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The impact of diet on water quality, histological changes and gut microbiota in ornamental fish *Pethia conchonius* (Rosy barb)

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ABSTRACT

The purpose of this study was to assess how a feeding plan affected the histological changes in the liver, gut, and gill tissues of *Pethia conchonius* (rosy barb). Fish were divided into five experimental groups. The control group was provided a regular food, while the D1, D2, D3, D4, and D5 groups were fed diets with varying concentrations of spirulina and beetroot, respectively. The water quality parameters like temperature, pH, TDS, total hardness and nitrate; were determined during the study period. From the water quality study, its concluded that all the parameters were within the optimum limits. Six fish per treatment were randomly selected, given anesthesia, and put down after ninety days. The tissues were then cut into histological sections. According to the histology investigations, the D5 group fish showed no symptoms of liver, gut, or gill damage, suggesting that the diets examined were effective in providing the fish with energy. The fish in the D2 group had a more diverse gut microbiota. Fish in the D4 and D5 groups possessed bacillus species, which might enhance their digestive systems, decrease waste, and aid in the better digestion and utilization of their nutrition. However, the results showed that the tested feeds were effective in providing the fish with energy.

Keywords: *bacillus*, beetroot, microflora, *Pethia conchonius*, spirulina

Introduction

The ornamental fish industry is now regarded as a sizable global market. In addition to the direct losses brought on by death, parasites may significantly affect ornamental fish development, behavior, resilience to stressors, and predation susceptibility. Additionally, parasites on the bodies of ornamental fish may make them less marketable (Brassard et al., 1982).

According to Mayer and Hendricks (1985), histological investigations are crucial for assessing the alterations in aquatic animals exposed to different risks in an aquatic environment. Probiotics have been widely used in aquaculture (Uma et al., 1999; Irianto and Austin 2003) and freshwater ornamental fish culture (Abraham, 2008). Ornamental fish have also been observed to benefit from the use of a feed probiotic for better health and reproductive outcomes (Ghosh et al., 2007). The histological changes of the gills, muscles, liver, and kidney in rohu fish administered probiotics were documented by Gobinath and Ramanibai (2014). Histopathology is a biomarker used to evaluate the effects of both internal (feed used) and external (aquatic) environmental conditions, along with other techniques like biochemical, growth, and disease diagnostic (Rajeshkumar and Munuswamy, 2011).

Many people refer to the gastrointestinal tract as a holobiont because it is one of the most densely inhabited ecosystems on Earth and the microbial communities that live there have formed close bonds with their hosts during millions of years of co-evolution (Brugman et al., 2018). Thus, through a variety of processes, including feed digestion, nutrient metabolism, energy homeostasis, immune system modulation, barrier function, and mucosal integrity, among others, the intestinal microbiota significantly affects the host's health. Furthermore, illnesses and imbalances in the digestive and systemic systems might result from a disruption in the microbial composition or in the host-microbe interactions (dysbiosis) (Sommer et al., 2017; Mills et al., 2019). These interactions between the microbiota and the host are mutual. The host influences the microbial composition, controlling the abundance of certain bacterial communities thought to be potential pathogens. For example, the bacterial community can activate the immune system through its pathogen-associated molecular patterns (PAMPs), which are identified by pattern recognition receptors (PRRs) and subsequently activate immune signaling pathways (Brugman et al., 2014). Fundamentally, it is believed that an animal's microbiota is adapted to its host species, but this is not a proven fact. Recent research distinguishes between the non-core microbiota, which is temporary and modifiable, and the core microbiota, which is made up of stable, permanent, and often extremely numerous individuals that endure throughout time and in spite of changing circumstances (Astudillo-Garcia et al., 2017). Therefore, knowing the complex interactions between the gut microbiota and the host and how to modify them may offer chances to maintain and encourage a healthy microbiota and assess its positive effects on the host.

In recent year, fish nutrition has improved with the creation of inexpensive and widely accessible balanced meals that support optimal growth and health of fish (Ahmed and Ahmed, 2020). In the present study, we attempted to determine how diet-associated microbial communities and histological change with the development of Rosy barb to reveal the possibility of using a diet for Rosy barb culture throughout their entire life.

MATERIALS AND METHODS

Animals and experimental condition

Pethia conchonius (Rosy barb) was purchased from Rose Aquarium, Arumanai, Kanyakumari, Tamil Nadu, India and kept for 15 days for acclimation in the laboratory. The final experiment lasted 90 days in an aquarium (50 ml size). Each treatment included 30 fish, and Rosy Barb was split into five treatments and one control (three replicates per treatment).

Experimental diets and feeding protocol

For a comparison analysis, six distinct diets with varying amounts of spirulina and beetroot powder were designed. The spirulina and beetroot powder were incorporated into the diet (Fish meal, rice bran, ground nut oil cake, soya bean meal, tapioca flour, vitamin and minerals) as: 0% (control), 0 and 50% (D1), 50 and 0% (D2), 25 and 25% (D3), 37.5 and 12.5% (D4) and 12.5 and 37.5% (D5). A diet with and without spirulina powder and beet root powder are used as a treatment group and control group, respectively.

Water quality parameters

Water quality parameters such as pH. Temperature, TDS, Total Hardness and Nitrate were analysed by standard methods.

Dissection of organs from fish

For appropriate tissue preparation and slide production, the fish were dissected, and the gills, gut, and liver were carefully removed from the body to prevent damage. The fish were then placed in labelled eppendorf bottles with Bouin's solution.

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Histopathological studies (Histopathology Procedures, 2011)

- Fixation
- Trimming
- Pre- Embedding
- Embedding
- Sectioning
- Staining and mounting

Isolation of gut microflora

Sample preparation

The fresh fish was thoroughly washed with sterile distilled water and scrubbed with 1% iodine solution after sacrifice (Trust and Sparrow, 1974). After dissection on ice the whole alimentary tract was removed and cleaned with chilled sterile physiological saline (0.9% NaCl in PBS buffer, pH 7.2). Subsequently, the pieces of digestive tract were homogenized with sterilized normal saline solution (NSS). The homogenate was used as inoculum for microbial culture (Das and Tripathi, 1991). Likewise, to study the microbial population the digestive tract/ gut was removed and homogenized. The pure colonies were screened and shortlisted based on the physiological and biochemical tests (Muthukumar and Kandeepan 2015; Saha *et al.*, 2006).

Isolation of Bacteria

One ml of aliquot of the gut homogenate was aseptically spread with 9 ml sterile double strength PBS and diluted up to 10^{-9}. The suspension was grown on Nutrient agar medium. The plates were then incubated at 37°C for 24 to 48 hours for the bacterial growth. All the procedures were carried out in sterile conditions. Colonies developed on the plate were counted and expressed as cfu/g. The isolates were purified and stored on the agar slants (Ritesh and Prasad, 2014).

Bacterial identification

Biochemical analysis of bacteria

The morphological characteristics of the isolates were identified by gram stain and biochemical reactions. The biochemical reactions include glucose fermentation, Indole, Methyl Red (MR), Voges-Proskauer (VP), Nitrate, Citrate, Urease, Triple sugar Iron (TSI), Catalase, Glucose, Fructose, Maltose, Gelatin and Starch were performed. Bacteria were isolated, identified and named based on morphological, physiological and biochemical

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characteristics presented in Bergey's Manual of Determinative Bacteriology and the APi Kit profiles (Holt *et al.*, 1994).

RESULTS

Water quality

Water quality plays an essential role in growth and survival of aquatic organisms. For improved growth and survival, the water's physico-chemical parameters should be within optimum limits. The physico-chemical parameter such as pH, temperature, total dissolved solids (TDS), hardness, and nitrate in the water recorded in both control and experimental tanks during the experimental period and are presented in the Table 1.

In the present study the pH of water in the control tank varied from 6.5 to 7.0. Similarly, there was 7.2, 7.0, 7.0, 6.5, and 6.5 in D1, D2, D3, D4, and D5 of treated tank, respectively. In the present experiment, the pH of experimental tanks was maintained at a desired level. During current study, it observed that, the water temperature was minimum in The temperature was recorded in the range of 17-21°C, 19-22°C, 17-20°C, 18-21°C, and 18-20°C in D1, D2, D3, D4, and D5 groups respectively. In the present investigation, the total dissolved solid (TDS) range from 24.28 mg/L in control group to 26.70 mg/L in the D1 fish tank. TDS values for D2, D3, D4, and D5 were 22.90 mg/L, 24.32 mg/L, and 24.83 mg/L, respectively. Water hardness is important factor in fish culture and a commonly reported water quality parameter. Water hardness measures the concentration of calcium and magnesium. Hardness values from this study not varied from tank to tank, it ranged from 18-20 mg/L. The nitrite content of water in the control tank was observed as 5 mg/l. at the same time, it increasing level on nitrite level of 6 mg/l, 7 mg/l, 6 mg/l, 9 mg/l, and 9 mg/l in D1, D2, D3, D4, and D5 groups respectively at the end of the experiment.

Table 1. Physico-chemical parameters of the fish tank water samples during the experimental period

Parameters	Control	D1	D2	D3	D4	D5
pH	6.5-7.0	7.2	7.0	7.0	6.5	6.5
Temperature (°C)	18-22	17-21	19-22	17-20	18-21	18-20
TDS (mg/l)	24.28	26.20	26.70	22.90	24. 32	24.83

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Total Hardness (mg/l)	18	18	20	19	19	20
Nitrate (mg/l)	5	6	7	6	9	9

Histopathological studies

In the present study histopathological changes were observed in gill, gut and liver tissue of fishes orally fed with natural food with the incorporation of different concentrations of spirulina and beetroot powder (0 and 50% (D1), 50 and 0% (D2), 25 and 25% (D3), 37.5 and 12.5% (D4) and 12.5 and 37.5% (D5)) and also the result was compared with the control group of fish with normal diet after 90 days of feeding experiment.

Gills histopathology

Histological sections of control group gills exhibited no morphological changes throughout the experimental period. Fish fed with D5 formulated feed revealed normal gill filament and lamellar structure. The lamellae form a consistent pattern along both sides of the filament. The pillar cells, which are contractile and divide adjacent lamellar capillaries, support the lamellae, which are covered by a one-cell thick lamellar epithelium.

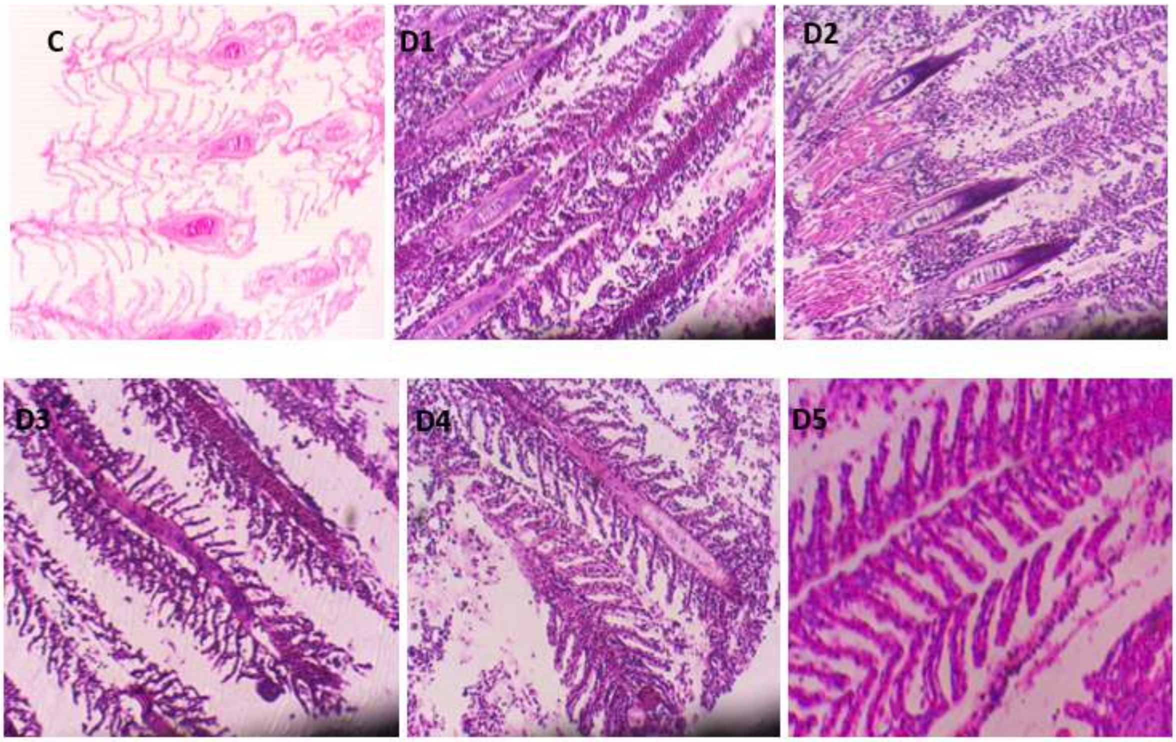

Fig 1. The histopathological examinations of gill tissue of *Pethia conchoniuss* fish fed different dietary treatments (D1, D2, D3, D4 and D5) in comparison with control group

Fish treated with D1, D2, D3, and D4 showed certain histological abnormalities in their gill tissue. Expanded cartilaginous tissue, epithelial hyperplasia, epithelial lifting in secondary lamellae linked to remarkable interstitial edema, lamellar fusion, increased space between filaments linked to secondary lamellae degeneration, decreased length of primary lamellae with secondary lamellae degeneration, and blood congestion were among the observed findings in the gills of D1, D2, and D3 experimental treatments (Fig. 1). The gill tissue of fish treated with D4 showed gill filament and lamellae morphology, but there was also secondary lamellae degeneration.

Gut histopathology

The control group shows normal gut histology with intact villi and crypts. There are no indications of inflammation or structural damage, and the mucosal layer seems to be in good health. There is no evidence of cellular infiltration or edema, and the epithelial cells are well-organized. The histological changes of the D5-fed animals' guts were not substantially impacted by the feed used in this study; their intestinal architecture, which included serosa, villi, circular muscles, and longitudinal muscles, was normal.

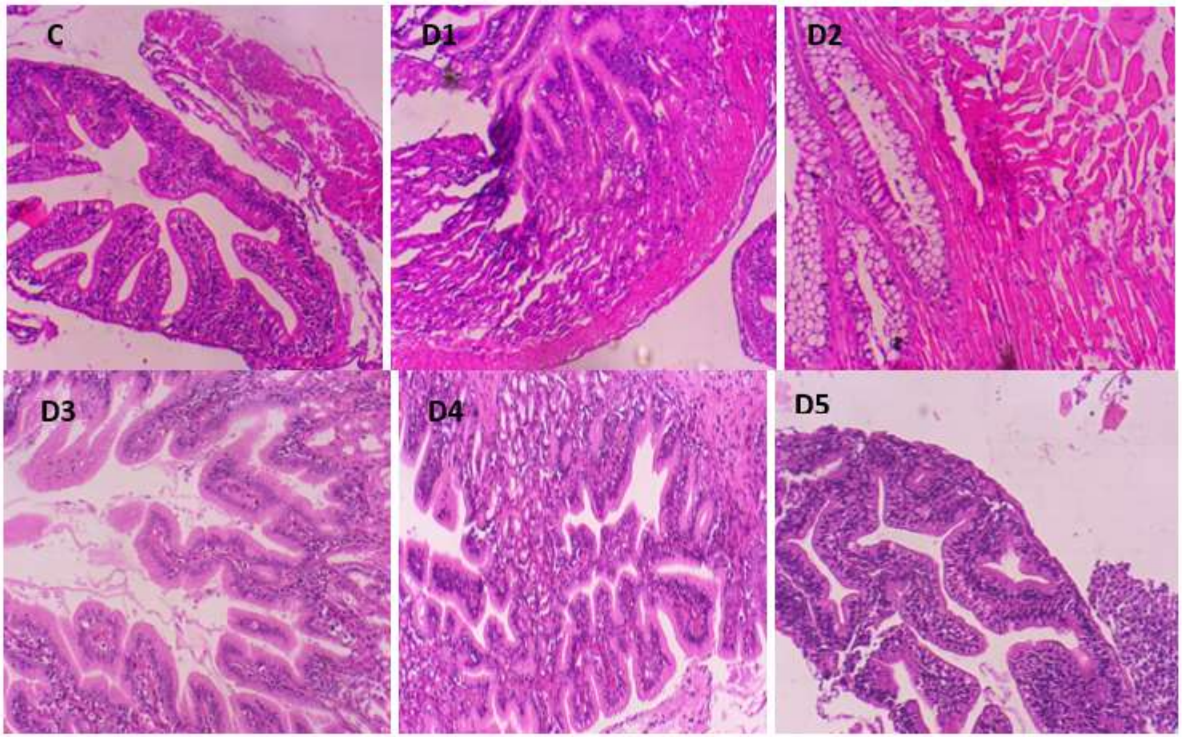

Fig 2. The histopathological examinations of gut tissue of *Pethia conchoniuss* fish fed different dietary treatments (D1, D2, D3, D4 and D5) in comparison with control group

The lumen of the villi of the gut given D1 is expanded. Larger circular muscles, longitudinal muscles, and serosa are common. The lumen of the villi of the gut given D2 is

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expanded. There are more gaps between the longitudinal and circular muscles, and the serosa layer is thinner, suggesting some dissociation of the villi's base. The serosa, longitudinal muscles, circular muscles, and villi in the fed D3's stomach appear to be normal. The fed intestine D4 exhibits the serosa, villi, longitudinal muscles, and circular muscles in their normal state. Near the base of the villi, there are further gaps (Fig. 2).

Liver histopathology

The hepatocytes in the liver tissue of the control fish were normal. When analyzing the liver cross-section of rose barbs fed different feeds in their individual diets, hepatocytes (H) with unique nuclei containing nucleoli, Kupffer cells (KCs), and hepatic sinusoids (HS) were seen. It's also crucial to remember that neither treatment damaged the liver, even though fish fed the D2 and D3 diets displayed some vacuolate in the cytoplasm of their hepatocytes (Fig. 3).

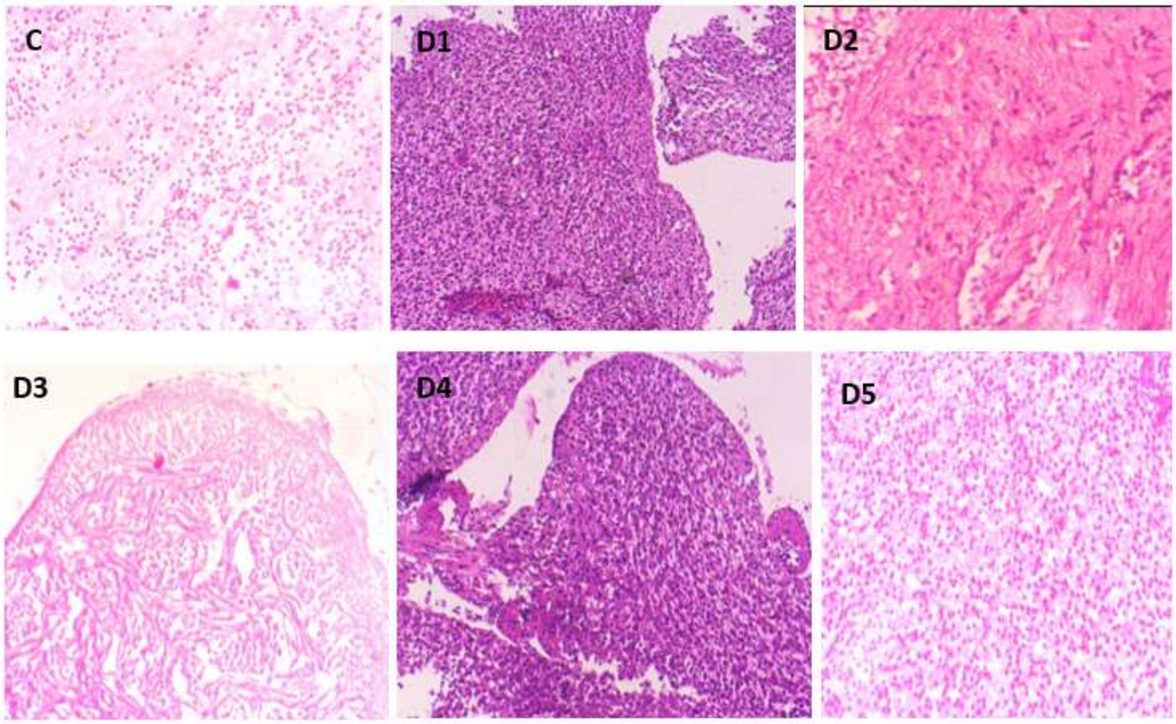

Fig 3. The histopathological investigation of different diet treated liver tissue of *Pethia conchoniuss*

Isolation of gut microflora

The bacterial cultures were isolated from the gut microflora of fishes. These bacteria were cultivated on nutrient media specified in Table 1. At a10^{-3} dilution, the control diet exhibited a CFU count of 31 $\times 10^4$ cfu/ml. Fish fed diets D1 and D3 showed similar CFU values of 28 $\times 10^4$ and 27 $\times 10^4$ cfu/ml respectively. Diets D2 and D3 resulted in lower CFU counts of 17 $\times 10^4$ and 16 $\times 10^4$ cfu/ml. Diet D4 contained an excessively high bacterial count

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at the 10^{-3} dilution TNTC (Too Numerous to Count). At a 10^{-5} dilution diet, D4 still exhibited the highest bacterial count at $102×10^{6}$ followed by the control diet at $18 ×10^{6}$, D1 at $21×10^{6}$, and D2 at $7×10^{6}$ cfu/ml. Diets D3 and D5 both showed a CFU count of $10×10^{6}$ cfu/ml. At a 10^{-7} dilution, the control diet and fish fed diets D1 and D4 showed similar CFU count of $15 ×10^{8}$, $17 ×10^{8}$ and $13 ×10^{8}$ cfu/ml, respectively. Diets D2 and D5 both showed a CFU count of $5×10^{8}$ cfu/ml. At 10^{-9} dilution, the control diet and fish fed diets D2, D3, D4 and D5 showed similar CFU count of $6 ×10^{10}$, $1 ×10^{10}$, $5 ×10^{10}$, $6 ×10^{10}$ and $3 ×10^{10}$ respectively. However, D2 exhibited the highest bacterial count at $16 ×10^{10}$ cfu/ml.

Table 1. Isolation of gut bacteria from fish fed different diets

Dilution	CFU Value (cfu/ml)					
	Control	D1	D2	D3	D4	D5
10^{-3}	$31 ×10^{4}$	$28 ×10^{4}$	$17 ×10^{4}$	$27 ×10^{4}$	TNTC	$16 ×10^{4}$
10^{-5}	$18 ×10^{6}$	$21×10^{6}$	$7×10^{6}$	$10×10^{6}$	$102×10^{6}$	$10×10^{6}$
10^{-7}	$15 ×10^{8}$	$17 ×10^{8}$	$5 ×10^{8}$	$7 ×10^{8}$	$13 ×10^{8}$	$5 ×10^{8}$
10^{-9}	$6 ×10^{10}$	$16 ×10^{10}$	$1 ×10^{10}$	$5 ×10^{10}$	$6 ×10^{10}$	$3 ×10^{10}$

Biochemical characteristics of gut bacteria

The bacterial isolates were characterized by various biochemical tests (Table 2). The results of the morphological, physiological, and biochemical tests revealed that the control, D1, and D3 were determined to be *Salmonella* sp. Sample D2 contained four bacterial species: *Clostridium* sp., *Aeromonas* sp. (two isolates), and *Salmonella* sp. Sample D4 comprised two species: *Bacillus* sp and *Salmonella* sp. Sample D5 comprised *Bacillus* sp as per Bergey's manual of determinative bacteriology.

DISCUSSION

The most important resource for human use is water. Water's diverse qualities makes it the most intriguing topic for countless studies. It is believed that thorough analysis of the physical and chemical characteristics of natural fresh water is necessary to comprehend the significance and quality of water. Determining the form and content of a water body's biotic population is largely dependent on its physical and chemical characteristics.

All experimental groups (D1 to D5) in the current study had their temperatures kept within the narrow range of 18 to 22°C. Many cold-water fish species are thought to benefit from this range as it encourages consistent metabolic activity without causing thermal stress.

The temperature in each of the following groups varied slightly: D1 (17–21°C), D2 (19–22°C), D3 (17–20°C), D4 (18–21°C), and D5 (18–20°C). According to Brianna Anderson (2023), the tank temperatures for rosy barb between 18 and 22°C. Fish growth and water temperature are related, with the observation that growth rates usually rise with temperature up to an ideal point, after which they fall (Brett, 1979, Fielder *et al.*, 2005).

pH is one of the most crucial factors to take into account while controlling the water quality in fish farming. The pH range that is ideal for most fish species is between 6.5 and 9. If they proceed outside of this range, fish may become stressed and slow down their growth. The pH of the control tank fluctuated between 6.5 and 7.0. By contrast, the pH values of the treated tanks were 7.2 (D1), 7.0 (D2 and D3), and 6.5 (D4 and D5). Our investigation revealed pH levels within the 6–8 range that Brianna Anderson (2023) suggested for rosy barb fish rearing. The experimental tanks' pH values show that the treatments were successful in keeping the pH within the desired range. This is especially important for preserving the health of the fish and encouraging the best possible output in the tanks. While D4 and D5 had somewhat more acidic pH values, they were still within acceptable bounds. In contrast, D1, D2, and D3 had pH values that were closer to neutral (7.0), which is typically beneficial for the majority of aquatic creatures. The study's treatments seem to have been effective in creating a stable environment, which is crucial since pH variations can impair metabolic functions and raise ammonia toxicity (Boyd, 1979). It is evident from the data that there was no discernible pH change in the fish that were fed.

TDS and suspended solids are present in all types of water. When combined, these two create total solids in water. TDS, or total dissolved solids, is a crucial metric for evaluating the quality of water. They consist of trace quantities of biological materials dissolved in water and inorganic salts. They are responsible for the water's overall salinity. The slight variations in TDS values observed across all samples suggest that the treatment parameters or any other outside factors had a small impact on altering the water's dissolved solids content. A somewhat higher abundance of dissolved chemicals is suggested by a raised TDS level in samples D1 and D2, but a lower value in sample D3 would reflect a lower concentration of dissolved materials. TDS levels are also significantly influenced by fish metabolic waste products and residues from uneaten feed. The breakdown of these organic components releases soluble chemicals into the water, which raises the concentration of dissolved solids even further. The amount that these residues contribute to TDS levels might

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vary greatly depending on the kind of feed and the rate of feed degradation (Omar and Sara, 2022).

The total amount of divalent salts in the water is measured quantitatively by hardness. Water hardness is mostly caused by the minerals calcium and magnesium. The physiological processes of aquatic animals, including the growth of fish scales and bones, depend heavily on calcium and magnesium (Wurts, 2002). All experimental tanks in this study had their water hardness tested regularly, and the results ranged from 18 to 20 mg/L. This small range denotes stable water conditions, indicating that there was little volatility in the calcium and magnesium levels between tanks and that they were enough to sustain the fish's physiological demands. There are no known negative health effects of hardness. The water's measured hardness readings in this investigation fell well below the WHO's recommended allowable range.

Fish health is seriously endangered by nitrate, a prominent water quality metric in aquaculture, since it may oxidize hemoglobin to methemoglobin. The nitrate content in the control tank was found to be 5 mg/L in the current investigation. Because it offers a point of comparison for evaluating the effects of nitrate levels in the experimental groups, this baseline value is significant. Nitrate levels rose in the experimental groups at the end of the trial; values in groups D1, D2, D3, D4, and D5 were 6 mg/L, 7 mg/L, 6 mg/L, 9 mg/L, and 9 mg/L, respectively. According to Meck (1996), nitrate concentrations in fish ponds ranging from 0 to 200 ppm are tolerable and typically low hazardous for some species, however marine species are particularly sensitive to its presence. According to a thorough investigation by Stone and Thomforde (2004), nitrate is generally harmless to fish and causes no health risks unless it is present in extremely high concentrations (over 90 mg/ml). According to Santhosh and Singh (2007), fish culture water should have a nitrate content between 0.1 and 4.0 mg/ml. According to the water quality investigation, providing fish food has no effect on the water's quality.

Fish eating habits are a direct reflection of their capacity to assimilate various food components. The anatomy of the digestive tract, the digestive enzymes released, and the composition, quantity, and variety of the intestinal microbiota all influence the fish's capacity to digest and use the various nutrients in their diet. In order to demonstrate the potential of adopting a diet for Rosy barb culture throughout their whole life, the study investigates the diet-associated microbial communities and histological changes with Rosy barb development.

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The discovered histopathological alterations suggest that fish health can be considerably impacted by different dietary amounts of beetroot powder and spirulina. These results demonstrate how dietary factors may affect fish health and stress how crucial it is to properly balance dietary supplements to prevent negative side effects.

According to Fernandes and Mazon (2003), the gills are thought to be the main target of the contaminants because they are involved in many vital processes in fish, including respiration, osmoregulation, and excretion, stay in close contact with the outside world, and are especially sensitive to changes in the water's quality. The fish in the D5 and control groups had typical lamellar and gill filament structure. Fish treated with D4 exhibited gill filament and lamellae morphology in their gill tissue, however secondary lamellae degeneration was also seen. However, the gill tissue of fish treated with D1, D2, D3, and D4 had specific histological abnormalities. Expanded cartilaginous tissue, epithelial hyperplasia, epithelial lifting in secondary lamellae linked to remarkable interstitial edema, lamellar fusion, increased space between filaments linked to secondary lamellae degeneration, decreased length of primary lamellae with secondary lamellae degeneration, and blood congestion were among the observed findings in the gills of D1, D2, and D3 experimental treatments. Long-term gill damage in fish can disrupt gas exchange, ammonia excretion, and osmotic balance (Mehrim et al., 2006). According to Roberts (2001), hyperplasia is a fish's defense mechanism against internal or external stimuli that irritate its tissue. It may have been the gills' first reaction to something toxic in the water or diet. The findings of Agbebi *et al.* (2013), who also noted hyperplasia in the gills of *Clarias gariepinus* fed various nutritional regimens, are consistent with this. Hyperplasia-induced secondary lamellar fusion may reduce free gas exchange, which would impact the fish's overall health (Skidmore and Tovel, 1972). In response to stresses, the fish in this research exhibited defensive behaviors such as lamellar fusion, oedema, epithelial lifting, and curling of the secondary epithelium (Parrmar and Shah, 2020).

Fish in the control group had normal gut histology, with intact crypts and villi. There are no indications of inflammation or structural damage, and the mucosal layer seems to be in good health. The histological changes of the D5-fed animals' guts were not substantially impacted by the feed used in this study; their intestinal architecture was normal, with serosa, villi, circular muscles, and longitudinal muscles that enabled them to mechanically break down the meal. The quality of the foods examined during raising is indicated by the growth of these intestinal villi (Goda *et al.,* 2020). The growth of the villi cells was nearly identical to that of the intestinal villi in fish fed artificial feed. While enterocytes are absorptive cells that serve as the primary structural element of the villous epithelium and control the flow of nutrient molecules in aquatic animals, intestinal villi enhance the absorptive surface area (Tabassum *et al.,* 2021). Goblet cells are intestinal epithelial cells that cover the digestive tract's surface with mucin, intestinal trefoil factor, and compounds that resemble resistin (Chen *et al.*, 2021). Mucus secretion will rise under specific circumstances, particularly those pertaining to the immunological response, which will help shield fish from illness (Tabassum *et al.,* 2021). In order to assess changes at the tissue level of fish during feed testing, histological examination of the intestinal tract is crucial (Yadav *et al.,* 2020). The findings imply that the fish's gut was not affected by the prepared food.

One of the body's essential organs, the liver is crucial to the metabolism of lipids, proteins, and carbohydrates. Histological analyses of the fishes' livers in the control and experimental groups showed no discernible pathological alterations. The primary determinant of larval development and liver metabolic function of a feed type is the accumulation of fat in the liver. A healthy liver with many sinusoids, central veins, and hepatocyte glycogen indicates that the diet is not causing stomach distress. Nevertheless, none of the fish showed any symptoms of liver damage, suggesting that the meals examined were effective in providing the fish with energy (Chen *et al.*, 2021).

Bacterial populations in the gut microflora of fish fed various diets, including control and experimental diets with different concentrations of spirulina and beetroot powder were assessed. The bacterial counts in the gut microflora varied significantly depending on the diet. The control diet showed a balanced bacterial population at most dilutions, indicating stable gut microbiota. In contrast, the experimental diets produced varied effects. Gut microbiota plays a crucial role in fish health and performance. Effective management of gut microbiota through dietary interventions, such as probiotics and prebiotics, can significantly benefit aquaculture practices (Romero *et al.*, 2014). The analysis of bacterial species present in the gut microflora of fish fed with different diets revealed a range of microbial communities, as identified through morphological, physiological, and biochemical tests. The results of the morphological, physiological, and biochemical tests revealed that the control, D1, and D3 were determined to be *Salmonella* sp. Sample D2 contained four bacterial species: *Clostridium* sp., *Aeromonas* sp. (two isolates), and *Salmonella* sp. Sample D4

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comprised two species: *Bacillus* sp and *Salmonella* sp. Sample D5 comprised *Bacillus* sp as per Bergey's manual of determinative bacteriology. Some beneficial bacteria were identified such as Bacillus and Clostridium. *Clostridium* species, as a predominant cluster of commensal bacteria in the gut, exert lots of salutary effects on our intestinal homeostasis. As a safer and more effective antibacterial product, the probiotic *Clostridium* has been increasingly used as additives in aquaculture to overcome the abuse of antibiotics and to promote fish health and their productivity (Cai *et al.*, 2025). Probiotics *Bacillus* is one of the many potential microbial possibilities that can synthesize compounds having anti-microbial properties, non-toxic as well as non-pathogenic to the fish and has the ability to sporulate (Ghosh, 2025).

CONCLUSION

Fish fed D4 and D5 formulated feed, which comprise diet with beetroot (12.5% and 37.5% respectively) and spirulina powder (37.5% and 12.5% respectively), showed no pathological changes in their gills, guts, or livers, according to the research. This suggests that rosy barb may be grown in an out-of-pond holding system using the researched foods without endangering the fish. According to gut microbiological research, the fish in the D4 and D5 groups contained beneficial microbes that might be added to aquaculture to combat antibiotic overuse and improve fish productivity and health.

Statements & Declarations

Compliance with Ethical Standard

This is an observational study. The Cochin University of Science and Technology (CUSAT) Research Ethics Committee has confirmed that no ethical approval is required.

Conflict of interest

The authors have declared no conflict of interest.

Consent to Publish

The author's obtained consent from all individual participants for whom identifying information is included in this article.

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Data availability statement

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The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Table 2

Biochemical characteristics of gut bacterial isolates from control and experimental fish

Biochemical test		Control Fish		D1 Fish		D2 Fish				D3 Fish	D4 Fish		D5 Fish
		Bac-1	Bac-2	Bac-1	Bac-2	Bac-1	Bac-2	Bac-3	Bac-4	Bac-1	Bac-1	Bac-2	Bac-1
Gram Staining		_	_	_	_	+	+	_	_	_	+	_	_
Motility		+	+	+	+	+	+	+	+	+	+	+	+
Indole		_	_	_	_	_	_	_	_	_	_	_	_
Methyl Red		+	+	+	+	+	+	+	+	+	+	+	+
VP		_	_	_	_	_	_	_	_	_	_	_	_
Nitrate		+	+	+	+	+	+	+	+	+	+	+	+
Citrate		_	_	_	_	_	_	_	_	_	+	_	_
Urease		_	_	_	_	_	_	_	_	_	_	_	_
Catalase		+	+	+	+	_	_	+	+	+	+	+	+
Oxidase		_	_	_	_	_	_	_	+	_	+	_	_
TSI		AK/A	A/-	_	AK/A	A/-	AK/A	A/A	AK/A	AK/A	AK/A	AK/A	AK/A
Starch hydrolysis		+	_	+	+	_	_	+	_	+	+	+	+
Gelatin hydrolysis		+	+	+	+	+	+	+	+	+	+	+	+
Carbohydrate Fermentation	Glucose	+	+	+	+	_	+	+	_	+	+	+	+
	Sucrose	_	+	+	_	_	+	+	_	+	+	+	+
	Maltose	+	+	+	+	_	_	+	_	+	+	+	_