Original Article

Immunological and Microbiological Responses of Rainbow Trout to Dietary Cottonseed Meal and Organic Acids

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ABSTRACT

Background: The use of plant-based feeds is increasing in the aquaculture industry. However, these diets have negative effects on fish, which must be addressed. Organic acids (OA) showed benefits in plant-based feed in aquaculture.

Objectives: This project aimed to investigate the effect of adding cottonseed (CS) meal and a mixture of OA to the diet on blood immunity indices, histopathology, and gut microbial population in rainbow trout.

Methods: To this end, 6 treatments consisting of 6 dietary formulations with 3 replications were designed in a 3×2 factorial arrangement. In this design, two levels of CS meal (0% and 15%) and three levels of the organic acid mixture (a mixture of lactic acid, citric acid, and potassium sorbate in equal proportions) at 0%, 0.5%, and 1% were added to the diet. A total of 270 rainbow trout with an Mean±SD weight of 0.14±0.35 g were stored in 18 aquariums containing 40 L of water, with a density of 15 fish per aquarium. The diets mentioned above were provided to the fish for 8 weeks at a daily rate of 3% to 4% of their biomass. After 8 weeks of rearing, blood and liver samples were collected from all treatments.

Results: CS meal and OA had no significant effect on white blood cells, total immunoglobulin levels, or intestinal histology. CS meal did not significantly affect lysozyme, complement, total protein, albumin, or plasma globulin. However, the OA led to a significant increase in these parameters. The addition of CS meal increased lactic acid bacteria (LAB) and *Vibrio* sp. in the fish's gut but did not affect the total number of gut bacteria. OA resulted in a decrease in total bacteria and *Vibrio* abundance while increasing LAB abundance in the gut.

Conclusion: In conclusion, adding 15% CS meal to the diet of rainbow trout does not impact blood immune responses or intestinal tissue structure. However, it increased both beneficial (lactic acid bacteria) and harmful (*Vibrio* sp.) gut populations. On the other hand, adding OA effectively addresses this issue by increasing LAB populations and decreasing *Vibrio* sp. population. Furthermore, regardless of the addition of CS meal to the diet, incorporating OA improves innate immune parameters in the blood.

Keywords: Cottonseed (CS), Blood, Immunity, Gut, Microbiota

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Introduction

Nutrition represents the largest component of production costs in fish farming, making the quality and availability of feed ingredients crucial for successful aquaculture. A primary ingredient in aquatic animal diets is soybean (SB) meal, known for its excellent digestibility and amino acid profile (Macusi et al., 2023). However, a significant portion of SB meal in Iran is imported (Tasnim, 2024), impacting its economic feasibility. Consequently, it is essential to explore domestic feed alternatives that can partially replace SB meal in aquatic diets.

One promising domestic ingredient is cottonseed (CS). In 2024, Iran's annual CS production was approximately 125000 tons. Depending on processing and oil extraction methods, CS meal can contain between 25% to 50% protein. This versatility has led to its successful use in the diets of various species, including Nile tilapia (*Oreochromis niloticus*) (Hassaan et al., 2019), channel catfish (*Ictalurus punctatus*) (Robinson & Li, 1994), common carp (*Cyprinus carpio*) (Wang et al., 2014), and red drum (*Sciaenops ocellatus*) (Wang et al., 2020). Research on rainbow trout (*Oncorhynchus mykiss*) indicated that while incorporating more than 10% CS meal could negatively impact the fish at early life stages (Cheng & Hardy, 2002), sub-adult and adult fish can tolerate up to 50% CS meal in their diets (Blom et al., 2001; Rinchard et al., 2003a; Rinchard et al., 2003b). However, studies have also shown that including CS meal may suppress immune function and induce dysbiosis in the fish gut (Liu et al., 2019; Chen et al., 2020; Shen et al., 2020; Wang et al., 2022). Therefore, it is vital to identify strategies to mitigate these drawbacks when using CS meal in fish diets.

The addition of organic acids (OA) to plant-based diets has been shown to enhance performance by balancing gut microbiota and improving fish immunity. For example, adding sodium acetate and sodium propionate to a plant-based diet significantly improved gut immunological parameters at transcription levels in yellowfin seabream (*Acanthopagrus latus*) (Sotoudeh et al., 2020). Dietary lactic acid supplementation significantly improved nutrient digestibility and gut lactic acid bacteria (LAB) density in beluga (*Huso huso*) fed plant-based diets (Matani Bour et al., 2018). An improvement in humoral and intestinal immunological parameters and gut microbial populations has been reported in rainbow trout fed a diet supplemented with lactic acid (Hoseini et al., 2022a) or sodium butyrate (Taheri Mirghaed et al., 2019).

According to what was discussed, incorporating dietary OA may help alleviate the adverse effects associated with CS meal inclusion in aquafeeds. To date, no research has explored this area; thus, this study aims to investigate the impact of adding OA to a diet containing 15% CS meal on humoral immunological parameters, gut microbial characteristics, and histopathology in rainbow trout fingerlings.

Materials and Methods

Diets

Lactic acid (85% purity, liquid form), anhydrous citric acid (99% purity), and potassium sorbate (99% purity) were sourced from the domestic market and imported from China. This study evaluated 6 experimental treatments, each consisting of distinct diets. Three diets were formulated without CS meal, comprising 25% SB meal and varying concentrations of OA—0% (SB), 0.5% (SB 0.5), and 1% (SB 1.0). The remaining three diets included 15% CS meal, with OA concentrations of 0% (CS), 0.5% (CS 0.5), and 1% (CS 1.0), following a 2×3 factorial design. The inclusion of CS meal was based on existing literature suggesting that a diet with up to 10% CS meal does not adversely affect the growth of rainbow trout. At the same time, higher levels may hinder growth and digestibility (Cheng & Hardy, 2002). Diet formulation was performed by WUFFDA (Microsoft Excel Workbook for least-cost feed formulation) software.

To prepare the diets, feed ingredients (Table 1) were accurately weighed and thoroughly mixed. Dietary oils were then incorporated into the mixture, followed by a 15-min mixing period. Subsequently, premixes and amino acids were measured, dissolved in 350 mL of water, and added to 1 kg of the initial mixture. At this point, the OA replaced a portion of the water. The resulting paste was then pelleted using a meat grinder fitted with a 3-mm die.

Chemical composition analysis of the diets was conducted according to AOAC (AOAC, 2005). Samples were dried at 105 ˚C for 24 h to assess moisture content. Protein content was determined using the Kjeldahl method, with nitrogen content calculated and multiplied by a factor of 6.25. Fat content was measured via ether extraction using a Soxhlet apparatus with petroleum ether as the solvent, while ash content was determined by incinerating samples at 550 ˚C for 8 hours.

Table 1. Feed ingredients and formulation of diets with and without CS meal

Feed ingredients	Without CS Meal	With CS Meal	Amino acids	Without CS Meal	With CS Meal
Fish meal[1]	16.4	17.3	Arg	2.8	2.9
Poultry slaughterhouse by-products[2]	25	25	Gly	2.8	2.8
SB meal[3]	25	12	Ser	2.1	2
Wheat meal	23.4	23.5	His	1	1
CS meal[4]	0	15	Ile	1.6	1.5
Canola oil	5.5	3.5	Leu	2.4	2.2
Sunflower oil	2.8	1.7	Lys	2.5	2.5
Vitamin premix	0.5	0.5	Met	1	1
Mineral premix	1	1	Cis	0.52	0.76
Methionine	0.25	0.25	Phe	1.7	1.7
Lysine	0.15	0.25	Tyr	1.1	0.94
Chemical composition			Thr	1.5	1.4
Crude protein	41.4	41.7	Trp	0.48	0.46
Crude fat	17	17.9	Val	1.9	1.9
Crude ash	7.7	7			
Crude fiber	3.2	4			

[1]Kilka fishmeal: Crude protein: 727 g/kg and crude fat: 96 g/kg; [2]Crude protein: 521 g/kg and crude fat: 229 g/kg; [3]Crude protein: 436 g/kg; [4]Crude protein: 323 g/kg, crude fat: 272 g/kg, crude ash: 35 g/kg, crude fiber: 134 g/kg.

Fish rearing and sampling procedures

Rainbow trout fingerlings were acquired from a private farm in Sari County, Iran, with an average weight of approximately 11 g. A total of 300 fish were transported to the Inland Waters Aquatic Resources Research Center in Gorgan, Iran. Initially, they were kept in a 500-L tank for 1 week and fed the SB diet to acclimatize to their new environment. Following this period, 270 healthy, similarly sized fish were distributed among 18 aquaria, with a water volume of 40 L per aquarium. Each aquarium housed 15 fingerlings weighing around 14 g, with 3 aquaria randomly assigned to each experimental treatment.

The fish were fed their respective diets over 8 weeks at a daily feeding rate of 3%-4%, divided into two meals. Throughout the study, water parameters—including temperature (12.3±0.88 °C), dissolved oxygen (7.33±0.65 mg/L), pH (7.89±0.28), un-ionized ammonia (0.023±0.001 mg/L), and nitrate (15.1±1.77 mg/L)—were monitored using Hach (HQ40D, Loveland, Colorado, USA) and Palintest (Model 7100, Palintest House, Kingsway, Team Valley, Gateshead, Tyne & Wear, NE11 0NS, United Kingdom) devices.

After the rearing period, three fish from each aquarium were anesthetized in a clove solution (3 g/L) (Hoseini et al., 2011). Blood samples were collected via heparinized syringes from the caudal vein; part of the sample was used immediately for leukocyte counting, while the remainder was centrifuged for 10 minutes at 5000 rpm to separate plasma, which was subsequently frozen for later analysis (Yousefi et al., 2012). Following blood collection, the fish were euthanized by blunt force trauma to the head and by severing the spinal cord. Their abdominal cavities were opened with scissors; a segment of the midgut was excised and fixed in 10% formalin for histopathological examination, while the hindgut was dissected for microbiological assessments.

Leukocyte counting

White blood cell (WBC) and differential WBC counting were performed based on the method of Dacie and Lewis (1996). Accordingly, blood samples were diluted using a Dacie solution, and cell counts were conducted under a microscope using a Neubauer chamber. For the differential WBC count, blood samples were fixed on slides and stained with Giemsa. Different WBC were counted based on their morphological characteristics under the microscope.

Plasma immunological parameters

Lysozyme was measured by the turbidimetric method using *Micrococcus luteus* and phosphate buffer at pH 6.2 at a wavelength of 530 nm (Ellis 1990). The activity of the alternative complement pathway (ACH50) was measured using a hemolytic method with sheep blood according to Yano (1992). Plasma total immunoglobulin (Ig) was determined according to Siwicki and Anderson (1993) and by precipitation with polyethylene glycol.

Total protein (Biuret method) and albumin (bromocresol green method) were determined using appropriate kits (Zistcehm Co., Tehran, Iran) and a spectrophotometer. Plasma globulin was calculated by subtracting plasma total protein and albumin.

Hindgut microbiological examination

Hindgut samples were freshly homogenized for microbiological assays. After preparing the homogenate using 0.9% physiological saline, various dilutions ranging from 10-1 to 10-7 were prepared. From the desired dilutions, an aliquot of 0.1 mL was taken under sterile conditions and transferred to tryptic soy agar for total viable bacteria (TVB) counting, de Man–Rogosa–Sharpe agar for LAB counting, and thiosulfate–citrate–bile salts–sucrose agar for *Vibrio* sp. counting. The plates were incubated for 24 h at room temperature under aerobic conditions. The number of bacteria in each sample was counted and determined based on the logarithm of colony-forming units (Log CFU=total colonies×inverse dilution factor).

Midgut section preparation and examination

To carry out the dehydration and paraffin embedding of the fixed gut samples, a tissue processor was used, and ultimately, the samples were placed in paraffin blocks. Then, using a microtome, sections with a thickness of 5 microns were prepared and placed on slides, where they were stained with eosin-hematoxylin. For each sample, two sections were prepared with intervals of 200 µ (Hoseini et al., 2022b). Finally, the prepared tissue sections were used to evaluate histological changes in the gut.

Statistical analysis

The data were analyzed using SPSS software, version 22. To this end, the normal distribution and homogeneity of variances were confirmed using the Shapiro-Wilk and Levene's tests. Subsequently, a 2-way analysis of variance (ANOVA) and Duncan test were utilized to examine the effects of CS meal and OA in the diet. Significant differences were assessed at a level of $P<0.05$, and the data were presented as Mean±SD.

Results

There were no significant effects of dietary CS and OA on WBC count and percentages of the blood lymphocyte, neutrophil, and monocyte (Figure 1).

Adding CS to the diet did not have significant effects on the plasma immune indicators of the fish (Figure 2; $P<0.05$). Plasma total Ig showed no significant responses to dietary CS and OA supplementation. However, the addition of OA to the diet resulted in a significant increase in lysozyme activity ($P=0.006$), the ACH50 ($P=0.004$), total protein ($P=0.006$), albumin ($P=0.019$), and globulin ($P=0.005$) in the plasma of the fish (Figure 2).

Dietary CS showed no significant effects on the gut TVB ($P=0.889$), but dietary OA significantly decreased it ($P=0.001$). There was a significant ($P=0.002$) interaction effect of dietary CS and OS on the gut LAB; the lowest gut LAB was observed in SB treatment, but the other treatments showed similar gut LAB. Dietary CS ($P=0.005$) and OA ($P<0.001$) had significant effects on the gut *Vibrio* sp. population. The gut *Vibrio* sp. population increased by dietary CS inclusion, but decreased by dietary OA addition (Figure 3).

Different letters above the bars show significant differences among the treatments (Duncan; n=3).

Histopathological sections of the fish gut are shown in Figure 4. There were no obvious lesions in any treatments.

Discussion

The number of WBC serves as a crucial indicator of immune status and stress in fish. In this study, we found that incorporating CS meal and OA into the diet of rainbow trout did not significantly affect WBC count. Notably, this research is the first to investigate these specific indicators in rainbow trout. Previous studies have shown

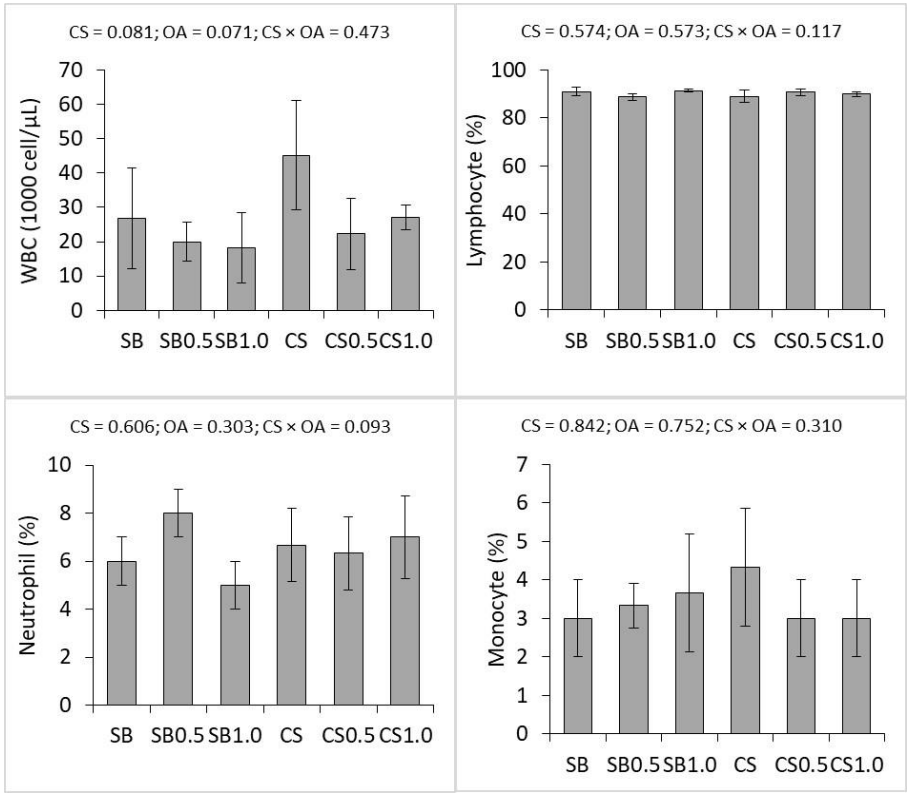

Figure 1. Blood leukocytes in different treatments (n=3)

varying effects of CS meal on WBC counts in different fish species. For instance, increasing CS meal levels to 17% and 25% in the diets of hybrid tilapia resulted in elevated WBC counts, whereas higher levels (34% to 56%) led to a decline (Yue & Zhou, 2008). Similarly, a study involving common carp revealed that adding 9% to 54% CS meal to the diet had no significant impact on WBC counts (Wang et al., 2014). In black carp (*Mylopharyngodon piceus*), WBC counts decreased linearly with increasing dietary CS meal from 10% to 40 % (Hu et al., 2015). In contrast, the inclusion of 1% lactic acid in the diet of rainbow trout resulted in increased WBC counts, while concentrations of 0.5% and 2% showed no significant effects (Hoseini et al., 2022a). Additionally, a mixture of malic acid and citric acid did not significantly influence WBC counts in crucian carp (Zhang et al., 2020). Thus, our findings suggest that adding 15% CS meal to trout diets does not adversely affect humoral cellular immunity.

Non-specific immunity functions as the first line of defense against pathogens and environmental stressors in fish. This immune system encompasses physical barriers like skin and mucosal membranes, along with soluble factors in the blood, such as lysozyme and complement proteins, which can recognize and respond to a wide array of invaders without prior exposure. Diet plays a significant role in modulating this immune response. For example, incorporating up to 59% CS meal into the diets of rainbow trout did not significantly affect plasma total protein levels after 130 days of rearing (Dabrowski et al., 2000). Based on our findings, the addition of 15% CS meal does not significantly impact non-specific plasma immune indicators. However, dietary OA appears to enhance these immune indicators, potentially offering better protection against pathogen invasions. These results align with previous studies on rainbow trout, which demonstrated that adding lactic acid (Hoseini et al., 2022a) or malic acid (Yousefi et al., 2023) to their diets improved plasma immune indicators.

Intestinal microbial communities have important roles in fish health, welfare, and nutrition. LAB are known as beneficial bacterial groups that improve fish immunity and nutrition; on the other hand, *Vibrio* sp. are mainly pathogenic. Studies are showing that CS meals have negative effects on gut microbiota. For example, adding 24% CS protein concentrate to the diet of golden pompano (*Trachinotus ovatus*) resulted in a decrease in the population of Firmicutes and an increase in the populations of *Vibrio* and *Proteobacteria* in the distal intestine (Shen et al., 2020). The same amount of CS protein concentrates in the diet of pearl grouper (♀*Epinephelus*

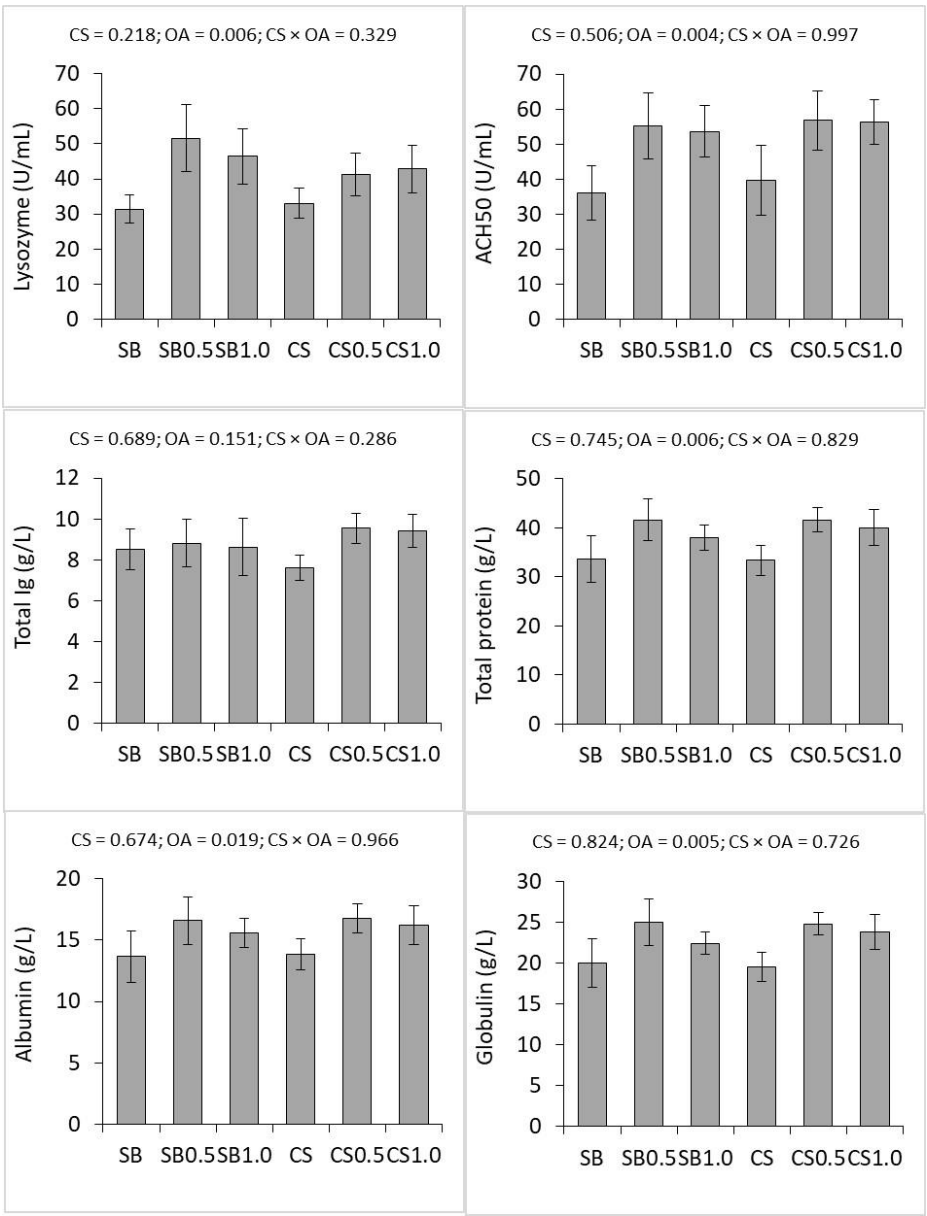

Figure 2. Plasma Immunological parameters in different treatments (n=3)

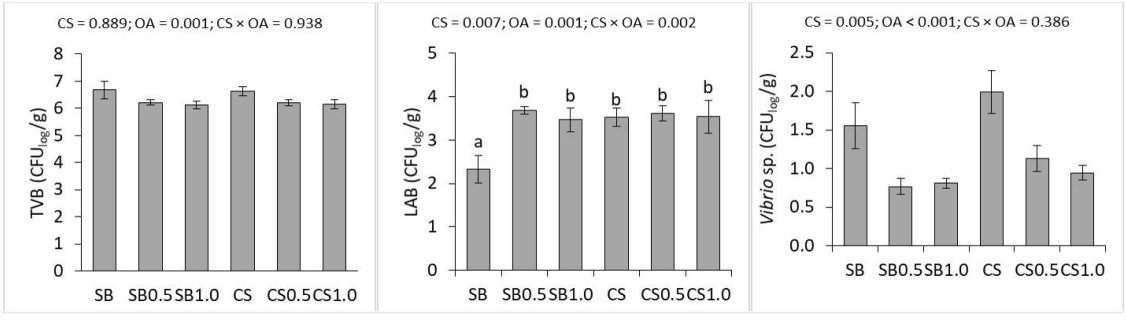

Figure 3. Gut TVB, LAB, and *Vibrio* sp. frequencies in different treatments

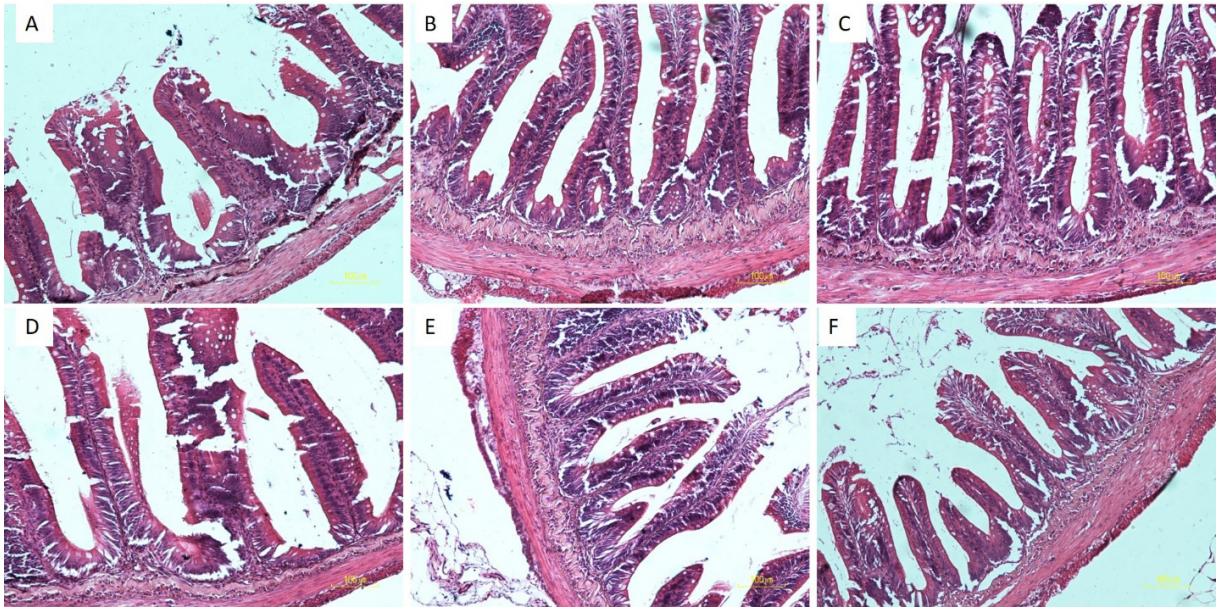

Figure 4. Histological section of the fish gut in different treatments

Note: A: SB; B: SB0.5; C: SB1.0; D: CS; E, CS0.5; F: CS1.0.

fuscoguttatus × ♂*Epinephelus lanceolatus*) led to a reduction in the density of *Bifidobacteria* and an increase in opportunistic pathogens such as *Vibrio*, *Proteobacteria*, and *Actinobacteria* in the distal intestine (Chen et al., 2020). An interesting finding in the present study was the increase in LAB in fish fed CS diets. Such an increase may be related to higher fiber contents in the CS diets that might serve as a fermentation substrate for LAB.

On the other hand, OA and its salts can facilitate the domination of LAB and inhibit harmful bacteria. LAB can utilize OA as an energy source, enabling dietary OA supplementation to increase gut LAB. Moreover, OA changes proton contents in the gut, which inhibits the domination of harmful bacteria (Hoseini et al., 2022a). Adding 326 mg of sodium butyrate per kilogram of the diet for grass carp (*Ctenopharyngodon idella*) increased the number of LAB in the intestine (Tian et al., 2017). Adding 5-10 g lactic acid to the diet significantly increased the population of *Lactobacillus* sp., but decreased *Streptococcus iniae* in rainbow trout gut (Hoseini et al., 2022a). Addam et al. (2019) showed that adding a mixture of OA (5 g per kilogram of diet) to the diet of Nile tilapia resulted in a decrease in the total number of bacteria and the genus *Pseudomonas* sp. in the fish intestine. Katya et al. (2018) also observed that adding two organic acid compounds to the diet of olive flounder (*Paralichthys olivaceus*) reduced the number of *Edwardsiella tarda* in the intestine. Overall, the present results show that adding OA to the diet may enhance gut health by dominating beneficial microbes and inhibiting harmful ones.

Our investigation revealed no pathological changes in the fish gut across various treatments, which contrasts with findings from previous studies. For instance, the inclusion of CS protein concentrates at levels exceeding 24% in the diet resulted in reduced gut villus height, thickness, and lamina propria thickness in pearl grouper (Chen et al., 2020). Similarly, golden pompano exhibited alterations in gut morphology when fed diets containing 40%-60% CS meal (Fu et al., 2022). These findings suggest that a 15% inclusion of CS meal is sufficiently low to avoid inducing pathological changes in the gut of rainbow trout.

In conclusion, incorporating 15% CS meal into the diet of rainbow trout does not affect humoral immunological responses or gut histomorphology. However, it does lead to an increase in both beneficial (LAB) and harmful (*Vibrio* sp.) populations within the fish gut. Conversely, adding OA to the diet effectively addresses this issue by enhancing LAB populations while inhibiting *Vibrio* sp. Furthermore, OA improves humoral innate immune parameters, regardless of the dietary inclusion of CS meal.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Scientific Committee of the Inland Waters Aquatics Resources Research Center, Gorgan, Iran (Code: 1402/3/1).

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Authors' contributions

Conceptualization: Seyyed Morteza Hoseini, Habibollah Kashiri, and Ali Taheri Mirghaed; Investigation and project administration: Seyyed Morteza Hoseini and Habibollah Kashiri; Methodology and writing the original draft and: Seyyed Morteza Hoseini and Ali Taheri Mirghaed; Writing and editing: Seyyed Morteza Hoseini.

Conflict of interest

The authors declared no conflict of interest.

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