et al., 2023ab; Mahmood et al. 2024). The world's rapidly increasing population faces a major challenge of food security, and fish is considered a sustainable diet for the future (Ullah et al., 2023ab; Rabbani et al., 2024). Aquaculture, particularly pond aquaculture, plays a significant role in Pakistan by increasing fish production and contributing to economic development. Several carp species are well-known but Common carp (*Cyprinus carpio*) and Grass carp (*Ctenopharyngodon idella*) play the most significant role in pond aquaculture (Shah et al., 2018). Both species have different feeding behaviors therefore its suitable polyculture candidates in the aquaculture sector (Mesallamy et al., 2016). Common carp and Grass carp lack a stomach and pyloric caeca, influencing their gut morphology and microbiota, which is crucial for cellulose metabolism and digestion (Van-Kessel et al., 2011; Yang et al., 2019). *C. carpio* is native to Eastern Europe and Asia, but it is also cultivated in Africa and North America (Emeish et al., 2023). Common carp are opportunistic feeders, while Grass carp are herbivorous, affecting their gut microbiota composition. This microbiota includes both gram-positive and gram-negative bacteria (Feher et al., 2021).

Factors like genetics, maturity, physiological state, disease, and environmental conditions influence fish gut microbiota (Feher et al., 2021). These microorganisms interact with the gut-brain axis, affecting host health, energy balance, and behaviors related to feeding, digestion, and immune responses (Ruzauskas et al., 2021). One of the major reasons for affected feeding, digestion, immune responses, and low growth performance in fish is infectious diseases caused by microorganisms (Prakoso et al., 2023). Similarly, the application of pesticides in or on the banks of fish ponds, such as insecticides, herbicides, and fungicides, is also an important factor contributing to issues related to feeding, digestion, and immune responses (Kazmi et al., 2023). Uncontrolled and frequent use of antibiotics is also major reason for affected immune response and infectious diseases in fish (Mansoor et al., 2023; Saad et al., 2023).

High-density and intensive aquaculture of Common carp and Grass carp can lead to disease outbreaks due to stress, affecting the intestinal mucosa and gut-associated lymphoid tissue (GALT), which reduces nutrient absorption and immunity (Yang et al., 2019). Farmed fish are vulnerable to various lethal diseases, including viruses, fungi, parasites, and bacteria (Butt and Helena, 2019). Although antibiotics have been used to manage these diseases, their abuse leads to antibiotic-resistant bacteria and transferable resistance genes, impacting both aquatic life and humans (Moustafa et al., 2021). Additionally, antibiotic residues in fish products pose health risks, and entail stricter regulation of antibiotic use in aquaculture (Rasul and Majumdar, 2017).

Disease outbreaks in aquaculture, are controlled by alternative management precautions like immunomodulators, have been implemented by the use of medicinal plants or herbal additives (Moustafa et al., 2021). Roselle or Hibiscus also known as *Hibiscus sabdariffa*, is an herbaceous plant in the *Malvaceae* family that is used for both gastronomic and medicinal purposes that is cultivated all over the world. Roselle contains a high concentration of flavonols and Anthocyanins, which have antibacterial and antioxidant properties (Carvalho et al., 2023). Anthocyanin accelerates growth, immunological responses, antioxidant defenses and cytokine gene expression (Jomeh et al., 2021).

Anthocyanin also known for its stress relief agent (Diab et al., 2023). And is also extracted from fruits such as blueberries, blackberries, and prickly pears. The primary antioxidant capability of Anthocyanin can be categorized into four groups, with delfinidine-3-sambubioside (D3S) and cyanidine-3-sambubioside (C3S) being the most prevalent, while delfinidine-3-glucoside and cyanidine-3-glucoside are of lesser significance. Among these, D3S accounts for 85% of all Anthocyanins, making it a vital source of antioxidant potential in *H. sabdariffa* extracts. This high content of D3S underscores its importance in contributing to the overall health benefits associated with Anthocyanins (Izquierdo-Vega et al., 2020).

One of the most rapidly increasing industries nowadays is the use of natural foods or herbal products to treat diseases, reduce stress and boost the immune system. Studies on *Carassius auratus*, *Oreochromis niloticus* and *Oncorhynchus mykiss* in dietary Roselle Anthocyanin extract dramatically increased growth, immune responses, plasma metabolic markers, survival and disease resistance (Jomeh et al., 2021). However, previously no study was done on the effect of Anthocyanin extract from the Roselle plant on the gut microbiota of *C. carpio* and *C. idella*. The main goal of this study was to evaluate the effect of Roselle Anthocyanin extract on the gut microbiota and immune response on *C. carpio* and *C. idella*.

Materials and Methods

Study area and duration

The current research work was conducted for two months from 8 October 2023 to 8 December 2023 at the Fisheries Research Farm located at the Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad.

Experimental design

Healthy fingerlings of Common carp and Grass carp, weighing on average between 5-8g, were obtained from freshwater research earthen ponds. These fingerlings underwent a period of acclimatization lasting 48 hours. Throughout the feeding trial, the fish in both groups were accommodated in 70-liter aquaria, with a stocking density of 40 fish per aquarium.

A total of 80 fish samples were divided into two groups, 20 specimens for each group for the specific research study, with;

T_0: received commercial feed 2% of their body weight

T_1: Received special feed containing Roselle Anthocyanin at 2% of their body weight

Each group included two species, *C. carpio* and *C. idella* because of polyculture behavior.

Preparation of Roselle anthocyanin

Fresh Roselle flowers and fruits were collected, washed, and separated into calyxes and sepals. The pieces were chopped and mixed with distilled water (2:1 ratio) in a glass container. A 2% citric acid solution was added, and the pH was adjusted to 2-4 to stabilize the anthocyanins, measured with a pH meter. After stirring well, the mixture was left at room temperature for 24-48 hours to allow anthocyanin extraction. The liquid was then filtered using cheesecloth, pressed to extract maximum liquid, and stored in a cool, dark place. Finally, the prepared Roselle anthocyanin was added to an aquarium at 2% of the total fish weight.

Sterilization

Sterilization, the process of eliminating microbial life in a clean environment, can be achieved through moist or dry methods. In this study, moist sterilization was utilized by placing culturing media and glassware in an autoclave at 200lb, 121ºC for 15 minutes while wrapped in aluminum foil, and sterilizing inoculation needles on a red-hot flame.

Sampling procedure

A total of 80 fish samples including 40 Common carp (*C. carpio*) and 40 Grass carp (*C.* idella) (thirteen from T_0 and thirteen from T_1 groups each) were collected, transported to the lab, and refrigerated for blood sampling. The blood samples were collected from fish subjected to euthanasia by adding clove powder solution in water. Blood was collected in vacutainer containing EDTA. The fish were then dissected, and their gut samples were stored in Eppendorf tubes with saline at 4°C.

Total bacterial count using different media

Solutions of nutrient agar (NA), Tryptic soy agar (TSA), and Eosin Methylene blue (EMB) were prepared by dissolving measured amounts (2.80g, 4.00g, and 3.75g respectively) in 100ml distilled water. The mixtures were autoclaved at 121ºC for 15 minutes according to the method of Ogunshe and Olabode, (2009) and Adejonwo et al., (2020). Fish gut bacteria were cultured on these media using the quadrant streaking method (Sanders, 2012). Petri plates were incubated at 37ºC for 24 hours, and then colony-forming units (CFUs) were counted using a digital colony counter by the method of Clarke et al., 2010. This process helped assess the impact of roselle anthocyanin feed versus commercial feed on gut microflora.

Immunological Parameters

Blood samples stained with Giemsa were used for leukocyte counts. A hemocytometer was used to count the cells under a light microscope for the count such as the different leukocyte counts (DLC), white blood cells (WBC) and thrombocytes (PLT) (Oluyemi et al., 2008). These counts were obtained from blood marks that were stained using a mixture of Giemsa, methanol, aqua distillate, and methylene blue. The samples were examined under oil immersion at a magnification of 100X (Hidayaturrahmah, 2015).

Physico-chemical parameters

The water index was kept in constant regulation. pH, temperature and DO were measured by using electronic instruments like a pH meter and HANNA HI-8424, respectively shown graphically measurements in Figure (1).

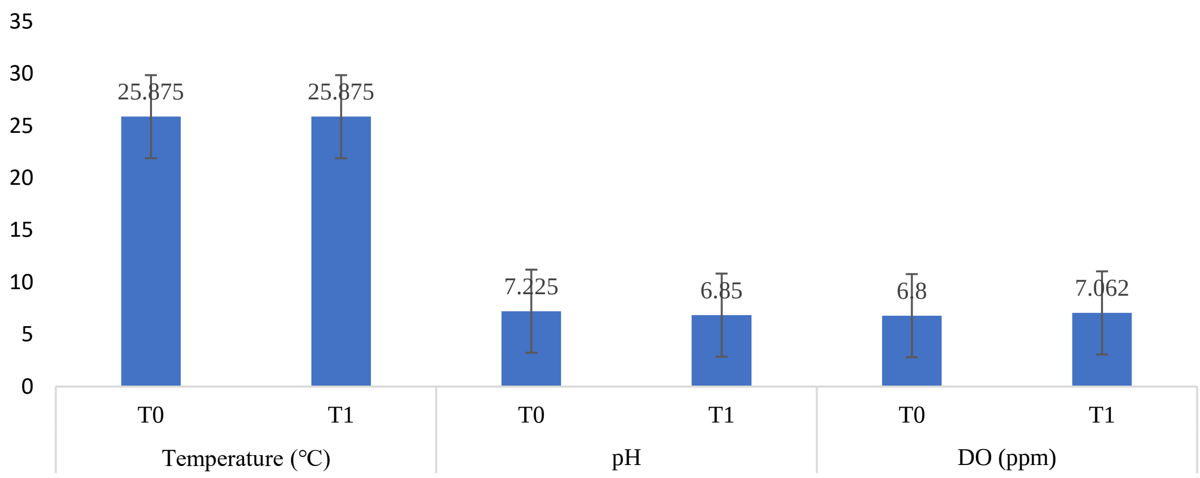


Figure 1: Physico-chemical parameters

Statistical analysis

Statistical analysis was conducted on the data after the research study. The Two-factor factorial ANOVA under complete randomized design (CRD) was employed, with a significance level of the *p*-value (<0.05), to assess the main and interaction effect of species and treatment.

Results

Total bacterial counting

The analysis of the overall microbiological composition found in intestinal samples obtained from control and experimental groups of Common and Grass carp, which were grown on various agar media, provided valuable insights into the diversity and abundance of microorganisms inhabiting the gastrointestinal tract of the fish. The gut microbiota undergoes alterations in response to variations in dietary intake, and these intricate microbial populations play a crucial role in influencing immune responses, consequently contributing to the implementation of effective aquaculture management approaches.

All main and interaction effects suggested significant results ($p<0.01$), which means that the antimicrobial effect of *H. sabdariffa* anthocyanin on fish guts is different between both species shown in ANOVA Table (1) and graphically represented in Figure (2).

Table 1: Analysis of main and interaction (S×T) effect between species (S) and treatment (T) and between treatment group by using Two-way factorial ANOVA

SOV	WBC ×103/μL		PLT ×103/μL		Microbial growth (NA)		Microbial growth (TSA)		Microbial growth (EMB)	
	F-Value	***p*-Value**	**F-Value**	***p*-Value**	**F-Value**	***p*-Value**	**F-Value**	***p*-Value**	**F-Value**	**p-Value**
S	17.22	<0.001	232.51	<0.001	10119.60	<0.001	21231.40	<0.001	45.67	<0.001
T	12.88	0.001	45.78	<0.001	20183.80	<0.001	43701.85	<0.001	1727.24	<0.001
S×T	1.15	0.290ns	15.64	<0.001	11360.76	<0.001	27410.20	<0.001	76.90	<0.001
Error	48									
Total	51									

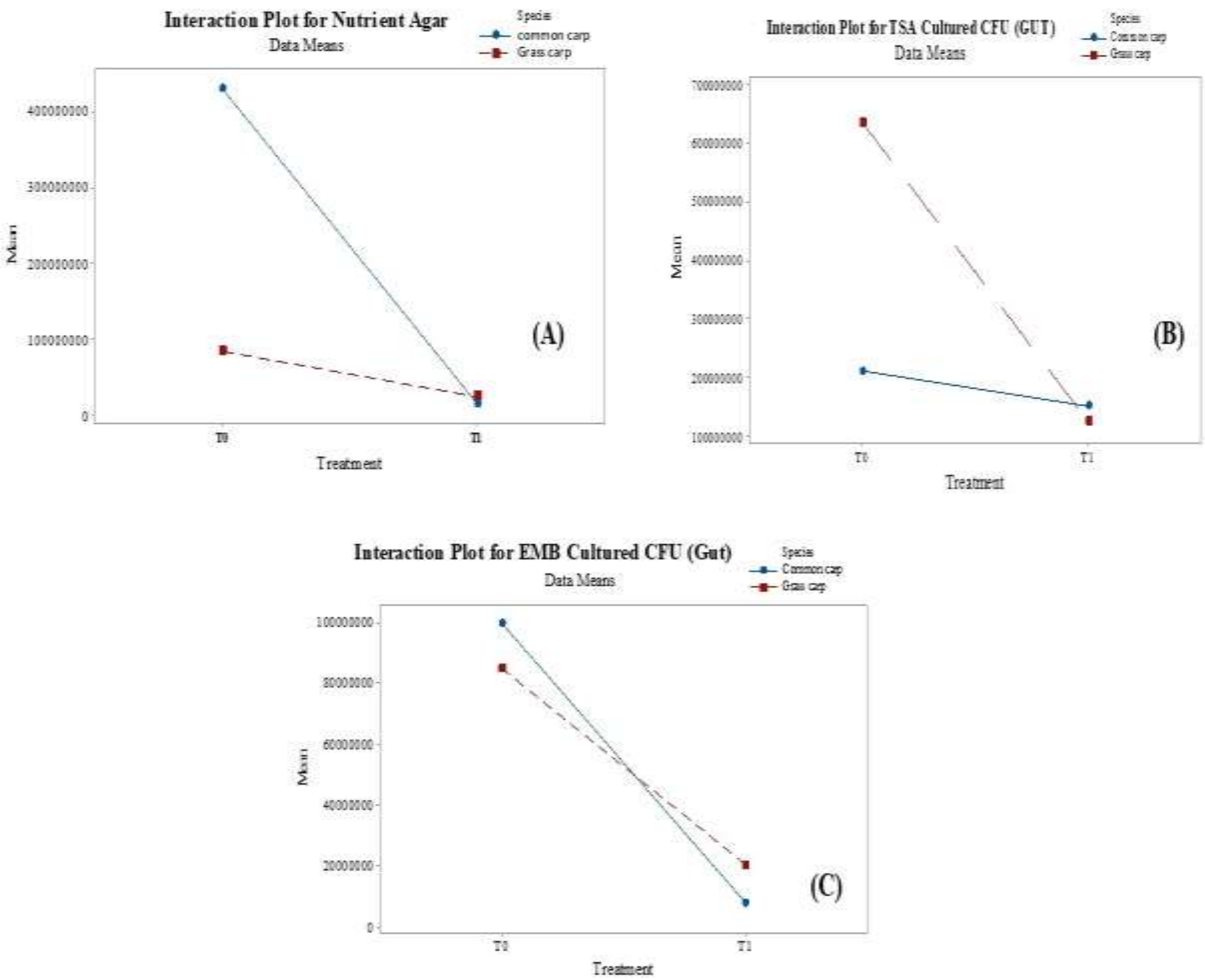


Figure 1: Interaction Plots for intestinal bacterial culture on (A) NA (B) TSA and (C) EMB

After cultured the bacteria on different media they were stained methods became essential for visualization and identification. Nutrient agar and Tryptone Soy Agar a common culture medium for non-fastidious microorganisms, provide a suitable growth environment for bacteria but Eosin Methylene Blue Agar (EMB) selective and differential culture mediums commonly were used in microbiology for the isolation and differentiation of gram-negative bacteria.

The growth of *Proteobacteria*, *Fusobacteriota*, *Gemmatimonadota*, *Bacteroidota*, *Chloroflexota* (*Chloroflexi*), *Verrucomicrobiota*, *Planctomycetota* and *Acidobacteriota* were significantly increased. The most significant growth was noted in the population of *Enterobacteriaceae* bacteria on the EMB medium. All gram-negative bacteria were pink-colored and gram-negative were purple colors shown in (Figure 3,4,5). The Table 2 shows different bacteria present in Common carp and Grass carp of control groups

Table 2: 3-4th of total gut bacteria of Common carp and Grass carp in T_0

Phylum	Class	Species and Morphology
***Proteobacteria* (*Pseudomonadota*)**	*Acidithiobacillia*	Rod shape, Gram-negative
	Betaproteobacteria	*Comamonadaceae ssp*. (oder; *Burkholderiales*) were Rod shape bacteria. Gram-negative
	Alphaproteobacteria	*Rhodobacter* (rod shape), *Methylobacterium* (straight rod shape) and *Roseomonas aquatica* (coccbacilli shape), Gram-negative
	Gammaproteobacteria	*Escherichia coli*, *Aeromonas spp*., *Shewanella spp*., *Pseudomonas spp*. and *Salmonella* belonging to the family *Enterobacteriaceae* were Rod-shaped bacteria but *Vibrio spp*. comma-shaped. Gram-negative
Fusobacteriota	*Fusobacteriia*	*Cetobacterium spp*. (rod shape), Gram-negative
***Firmicutes* (*Bacillota*)**	*Clostridia*	*Clostridium*; *Clostridium histolyticum* (rod shape but slightly curved edges), Gram-Positive
	Bacilli	*Bacillus spp*. also rod shape. In Grass carp, the most abundant diversity of *Firmicutes* was recorded as *Bacillus spp*. like *Bacillus megaterium* uniquely present. Gram-Positive bacteria *Lactobacillus spp*. like *Lactococcus*, *Streptococcus* (diplococci), *Staphylococcus aureus* and *Enterococcus* were Spherical shape or cocci-shaped bacteria recorded. Gram-Positive bacteria

Table 3: Other gut bacteria of Common carp and Grass carp in the T_0 group

Phylum	Class	Species and Morphology
Actinomycetota (Actinobacteria)	*Actinomycetes*	*Mycobacterium* (rod-shaped) and *Streptomyces spp*. (Branched Filamentous), Gram-positive
Gemmatimonadota	*Gemmatimonadetes*	It is oval and rod shape bacteria, Gram-negative
Bacteroidota	*Bacteroidia*	*Barnesiellaceae* and *Bacteroides* elongated and rod-shaped but *Bacteroides eggerthii* were straight and slightly curved. Gram-negative bacteria
Chloroflexota (Chloroflexi)		filamentous shape, Gram-negative
Verrucomicrobiota	*Verrucomicrobiae*	*Akkermansia ssp*. (Cylindrical shape), Gram-negative
Planctomycetota		Elongated shape, Gram-negative
Mycoplasmatota	*Mollicutes*	*Tenericutes* bacteria (Filamentous shape), acellular not positive nor negative

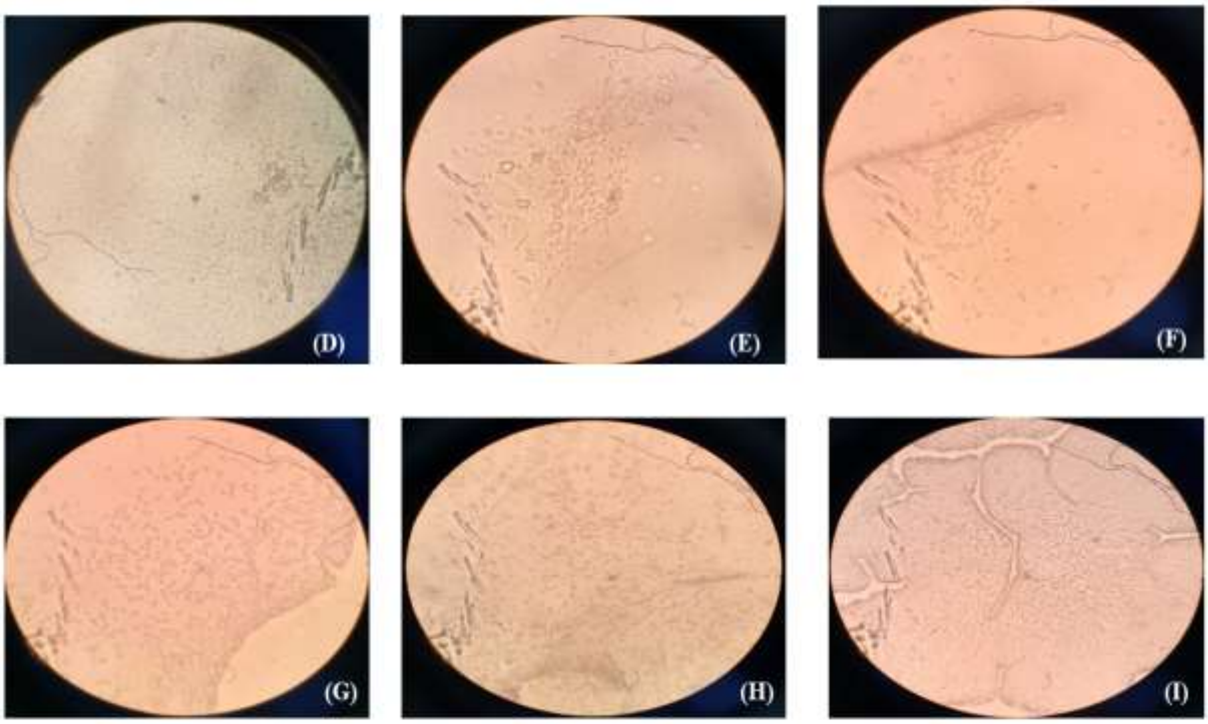


Figure 2: (D); T_0 Gram-positive of Common carp, (E); is Grass carp, (F); is Gram-negative of Grass carp, (G, H and I); Gram-negative of T_1 Common carp (G and H); Grass carp, (I); Gram- positive of Grass carp (100X)

In the experimental group (T_1), it was observed they had decreased colony formation and the number of bacteria due to Anthocyanin antimicrobial feed; they killed harmful bacteria. In the T_1 group, *Firmicutes* phyla increased i.e., *Lactobacillus spp*. and *Clostridium*. Anthocyanin inhibited the growth of *Clostridium histolyticum*. However, the phyla of *Proteobacteria* had some species like *Shewanella spp*. and *Pseudomonas spp*. decreased due to Anthocyanins. Due to the effect of Anthocyanin some bacterial growth was inhibited in Common carp and Grass carp, like *E. coli*, *Salmonella sp*. some *Bacillus spp*. *Chloroflexota* (*Chloroflexi*), and *Roseomonas aquatica*, but proliferated beneficial microbes were observed.

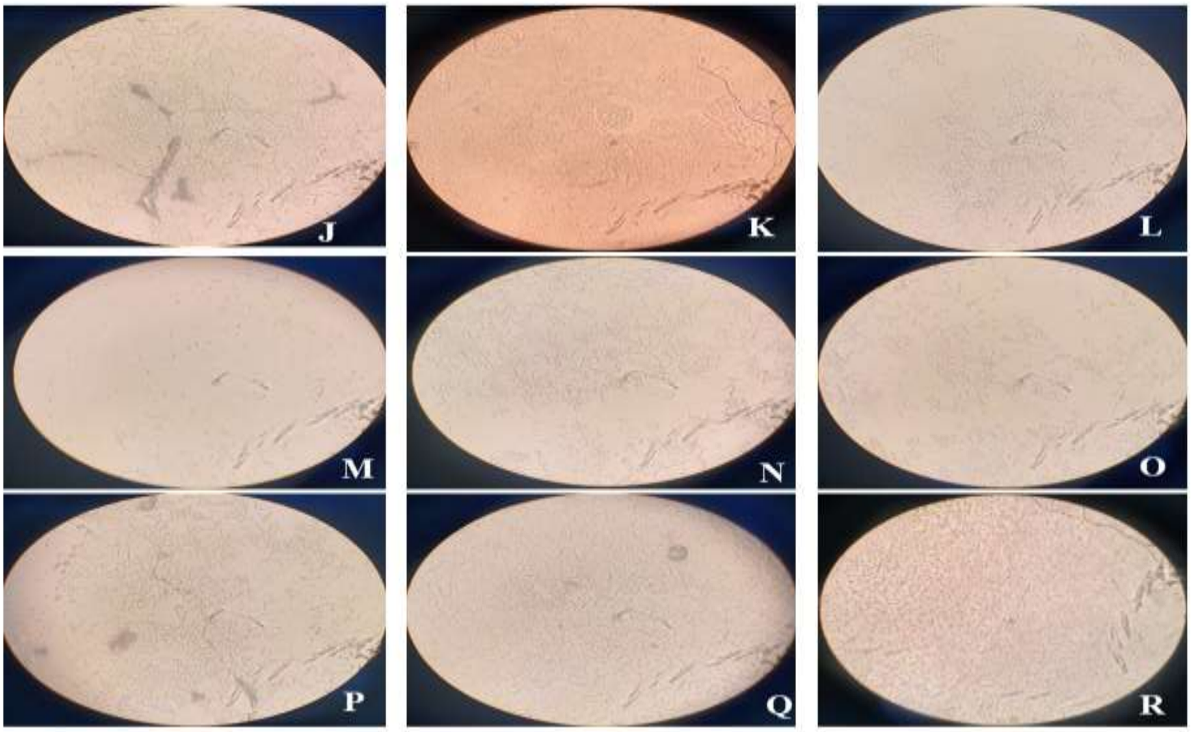


Figure 3: (J, K); Gram-negative of Common carp and Grass carp, (L, M); Gram-positive, (N, O, P, N and Q); Gram-negative of Common carp and Grass carp (N, O); Gram-positive (M, Q) but (R) *Acidobacteriota* was spherical shape and Gram-negative

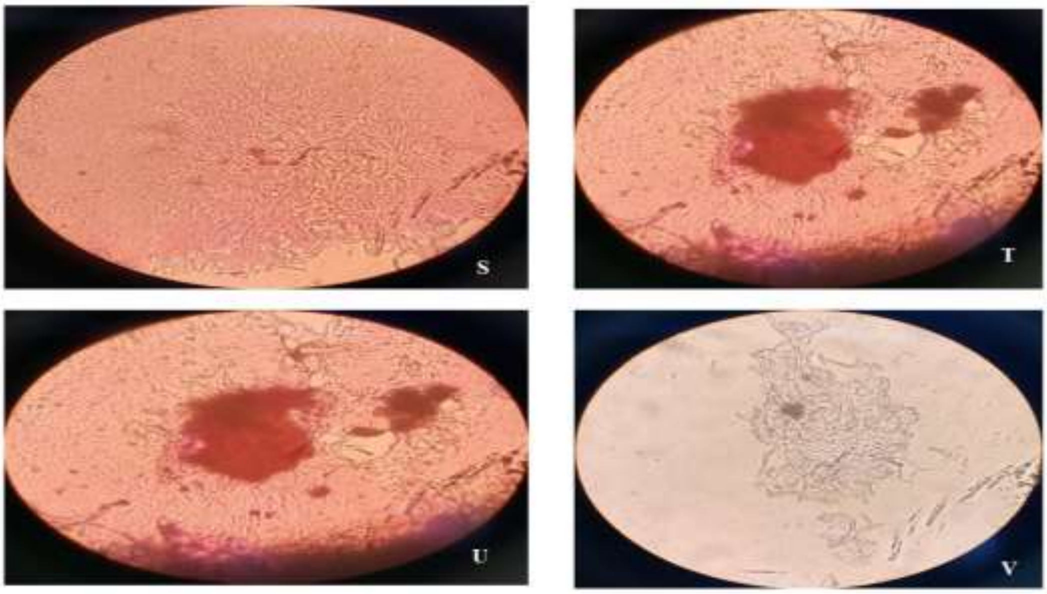


Figure 4: (S); Gram-negative bacteria of Common carp, (T); Grass carp of T_0 group on EMB, (U); Gram-negative bacteria of Common carp, (V); Grass carp of T_1 group on EMB

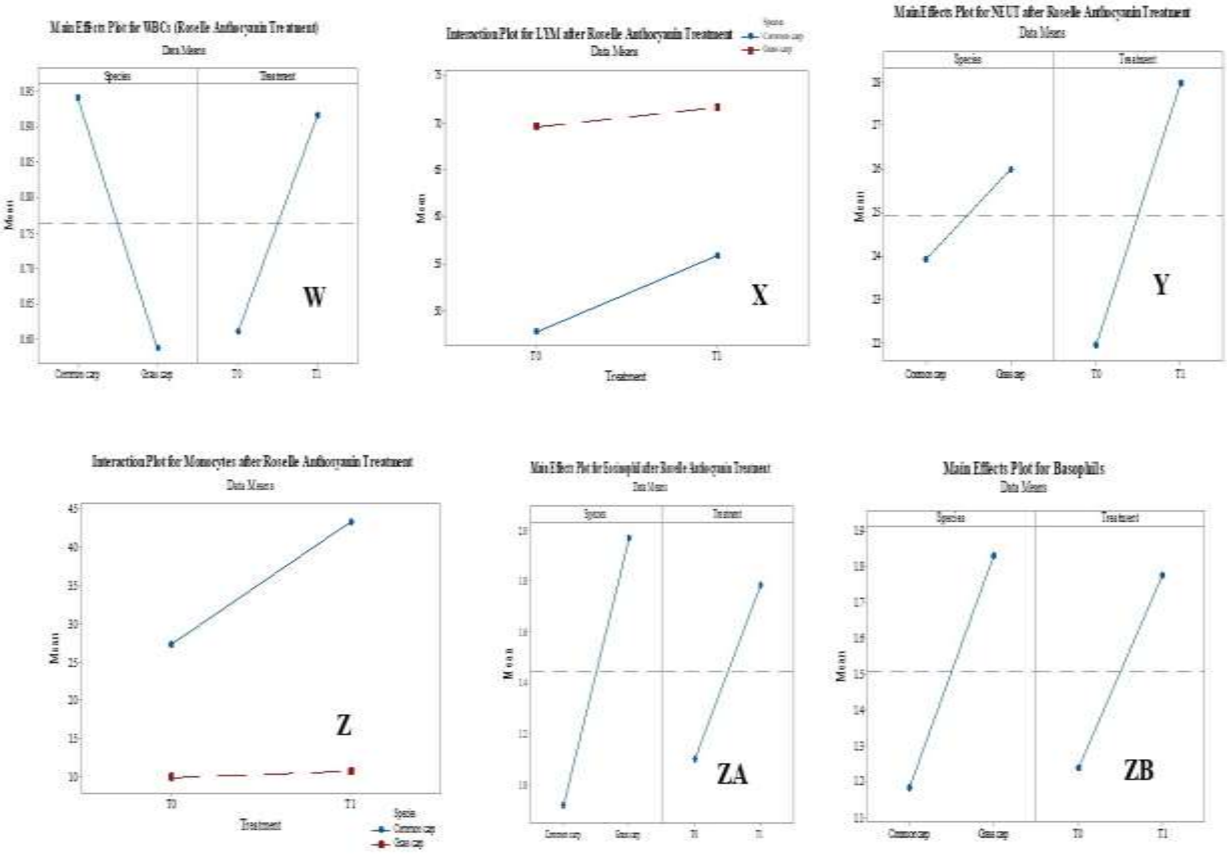
Immunological Parameters

White blood cells also known as Leukocytes are essential components of the immune system that protect the body against infectious diseases and foreign substances. These specialized cells rapidly recognize and neutralize foreign substances, hence maintaining overall health and well-being. The production of a targeted defense mechanism for long-term protection against specific diseases is maintained by the immune system such as agranulocytes (lymphocytes and monocytes) and granulocytes (neutrophils, eosinophils, and basophils).

Table 4: Analysis of main and interaction (S×T) effect between species (S) and treatment (T) and between treatment group by using Two-way factorial ANOVA

SOV	LYM%		NEUT %		Monocytes %		Eosinophils %		Basophil %	
	F-Value	*p*-Value	F-Value	*p*-Value	F-Value	*p*-Value	F-Value	*p*-Value	F-Value	*p*-Value
S	176.07	<0.001	2.03	0.161^{ns}	88.42	<0.001	68.77	<0.001	34.45	<0.001
T	12.80	0.001	17.18	<0.001	9.89	0.003^{*}	28.86	<0.001	23.89	<0.001
S×T	4.62	0.037^{*}	0.32	0.573^{ns}	8.26	0.006^{*}	0.38	0.538^{ns}	2.89	0.096^{ns}
Error	48									
Total	51									

All main and interaction effects suggested non-significant results (*p*>0.05) in WBCs, neutrophils eosinophils, and basophils which means that *H. sabdariffa* anthocyanin same effect on Common carp and Grass carp but as compared to control group, experimental group had significantly (*p*<0.05) results. However, agranulocytes (lymphocytes and monocytes) and platelets had main and interaction effects that suggested significant results shown in ANOVA (Table 1, 2). Due to their strong antioxidant properties, anthocyanin prevents thrombosis by stabilizing platelet activity. All these significant and non-significant effect showed graphically in Figure (2,6).



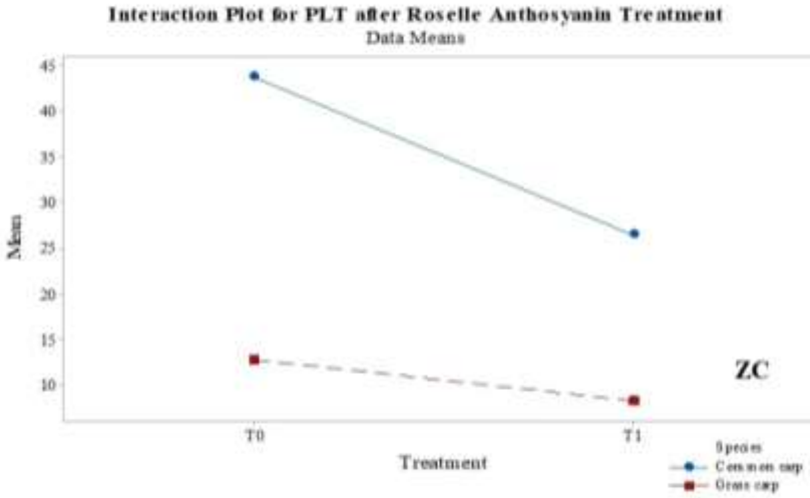


Figure 5: (W); Main effect plot of WBCs, (X); NEUT, (Y); Eosinophils, (Z); Basophils and interaction plot of (ZA); LYM, (ZB and ZC); PLT of Common and Grass carp of T_0 and T_1

Discussion

We conducted the first study investigating the effect of Roselle anthocyanin extract (RE) on Common carp and Grass carp. However, Jomeh et al. (2021) investigated the hematological and immunological parameters of Rainbow Trout (*Oncorhynchus mykiss*) after being fed with varied concentrations of RE. According to their revealed results, while not always, RE level showed non-significant results on WBCs and leukocytes (basophils, neutrophils, and eosinophils) count on different concentrations of diet, however, it improved the immunity level of Rainbow Trout. The results of Abubakar et al. (2023) study on the African Sharptooth Catfish (*Clarias gariepinus*) supported our findings, with significant variations in leukocyte counts and WBCs when compared to the control group. In our study, we observed that the count of WBCs and leukocytes were non-significant ($p>0.05$) between Common carp and Grass carp, which means that all main and interaction effects suggested non-significant results but significant compared with the control group ($p<0.05$). Common carp and Grass carp had the same response to the anthocyanin diet shown in (Figures: 6). Since RE increases the count of neutrophils and WBCs, fed after Roselle showed higher phagocytic activity and a higher WBC count as compared to the control diet, which became the reason for increasing the immunity of the fish.

White blood cells (WBCs) and agranulocytes (lymphocytes and monocytes) in tilapia and catfish are significantly affected by Anthocyanin feed intake, as described by Diab et al. (2023); Abubakar et al. (2023) and Khan et al. (2023). In trout fish, the presence of Roselle Anthocyanin also affects several immunological and hematological parameters due to its antioxidant properties. Yousefi *et al*. (2021) observed that when trout fish were fed an Anthocyanins diet, their white blood cells (WBCs), leukocytes, and agranulocytes significantly increased which improved the fish's immune system. Comparably, our study revealed that agranulocytes significantly increased in specimens of both Grass carp and common carp. The ANOVA (Tables 4) suggested that all main and interaction effects had significant results ($p<0.05$). There was a significant difference in agranulocyte levels between the experimental and control groups, as well as between the Common and Grass carp in each group shown in (Figure 6). Due to having antioxidant properties in Anthocyanin, the consumption of Roselle which has a high Anthocyanin concentration helped protect against several kinds of diseases.

Previous studies have also investigated the effect of Roselle Anthocyanin on platelets of different animals and humans (Tian et al., 2021; Amer et al., 2022; Liang et al., 2023). These studies revealed that Anthocyanin prevented thrombosis by stabilizing platelet activity. We observed in our study that platelets significantly decreased as compared to the control group. The ANOVA (Table 1) suggested that all main and interaction effects had significant results ($p<0.05$). We detected that strong antioxidant properties are a well-known characteristic of Anthocyanins. They eliminated oxidative stress and scavenged free radicals, both of which had factors in platelet activation and aggregation. Therefore, Anthocyanins indirectly inhibit platelet activation and function by reducing oxidative stress.

Common carp is an opportunistic feeder and Grass carp are primarily herbivores, their microbiota depends upon the diet of fish. These mostly consist of aerobes like *Aeromonas*, *Enterobacteriaceae*, *Pseudomonas, Acinetobacter, Flavobacterium*, *Staphylococcus*, *Streptococcus, Bacillus, Vibrio* and anaerobic bacterial species such as *Bacteriodetes, Clostridium, Cetobacterium* play a role in fish gut health (Van-Kessel et al., 2011; Feher et al., (2021). Similarly, previous studies observed the most abundant bacteria present in the Common carp and Grass carp gut were such as *Proteobacteria*, *Fusobacteriota*, and *Firmicutes*, which making around 79.9% of the gut bacterial community in the natural environment which supported the present research (Ni et al., 2014; Yang et al., 2019; Nugrahi et al., 2021). In the current study, we observed that *Proteobacteria*, *Fusobacteriota*, and *Firmicutes*, make up 3 and 4th total of the gut bacteria in Common carp and Grass carp. Additionally, we observed *Actinobacteraeota*, *Gemmatimonadota*, *Bacteroidota*, *Chloroflexi*, *Verrucomicrobiota*, *Planctomycetota*, and *Tenericutes* bacterial colonies grew on Nutrient and Tryptone Soy Agar (TSA) in the control group of Common carp and Grass carp shown in (Table: 1). Some bacteria grew only on TSA such as some bacteria of Grass carp (*Acidobacteriota* and *Ruminococcus albus*), indicating distinct microbial compositions in the gut microbiota of these two species. With the support of gram staining and bacteria cultured on selective media Eosin Methylene Blue Agar (EMB), we observed the gram-negative bacteria and morphological study conducted on bacterial colonies of both species kept in control groups as shown in (Table 2,3). All gram-negative bacteria were pink-colored in (Figure: 5) and gram-positive were purple color.

The current study observed that supplementation of Roselle anthocyanin significantly decreased (p<0.05) the gram-negative gut microbes of Common carp and Grass carp but promoted the gram-positive bacteria which were less harmful than gram-negative bacteria. Anthocyanin has promoted the *Firmicutes* phyla increased i.e., *Enterococcus, Bacillus, Lactobacillus spp*. and *Clostridium* which play a critical role in immune balance. Because Grass carp are herbivorous, *Ruminococcus* and *Bacillus* bacteria were found in their gut and could ability cellulose breakdown. In Immunological parameters, *Bacillus* increased the count of WBCs significantly to improve the protein profile of blood. The research work of Ni et al. (2014) and Igwe et al. (2019) supported our result, according to their findings Anthocyanin promoted gram-positive *Firmicutes* phyla (*Bacillus*) and *Ruminococcus* in the gut of Common carp and Grass carp which helped to enhance the immunity and breakdown of cellulose. Van-Kessel et al. (2011) and Ruzauskas et al. (2021) investigated the gut of Common carp that *Clostridium* played a vital role in vitamins and fatty acids. Moustafa et al. (2021) investigated the challenge of *Streptococcus iniae* infection on Nile Tilapia. Infected tilapia treated with *Bacillus* probiotics which increased the count of WBCs. The higher WBC count in tilapia treated with *Bacillus* significantly improved immune responses (such as phagocytosis) against the infection. The increased systemic immune response in the presence of infection is shown by the elevated blood protein profile, which includes total protein, albumin, and globulin. This also shows an increase in humoral immunity.

Recent studies investigated the effect of Roselle anthocyanin on *E. coli* and *Salmonella spp*. bacteria. Their findings revealed that Roselle Anthocyanin inhibited the growth of harmful *E. coli*, *Salmonella spp.* and *Pseudomonas spp*. Bacteria (Carvalho et al., 2023; Wang et al., 2022). *Pseudomonas flourescens* infection in Common carp can cause the loss of economic value of Common carp. Roselle anthocyanin supplementation significantly inhibited the *P. flourescens* and enhanced the immunity of Common carp (Prajitno 2020). These findings supported our studies that the *Proteobacteria* phyla had some species like *Shewanella spp*. and *Pseudomonas spp*. decreased and some bacterial growth was inhibited in Common carp and Grass carp due to anthocyanins, like *E. coli*, some *Bacillus spp*., *Chloroflexota* (*Chloroflexi*), and *Roseomonas aquatic* microbes. A prior study investigated that *Planctomycetota* were present in Common carp to help in the nitrogen cycle (Van-Kessel et al. 2011). Our findings in current research work, the effect of Anthocyanins on *Planctomycetota* in Common carp and *Acidobacteria* in Grass carp was very low but significantly decreased compared to the control group. Ni et al. (2014) observed *Acidobacteria* only in Grass carp which was evidence of our fine work.

A recent study revealed that the phylum *Bacteroidetes* is well known for its important role in the digestion of plant-based foods in fish gastrointestinal tracts and *Akkermansia ssp.* were immunomodulatory bacteria that stimulate activity. Anthocyanin enhanced the growth of *Bacteroidetes ssp.* and *Akkermansia ssp.* (Wang et al., 2022; Liang et al., 2023). Similarly, in the study, the *Bacteroidetes ssp.* and *Akkermansia ssp.* were gram-negative bacteria but Anthocyanin enhanced the growth in Common carp and grass crap. Previous studies investigated the effect of blueberries, blackberries red grapes and maqui berry Anthocyanin on fish gut microbiota. According to their findings, Anthocyanin improved the *Actinobacteria* population's beneficial for fish health (Overall et al., 2017; Liang et al., 2023). These findings were similar to our research work. Roselle Anthocyanin also significantly promoted the *Actinobacteria* population (Figure 4).

Conclusion

The effect of dietary Roselle anthocyanin significantly increased WBCs, granulocytes, and agranulocytes, ultimately enhancing the immunity of *C. carpio* and *C. idella*. Additionally, it prevented thrombosis by stabilizing platelet activity due to its potent antioxidant properties. Roselle's enhanced the intestine morphology of Common carp and Grass carp but also stimulated immunity by increasing beneficial microbes and inhibiting harmful bacteria. Roselle anthocyanin acts as an immunomodulator that protects against various diseases. From a future perspective, roselle anthocyanin is a viable alternative to antibiotics in aquaculture, providing beneficial effects for immunomodulation and disease prevention in the aquaculture sector. Further studies can be performed to find its application across diverse aquatic species for sustainable health management.

References

1. Abubakar HS, Oshoke JO, Shehu SU. The effects of Roselle (*Hibiscus sabdariffa*) calyx as dietary additive on the growth performance, nutrient utilization, and haematological parameters of *Clarias gariepinus* fingerlings. UMYU Sci 2023. 2:68-75.
2. Adejonwo OA, Omitoyin BO, Ajani EK, et al. Growth, gut morphology and microflora of African catfish *Clarias gariepinus* fed mushroom (*Pleurotus pulmonarius*) stalk meal supplemented diets. Croat J Fish 2020. 78:79-90.
3. Amer SA, Al-Khalaifah HS, Gouda A, et al. Potential effects of anthocyanin-rich roselle (*Hibiscus sabdariffa* L.) extract on the growth, intestinal histomorphology, blood biochemical parameters, and the immune status of Broiler chickens. Antioxidants 2022. 11:544.
4. Butt RL, Volkoff H. Gut microbiota and energy homeostasis in fish. Front. Endocrinol 2019. 10:9.
5. Carvalho A, Domingues I, Carvalho C, et al. In vitro antiprotozoal activity of *Hibiscus sabdariffa* extract against a ciliate causing high mortalities in turbot aquaculture. Biology 2023. 12:912.
6. Diab AM, Eldeghaidy EE, Abo-Raya MH, et al. Assessment of growth-related parameters, immune-biochemical profile and expression of selected genes of Red Tilapia fed with Roselle calyces (*Hibiscus sabdariffa*) extract. Fishes 2023. 8:172.
7. El Mesallamy AM, Ahmad MH, Souleman AM, et al. Effects of Roselle calyx (*Hibiscus sabdariffa l.*)-supplemented diets on growth and disease (*Aeromonas hydrophila*) resistance in Nile Tilapia (*Oreochromis niloticus* L.). Egypt J Pharm 2016. 15:78.
8. Emeish WFA, Soliman SA, Ahmed AE, et al. Osmoregulatory genes expression analysis and chloride cells activity in common carp: insight from fish exposed to salinity. Pak Vet J 2023. 43(3): 515-522. http://dx.doi.org/10.29261/pakvetj/2023.069.
9. Feher M, Fauszt P, Tolnai E, et al. Effects of phytonutrient-supplemented diets on the intestinal microbiota of *Cyprinus carpio*. PloS One 2021. 16:0248537.

10. Rabbani G, Rasool F, Fatima M, Majeed MB. Morphometric and molecular characterization of *Channa marulius* from Riverine system of Punjab, Pakistan. Mol Biol Rep 2024. 51(1):1-9.
11. Igwe EO, Charlton KE, Probst YC, et al. A systematic literature review of the effect of anthocyanins on gut microbiota populations. J Hum Nutr Diet 2019. 32:53-62.
12. Izquierdo-Vega JA, Arteaga-Badillo DA, Sanchez-Gutierrez M, et al. Organic acids from roselle (*Hibiscus sabdariffa* L.) a brief review of its pharmacological effects. Biomedicines 2020. 8:100.
13. Jomeh R, Chitsaz H, Akrami R. Effect of Anthocyanin extract from roselle, *Hibiscus sabdariffa*, calyx on hematological, biochemical and immunological parameters of Rainbow Trout, *Oncorhynchus mykiss*. Aquac Res 2021. 52:3736-3744.
14. Kazmi SAH, Iqbal R, Al-Doaiss AA, et al. Azoxystrobin-induced oxidative stress in gills, hematological biomarkers and histopathological ailments in fresh water fish. Pak Vet J 2023. 43(2): 321-326. http://dx.doi.org/10.29261/pakvetj/2023.025.
15. Khan M, Mortuza A, Blumenthal E, Mustafa A. Role of Elderberry (*Sambucus nigra*) on the modulation of stress and immune response of Nile tilapia, *Oreochromis niloticus*. J Appl Aquac 2023. 35:765-787.
16. Liang A, Leonard W, Beasley JT, et al. Anthocyanins-gut microbiota-health axis: A review. Crit. Rev Food Sci Nutr 2023. 9:1-26.
17. Liang A, Leonard W, Beasley JT, et al. Anthocyanins-gut microbiota-health axis: A review. Crit. Rev. Food Sci Nutr 2023. 9:1-26.
18. Mahmood S, Rasool F, Hafeez-urRehman M, Anjum KM. Molecular characterization of *Aeromonas hydrophila* detected in *Channa marulius* and *Sperata sarwari* sampled from rivers of Punjab in Pakistan. PLoS ONE 2024. 19(3): e0297979. https://doi.org/10.1371/journal.pone.0297979.
19. Mansoor A, Mehmood M, Hassan SMU, et al. Anti-bacterial effect of titanium-oxide nanoparticles and their application as alternative to antibiotics. Pak Vet J 2023. 43(2): 269-275. http://dx.doi.org/10.29261/pakvetj/2023.039.
20. Manzoor K, Rasool F, Khan N, Anjum KM, Parveen S. Prevalence and molecular detection of *Edwardsiella tarda* in cultured tilapia species of fish farms of Punjab in Pakistan and their postmortem examination. Pak Vet J 2023a. 43(2): 309-314. http://dx.doi.org/10.29261/pakvetj/2023.015.
21. Manzoor K, Rasool F, Khan N, Anjum KM, Parveen S. Resistance patterns of frequently applied antimicrobials and occurrence of antibiotic resistance genes in *Edwardsiella tarda* detected in edwardsiellosis-infected Tilapia species of fish farms of Punjab in Pakistan. J Microbiol Biotech 2023b. 33(5):668.
22. Moustafa EM, Farrag FA, Dawood MA, et al. Efficacy of *bacillus* probiotic mixture on the immunological responses and histopathological changes of Nile Tilapia (*Oreochromis niloticus*, L) challenged with *Streptococcus iniae*. Aquac Res 2021. 52:2205-2219.
23. Ni J, Yan Q, Yu Y, Zhang T. Factors influencing the Grass carp gut microbiome and its effect on metabolism. FEMS Microbiol Ecol 2014. 87:704-714.
24. Nugrahi TNU, Mulyani Y, Agung MUK. Community structure of Common carp (*Cyprinus carpio* Linnaeus, 1758) gut bacteria in the Cirata Reservoir, West Java Province, Indonesia. World Sci News 2021.161:130-142.
25. Ogunshe AA, Olabode OP. Antimicrobial potentials of indigenous lactobacillus strains on gram-negative indicator bacterial species from *Clarias gariepinus* (Burchell.) microbial inhibition of fish-borne pathogens. Afr J Microbiol Res 2009. 3:870-876.
26. Oluyemi KG, Adeparusi E, Olanrewaju J. Basic haematological parameters in African catfish, *Clarias gariepinus* (Burchell, 1822) fed ascorbic acid supplemented diets. Res J Anim Sci. 2008. 2:17-21.
27. Overall J, Bonney SA, Wilson M, et al. Metabolic effects of berries with structurally diverse anthocyanins. Int J Mol Sci 2017. 18:422.

28. Prajitno A. Change of hematology and histopathology of Common carp (*Cyprinus carpio* L.) infected by pseudomonas flourescens by natural bioactive treatment of rosella petals (*Hibiscus sabdariffa* L.). Eco Env Cons 2020. 26:1500-1508.
29. Prakoso YA, Babazadeh D, Wijayanti AD. Potency of desert rose (*Adenium obesum* (Forssk.) Roem. & Schult.) flower extract against artificially induced furunculosis in oranda goldfish (*Carassius auratus auratus*). Pak Vet J 2023. 43(2): 339-344. http://dx.doi.org/10.29261/pakvetj/2023.024.
30. Rasul M, Majumdar B. Abuse of antibiotics in aquaculture and it's effects on human, aquatic animal and environment. Saudi J Biol Sci 2017. 2:81-88.
31. Ruzauskas M, Armalyte J, Lastauskiene E, et al. Microbial and antimicrobial resistance profiles of microbiota in Common carps (*Cyprinus carpio*) from aquacultured and wild fish populations. Anim 2021. 11:929.
32. Saad N, El-Abasy MA, El-Khayat F, et al. Efficacy of chitosan nanoparticles as a natural antibacterial agent against pathogenic bacteria causing omphalitis in poultry. Pak Vet J 2023. 43(3): 573-578. http://dx.doi.org/10.29261/pakvetj/2023.065.
33. Sanders, E.R. 2012. Aseptic laboratory techniques: Plating methods. J Vis Exp 63: 3064.
34. Shah SBH, Mu Y, Abbas G, et al. An economic analysis of the fisheries sector of Pakistan (1950-2017): Challenges, opportunities and development strategies. Int. J Fish Aquat Stud 2018. 6:515-524.
35. Tian Z, Li K, Fan D, et al. Dose-dependent effects of anthocyanin supplementation on platelet function in subjects with dyslipidemia: A randomized clinical trial. EBioMedicine 2021. 70. 103533.
36. Ullah M, Rasool F, Khan N, Ali S, Sheikh AA. Antibiotic resistance and its gene profile in Escherichia coli isolated from diseased farm-raised carps in Punjab, Pakistan. Pak Vet J 2023a. 43(3): 470-476. http://dx.doi.org/10.29261/pakvetj/2023.041.
37. Ullah M, Rasool F, Khan N, Ali S, Sheikh AA. Molecular characterization and antimicrobial resistance among diarrheagenic and non-diarrheagenic *Escherichia coli* from healthy and diseased freshwater farmed fish. Pak J Agri Sci 2023b Jul 1;60(4).
38. van Kessel MA, Dutilh BE, Neveling K, et al. Pyrosequencing of 16s rRNA gene amplicons to study the microbiota in the gastrointestinal tract of carp (*Cyprinus carpio* L.). Appl Microbiol Biotechnol Exp 2011. 1:1-9.
39. Wang M, Zhang Z, Sun H, et al. Research progress of Anthocyanin prebiotic activity: A review. Phytomedicine 2022. 102:154145.
40. Yang G, Jian SQ, Cao H, et al. Changes in microbiota along the intestine of Grass carp (*Ctenopharyngodon idella*): Community, interspecific interactions, and functions. Aquac 2019. 498:151-161.
41. Yang S, Du J, Luo J, Zhou Y, et al. Effects of different diets on the intestinal microbiota and immunity of Common Carp (*Cyprinus carpio*). J Appl Microbiol 2019. 127:1327-1338.
42. Yousefi M, Vatnikov YA, Kulikov EV, Ahmadifar E, et al. Effects of dietary *Hibiscus sabdariffa* supplementation on biochemical responses and inflammatory-related genes expression of Rainbow Trout, *Oncorhynchus mykiss*, to ammonia toxicity. Aquac 2021. 533:736095.