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Research Article

Supplementation of a Multienzyme Complex, an Organic Acid-Essential Oil Complex, and Prebiotic Alone or in Combination Affects Growth, Nutrient Utilization, and Immune Function of Rainbow Trout (*Oncorhynchus mykiss*)

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Dietary supplementation of functional additives is an effective strategy to improve the nutritive value of low fish meal diets for fishes. The present study is aimed at investigating the dietary effects of a multienzyme complex, an organic acid-essential oil complex and prebiotic on growth, immune function, and intestinal health of rainbow trout (*Oncorhynchus mykiss*). A positive control diet (PC) and a negative control diet (NC) were formulated to contain 200 g/kg and 100 g/kg of fishmeal, respectively. Seven other diets were formulated by supplementing a multienzyme complex (MC, 0.125 g/kg), organic acid-essential oil complex (OEC, 0.5 g/kg), and prebiotic (P, 2 g/kg) alone or in combination to the NC diet (MC, OEC, P, MC+OEC, MC+P, OEC+P, MC+OEC+P). All diets were formulated to be isoproteic (434.3 g/kg-439.1 g/kg) and isolipidic (119.2 g/kg-125.9 g/kg) and fed to rainbow trout of 7.66 ± 0.05 g for 56 days. The weight gain, apparent digestibility coefficient of dry matter, protein efficiency ratio, and protein retention efficiency were significantly increased, and feed conversion ratio was significantly decreased by the three additives alone or in combination ($P < 0.05$) compared to the NC. No significant differences in growth performance and feed utilization were observed among the treatment groups. Compared to the NC group, serum alanine transaminase, aspartate transferase activities, and malondialdehyde content reduced significantly, while serum superoxide dismutase increased in all except for the MC group, and alkaline phosphatase and lysozyme activity increased in all except for the MC and MC+P groups ($P < 0.05$). The foregut protease activity of the MC+P and OEC+P groups and the amylase activity of the OEC and MC+OEC groups were significantly higher than those of the NC group ($P < 0.05$). The villus height of P, MC+OEC, and OEC+P groups, the villus width of MC group, and the muscle thickness of MC, MC+OEC, and MC+OEC+P groups were also significantly higher than those of the NC group ($P < 0.05$). Compared to the PC group, the richness and diversity of intestinal microorganisms in the NC group and all the supplemented groups were significantly reduced ($P < 0.05$), but no differences among them ($P > 0.05$). In conclusion, the individual or combined supplementation of multienzyme complex, organic acid-essential oil complex, and prebiotic in a low fish meal diet (100 g/kg) improved the growth performance, nutrient utilization, and immune function of rainbow trout, but no synergistic effects were observed in the combination of the three supplements.

1. Introduction

Due to the shortage and high price of fish meal, fish meal replacement studies have been a common research focus in carnivorous fish feed for the last two decades. At present, soybean meal [1, 2], insect meal [3, 4], poultry by-product meal [5, 6], and rapeseed meal [7–9] have been reported to substitute the fish meal inclusion in the diets of rainbow trout. Replacement of fish meal with these alternative sources created problems such as reduced digestibility, palatability, and intestinal damage. They can be addressed implementing some nutritional strategies, e.g., use of enzymes, organic acids, essential oils, and prebiotic.

Dietary enzyme supplementation can eliminate or reduce plant antinutritional factors (ANFs), increase endogenous enzyme activity, and improve nutrient utilization. The commonly used feed enzymes include protease (acid, alkaline, neutral, monocomponent, multicomponent, multicomponent alkaline, etc.), carbohydrase (amylase, cellulase, β-glucanase, chitinase, etc.), lipase, and phytase. Several studies have confirmed the positive effects of dietary enzymes in rainbow trout. For example, supplementation of 250 mg/kg protease [10] or the inclusion of 67 mg/kg β-glucan and 228 mg/kg protease in high plant protein diets [11] improved crude protein digestibility of rainbow trout.

Dietary organic acids can lower the pH of feed and chime, promote nutrient digestibility, inhibit specific microbial growth, and improve intestinal microbiome. Fumaric acid, malic acid, formic acid, citric acid, lactic acid, and butyric acid are the commonly used organic acids in animal diets. The supplementation of citric acid in diets has been reported to improve the growth performance of rainbow trout [12, 13]. Essential oils are volatile oily substances extracted from root, branch, and leaf tissues of plants rich in essential oil. They have natural antioxidant and antibacterial activity and can improve the palatability of feed, thereby improving the growth performance, gut health, and antioxidant capacity of farmed animals [14]. Rafieepour et al. [15] reported that the supplementation of 6 g/kg and 10 g/kg oregano essential oil in feed promoted the growth of rainbow trout. Rainbow trout fed diet containing 3 g/kg oregano essential oil showed higher growth performance and immunity than those fed the control diet [16]. However, the direct addition of essential oils and organic acids to feeds still has some problems. For example, essential oils are easy to evaporate, and most free organic acids have been disassociated and lose their efficacy in the stomach and foregut. The microencapsulation technology can slow the release of organic acids in the gastrointestinal tract and prevent the loss of essential oils during the feed processing, thus improving the effect of organic acids and essential oils in fish diets.

Prebiotic are dietary ingredients that cannot be digested by the animal but utilized by the beneficial bacteria in the gut to improve host health. At present, the main prebiotic used in feed are oligosaccharides, including mannan-oligosaccharides, xylo-oligosaccharides, fructo-oligosaccharide, and inulin. Ortiz et al. [17] reported that adding 5 g/kg and 10 g/kg fructo-oligosaccharides in diets improved the growth performance and calcium absorption of rainbow trout. Yilmaz et al. [18] found that dietary supplementation of 1.5 g/kg mannan oligosaccharides improved the growth performance of rainbow trout. Similar results were also reported in red drum (*Sciaenops ocellatus*) [19], gilthead seabream (*Sparus aurata*) [20], and European seabass (*Dicentrarchus labrax*) [21].

The above studies have shown the positive effects of enzymes, organic acids, essential oils, and prebiotic supplementation on the growth, feed utilization, immunity, and health of rainbow trout. It is hypothesized that the combination of these supplements may have some synergistic effects. Therefore, the present study was conducted to investigate the supplemental effects of multienzyme complex, organic acids-essential oil complex, and prebiotic individually or in combination in low fish meal diet on growth, immune function, and intestinal health of rainbow trout.

2. Material and Methods

2.1. Ethical Statement.
The procedures have been authorized by the Animal Ethics Committee for Experiments on Animals of Shanghai Ocean University and implemented in accordance with the experiment animal welfare regulation formulated by the Chinese Association for Laboratory Animal Science.

2.2. Experimental Diets and Design.
A total of 9 isoproteic and isolipidic diets were prepared, including a positive control (PC) diet and a negative control (NC) diet containing 200 g/kg and 100 g/kg fish meal, respectively. In NC diet, DL-methionine and L-lysine were supplemented to balance the amino acids composition as the PC diet. Then, 0.125 g/kg multienzyme complex (MC), 0.5 g/kg organic acid-essential oil complex (OEC), and 2 g/kg prebiotic (P) were added into the NC diet individually or in combination to formulate another seven diets (MC, OEC, P, MC+OEC, MC+P, OEC+P, and MC+OEC+P). Y_2O_3 (0.5 g/kg) was added in all diets as inert marker to detect apparent digestibility.

The feed ingredients with large particles were pulverized and screened by 60-mesh sieve. Then, all feedstuff including premix and oil were thoroughly mixed to form a uniform mixture. An extruder was used to make the diets with pelleting temperature of $85 \pm 5°C$ and a diameter of 2 mm (SLP-45, Fishery Machinery and Instruments Research Institute, China). The formulation, proximate composition and amino acid profiles of experimental diets are shown in Tables 1 and 2, respectively.

Multienzyme complex and organic acid-essential oil complex were supplied by JEFO Nutrition Inc., Canada. The multienzyme complex was composed of protease (5000000 U/kg) and carbohydrase (xylanase, 200000 U/kg). The organic acid-essential oil complex was microencapsulated product containing fumaric acid (16%), sorbic acid (8%), malic acid (7%), citric acid (7%), and essential oil as thymol, vanillin, and eugenol. Prebiotic consisted of xylo-oligosaccharide and fructo-oligosaccharide (2 : 1), which was provided by Shanghai Yuanye Biological Company.

TABLE 1: Ingredients and proximate composition of experimental diets (air-dried basis, g/kg).

Items	PC	NC	MC	OEC	P	MC+OEC	MC+P	OEC+P	MC+OEC+P
Fish meal	200.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Soy bean meal	50.0	225.0	225.0	225.0	225.0	225.0	225.0	225.0	225.0
Soy protein concentrate	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Wheat flour	259.5	175.9	175.8	175.4	173.9	175.3	173.8	173.4	173.3
Corn gluten meal	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Cottonseed protein concentrate	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Meat and bone meal	90.0	90.0	90.0	90.0	90.0	90.0	90.0	90.0	90.0
Brewers dried yeast	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Fish oil	30.0	37.0	37.0	37.0	37.0	37.0	37.0	37.0	37.0
Soybean oil	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
DL-methionine	0.0	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
L-lysine	0.0	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Vitamin premix[a]	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Mineral premix[b]	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Y_2O_3	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Multienzyme complex	0.0	0.0	0.125	0.0	0.0	0.125	0.125	0.0	0.125
Organic acid-essential oil complex	0.0	0.0	0.0	0.5	0.0	0.5	0.0	0.5	0.5
Prebiotics	0.0	0.0	0.0	0.0	2.0	0.0	2.0	2.0	2.0
Total	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0
Proximate composition									
Crude protein	439.1	437.7	437.8	438.5	438.9	436.9	434.3	436.9	436.9
Crude fat	119.2	123.1	125.9	125.4	125.1	120.3	122.0	122.7	121.7
Crude ash	118.9	116.3	114.4	116.9	116.3	117.1	116.7	117.3	114.4
Moisture	86.6	89.7	86.5	83.4	80.3	81.0	82.7	79.9	89.5

[a]Vitamin premix (mg or IU/kg diet): VA, 10000 IU; VD_3, 3000 IU; VE, 150 IU; VK_3, 12.17 mg; VB_1, 20 mg; VB_2, 20 mg; VB_3, 100 mg; VB_6, 22 mg; VB_{12}, 0.15 mg; VC, 1000 mg; biotin, 0.6 mg; folic acid, 8 mg; inositol, 500 mg. [b]Mineral premix(mg/kg diet): iodine, 1.5 mg; cobalt, 0.6 mg; cuprum, 3 mg; iron, 63 mg; manganese, 11.45 mg; selenium, 0.24 mg; magnesium, 180 mg; monocalcium phosphate, 2000 mg.

2.3. Experimental Fish and Feeding Management. The experimental rainbow trout were purchased from Tanghao Aquaculture Company (Kunming, China). After the transport to the lab, rainbow trout were temporarily stocked for 2 weeks. Then, 702 fish with a mean weight of 7.66 ± 0.05 g were randomly distributed to 27 barrels with circulating water, and the water volume in each barrel was 650 L. There were 9 treatments with triplicate (barrels) per treatment and 26 fish per barrel. During the feeding period, all fish were fed twice daily (8:30 am and 15:30 pm) with feeding rate of 3%-5% of body weight. Feed intake was adjusted according to weather and feeding behavior to ensure no feed residue left in 5 min after the feeding. During the feeding period, the water temperature (12-16°C), dissolved oxygen (6-7 mg/L), pH (7.0-7.5), and ammonia (≤ 0.2 mg/L) were measured daily. The feces were siphoned out from the bottom in 2 hours after feeding every morning. About 1/3 of the water was renewed with aerated tap water every three days. The feeding trial was carried out in Binhai Aquaculture Station of Shanghai Ocean University and lasted for 56 days from December 2020 to February 2021.

2.4. Sample Collection. Before the feeding trial, 9 fish were randomly selected for the determination of the initial whole body composition. At the end of feeding trial, all the fish were stopped feeding for 24 hours and then bulk weighed. Three fish were collected from each barrel and stored at -20°C for whole-body proximate composition analysis. Another three fish were used to draw blood from caudal vein. Blood samples were centrifuged at 1512 ×g for 10 min at 4°C; then, the serum was collected and preserved at -80°C for the further measurement. Then, the three fish were immediately dissected, and the visceral mass and liver were weighed. The foregut tissue (1-2 cm) and liver tissue were collected and stored in Born's solution for the histological analysis. The other 3 fish were used to collect the hindgut (without chyme), which was stored in liquid nitrogen to determine the intestinal flora. All the fish were anesthetized with MS-222 (Nanhua Qianmu Biotechnology Co., Ltd.) at 0.1 g/L before sampling. In the fourth and fifth week, the intact feces were collected by siphoning, then preserved at -20°C for the nutrient digestibility analysis.

2.5. Measurement Indexes

2.5.1. Growth and Physical Indicators. According to the initial weight, final weight, fish number, feed intake, and tissue (organ) weight, the survival, weight gain (WG), and feed

TABLE 2: Amino acid profiles of experimental diets (dry matter, g/kg).

Items	PC	NC	MC	OEC	P	MC+OEC	MC+P	OEC+P	MC+OEC+P
Essential amino acid									
Arginine	32.0	33.6	33.8	32.9	33.5	33.1	32.3	33.4	33.2
Histidine	14.1	13.7	13.7	14.0	13.8	13.6	13.8	13.2	13.8
Isoleucine	16.6	16.4	16.0	16.5	16.0	15.9	16.1	16.3	17.5
Leucine	35.4	34.1	34.0	31.7	33.9	33.9	33.9	34.0	33.5
Lysine	23.5	23.8	23.9	24.6	23.7	24.3	24.4	23.6	24.4
Methionine	8.1	8.1	8.2	8.2	8.1	8.1	8.1	8.1	8.1
Phenylalanine	19.9	18.8	18.7	18.3	18.7	19.0	18.2	19.1	18.1
Threonine	19.5	18.6	18.6	18.5	18.6	18.4	18.6	18.5	18.5
Valine	19.1	18.2	17.8	18.3	18.0	18.0	18.2	18.0	17.7
Nonessential amino acid									
Alanine	25.0	22.4	22.4	23.5	22.3	22.2	22.4	22.1	22.2
Aspartic acid	29.0	34.9	36.2	35.2	34.9	35.8	35.1	35.6	36.0
Cysteine	4.6	4.3	4.6	4.4	4.8	4.5	4.8	4.3	4.5
Glutamic acid	83.7	82.1	82.3	82.0	82.8	81.5	81.0	82.8	81.6
Glycine	25.5	23.1	23.1	23.6	23.3	23.0	23.3	22.8	23.1
Serine	24.2	23.0	23.2	23.4	23.2	22.8	23.1	22.7	22.9
Tyrosine	14.8	14.3	14.3	15.2	14.2	14.3	13.9	14.4	14.5
TAA	394.8	389.4	390.7	390.3	389.9	388.5	387.2	389.1	389.6

TAA: total amino acids.

conversion ratio (FCR) are calculated as follows:

$$WG\,(\%) = 100 \times \frac{(\text{final body weight (g)} - \text{initial body weight (g)})}{\text{initial body weight (g)}},$$

$$FCR = \frac{\text{feed intake (g)}}{(\text{final body weight (g)} - \text{initial body weight (g)})},$$

$$Survival\,(\%) = 100 \times \frac{\text{final number of fish}}{\text{initial number of fish}},$$

$$Hepatosomatic\ index\ (HSI,\%) = 100 \times \frac{\text{liver weight (g)}}{\text{body weight (g)}},$$

$$Viscerosomatic\ index\ (VSI,\%) = 100 \times \frac{\text{visceral weight (g)}}{\text{body weight (g)}},$$

$$Condition\ factor\ (CF, g/cm^3) = 100 \times \frac{\text{body weight (g)}}{\text{body length (cm)}^3}.$$

(1)

2.5.2. Composition of Whole Body and Diet. The proximate composition of whole body and diets was determined according to AOAC [22]. Moisture content was determined by 105°C drying method, and crude protein content was determined by automatic Kjeldahl nitrogen analyzer (2300-Auto-Analyzer, Fosstecator, Sweden). Crude fat content and crude ash content were detected by methanol extraction with chloroform, and by burning method at 550°C for 4 h, respectively. Amino acid content was determined by Sykam S-433D amino acid automatic analyzer (Sekam, Germany).

2.5.3. Liver and Intestinal Histology. The samples were dehydrated in alcohol with different concentrations; then, the sections were stained with hematoxylin-eosin. The optical microscope equipped with a photographic system was used to observe the liver morphology and intestine morphology as intestinal villus height, width, and muscle thickness.

2.5.4. Intestinal Enzyme Activities. After the thawing, intestine sample (1.0 g) was homogenized with 10 times saline, then centrifuged at 4°C and 1512×g for 10 minutes. The supernatant was collected to determine the enzyme activity. Protease activity was detected with folin–phenol method [23], and the amount of enzyme decomposing casein into 1 μg tyrosine per minute at pH 7.2 and 37°C per microgram tissue protein is defined as one unit. The determination of amylase activity was performed with starch-iodine colorimetry. The kits used for the determination were provided by Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China).

2.5.5. Serum Antioxidant and Immune Indexes. Serum alanine transferase (ALT) and aspartate transferase (AST) activities were determined by the kits provided by Shanghai Haring Biotechnology Co., Ltd. The amount of 1 μmol pyruvate catalyzed by 1 mL serum sample per hour was defined as one activity unit of ALT and AST. Serum superoxide dismutase (SOD) activity was measured by xanthine oxidase method, and the amount of SOD corresponding to the SOD inhibition rate of 50% per mL of the reaction solution was defined as one SOD activity unit. Total nitric oxide synthase (T-NOS) was measured by colorimetric method, and one unit of enzyme activity was defined as 1 nmol NO produced per minute per mL of serum. Alkaline phosphatase

(AKP) was measured by p-nitrophenyl phosphate method, and the 100 mL of serum reacted with the matrix for 15 minutes at 37°C yielded 1 mg of phenol as one viability unit. Malondialdehyde (MDA) can condense with thiobarbituric acid to form a red product with a maximum absorption peak at 532 nm. Bacteria can be lysed by lysozyme (LZM) to increase the light transmittance, and the content of LZM can be estimated according to the change of light transmittance. All the kits for the determination of the above indica-tors are provided by the Nanjing Jiancheng Institute of Biological Engineering (Nanjing, China).

2.5.6. Nutrient Utilization. The content of yttrium in feed and feces was determined by plasma atomic emission spec-trometry (Vista MPX, Varian Alo Alto, California, Ameri-can). The formulas for calculating dry matter and crude protein apparent digestibility, protein efficiency ratio, and protein retention are as follows:

$$\text{Apparent digestibility coefficient of dry matter (ADCDM, \%)} = 100 \times \left(\frac{1 - \text{dietary yttrium content}}{\text{fecal yttrium content}} \right),$$

$$\text{Apparent digestibility coefficient of crude protein (ADCCP, \%)} = 100 \times \left(\frac{1 - (\text{dietary yttrium content} \times \text{fecal crude protein content})}{(\text{fecal yttrium content} \times \text{dietary crude protein content})} \right),$$

$$\text{Protein efficiency ratio (PER)} = \frac{(\text{final weight (g)} - \text{initial weight (g)})}{\text{protein intake (g)}},$$

$$\text{Protein retention (PR, \%)} = 100 \times \frac{\text{protein gain (g)}}{\text{protein intake (g)}}.$$

$$(2)$$

2.5.7. Gut Microbes. The intestinal samples were sent to Shanghai Majorbio Bio-pharm Technology Co., Ltd. for extraction of DNA and PCR amplification by Illumina MiSeq Sequencing platform. Trimmomatic was used for software quality control. OTU cluster and species classifica-tion was analyzed by using UPARSE and the RDP classifier Bayesian algorithm, respectively. The microbiota structure was analyzed at the phylum and genus levels on the platform of Majorbio Cloud Platform.

2.5.8. Data Analysis. The data were expressed as mean ± standard deviation (mean ± SD) and statistically analyzed by SPSS 26.0 software. One-way analysis of variance (one-way ANOVA) was used, and the multiple comparisons of the data were conducted with Tukey's method. $P < 0.05$ was the standard of significant difference.

The effects of different treatments on weight gain, feed conversion ratio, apparent digestibility coefficient of dry matter, protein efficiency ratio, and protein retention of rainbow trout were analyzed by principal component analy-sis (PCA) using SPSS 26.0.

3. Results

3.1. Growth Performance and Physical Indicators. As shown in Table 3, the PC group had the highest WG and the lowest FCR among all the groups. The WG of the NC group was 8.1% lower, and the FCR was 0.06 higher than those of the PC group ($P < 0.05$). Compared to the NC group, the sup-plementation of MC, OEC, and P alone or in combination significantly increased the WG and reduced the FCR ($P < 0.05$), but the WG of these groups was still lower, and FCR was higher than those of the PC group ($P < 0.05$).

There was no significant difference in the WG and FCR among the additive-supplemented groups ($P > 0.05$). No sig-nificant differences in CF, SR, HSI, and VSI were found among all the groups ($P > 0.05$).

3.2. Whole Body Composition. In Table 4, there was no sig-nificant difference in moisture and crude ash contents among all the groups ($P > 0.05$). Except P and MC+OEC +P groups, all the other groups showed significantly higher crude protein than the NC group ($P < 0.05$). In crude lipid, only MC+P group presented significantly higher value than the NC group ($P < 0.05$), and no difference was detected among other groups.

3.3. Nutrient Utilization and Intestinal Enzyme Activity. In Table 5, the supplementation of MC, OEC, and P to the NC diet alone or in combination significantly improved the ADCDM, PR, and PER ($P < 0.05$), and the ADCDM of these groups reached the similar level to the PC group. There was no significant difference in ADCCP among all the groups ($P > 0.05$).

Compared to the NC group, the supplementation of MC +P and OEC+P significantly increased the foregut protease activity ($P < 0.05$), and the supplementation of OEC and MC+OEC increased the amylase activity ($P < 0.05$). The other supplements did not significantly affect the enzyme activities ($P > 0.05$) (Table 5).

3.4. Serum Indicators. Compared to the NC group, the sup-plementation of the three additives alone or in combination significantly reduced serum ALT and AST activities and increased SOD (except MC group) and AKP activities (except MC and MC+OEC+P groups) ($P < 0.05$). Serum

Table 3: Effects of multienzyme complex, organic acid-essential oil complex, and prebiotic on the growth performance of rainbow trout.

Groups	IBW/g	FBW/g	WG/%	FCR	Survival/%	CF/(g/cm^3)	VSI/%	HSI/%
PC	7.66 ± 0.02	84.08 ± 1.35^a	997.6 ± 20.0^a	0.90 ± 0.01^a	100.0 ± 0.0	1.42 ± 0.02	8.88 ± 0.76	1.32 ± 0.05
NC	7.67 ± 0.03	77.94 ± 0.75^c	916.6 ± 8.7^c	0.96 ± 0.01^c	98.7 ± 2.2	1.42 ± 0.09	8.50 ± 0.63	1.23 ± 0.02
MC	7.64 ± 0.02	81.10 ± 0.84^b	961.4 ± 8.2^b	0.93 ± 0.00^b	100.0 ± 0.0	1.38 ± 0.06	8.74 ± 0.32	1.24 ± 0.07
OEC	7.67 ± 0.03	80.27 ± 0.92^b	947.0 ± 8.1^b	0.93 ± 0.01^b	98.7 ± 2.2	1.35 ± 0.05	8.69 ± 0.77	1.27 ± 0.07
P	7.68 ± 0.02	80.74 ± 0.56^b	951.4 ± 4.9^b	0.93 ± 0.01^b	98.7 ± 2.2	1.38 ± 0.05	8.68 ± 0.36	1.31 ± 0.10
MC+OEC	7.68 ± 0.02	80.04 ± 0.57^b	942.2 ± 6.0^b	0.93 ± 0.01^b	100.0 ± 0.0	1.39 ± 0.03	8.78 ± 0.23	1.27 ± 0.02
MC+P	7.68 ± 0.02	80.72 ± 0.46^b	951.1 ± 9.0^b	0.93 ± 0.01^b	98.7 ± 2.2	1.42 ± 0.06	9.08 ± 0.37	1.27 ± 0.04
OEC+P	7.66 ± 0.04	79.78 ± 1.81^b	942.0 ± 18.9^b	0.94 ± 0.01^b	100.0 ± 0.0	1.34 ± 0.12	8.63 ± 0.40	1.25 ± 0.02
MC+OEC+P	7.67 ± 0.02	80.74 ± 1.06^b	953.2 ± 16.8^b	0.93 ± 0.01^b	100.0 ± 0.0	1.40 ± 0.07	8.49 ± 0.28	1.29 ± 0.01

IBW: initial body weight; FBW: final body weight; WG: weight gain; FCR: feed conversion ratio; CF: condition factor; VSI: viscerosomatic index; HSI: hepatosomatic index; in the same column, values without letter or with the same letter superscripts mean no significant difference ($P > 0.05$), while with different small letter superscripts mean significant difference ($P < 0.05$). The same as the following tables.

Table 4: Effects of multienzyme complex, organic acid-essential oil complex, and prebiotic on body composition of rainbow trout (g/kg, fresh weight).

Groups	Moisture	Crude ash	Crude protein	Crude lipid
PC	732.6 ± 6.1	21.91 ± 1.46	167.4 ± 0.2^{ab}	62.3 ± 3.4^b
NC	730.7 ± 2.5	21.76 ± 0.43	158.1 ± 2.3^d	60.7 ± 2.4^b
MC	730.3 ± 8.9	21.14 ± 1.24	165.6 ± 2.7^{abc}	60.0 ± 0.4^b
OEC	711.3 ± 10.7	21.93 ± 0.72	166.7 ± 4.6^{abc}	66.7 ± 5.7^{ab}
P	719.0 ± 27.6	21.44 ± 1.03	161.8 ± 3.0^{bcd}	62.6 ± 4.0^b
MC+OEC	719.0 ± 15.8	21.47 ± 1.45	169.8 ± 3.6^a	63.1 ± 2.9^b
MC+P	715.0 ± 9.5	22.67 ± 1.71	168.2 ± 3.4^a	72.8 ± 2.5^a
OEC+P	726.1 ± 11.6	22.47 ± 1.63	164.8 ± 3.3^{abc}	62.4 ± 4.6^b
MC+OEC+P	723.3 ± 31.1	20.49 ± 1.67	161.1 ± 3.1^{cd}	67.0 ± 6.4^{ab}

Table 5: Effects of multienzyme complex, organic acid-essential oil complex, and prebiotic on nutrient utilization and intestinal enzyme activities of rainbow trout.

Groups	ADCDM/%	ADCCP/%	PR/%	PER	Protease (U/mg prot)	Amylase (U/mg prot)
PC	83.10 ± 1.65^a	92.75 ± 1.02	46.83 ± 0.56^a	2.54 ± 0.03^a	14.16 ± 1.28^{ab}	0.60 ± 0.03^b
NC	78.72 ± 3.76^b	91.83 ± 1.01	41.71 ± 0.81^d	2.38 ± 0.02^c	11.80 ± 1.69^{bc}	0.51 ± 0.09^b
MC	83.71 ± 1.14^a	91.65 ± 1.01	44.92 ± 0.67^{bc}	2.46 ± 0.01^b	11.32 ± 1.73^c	0.50 ± 0.05^b
OEC	82.23 ± 0.82^a	91.53 ± 1.10	45.06 ± 1.66^{abc}	2.45 ± 0.03^b	12.70 ± 1.24^{abc}	0.73 ± 0.05^a
P	82.07 ± 0.54^a	91.95 ± 0.72	43.92 ± 0.65^c	2.46 ± 0.02^b	11.95 ± 1.14^{bc}	0.52 ± 0.04^b
MC+OEC	83.82 ± 0.43^a	92.75 ± 0.06	45.95 ± 0.84^{ab}	2.45 ± 0.02^b	14.10 ± 0.30^{ab}	0.72 ± 0.03^a
MC+P	84.79 ± 0.77^a	92.70 ± 0.78	45.89 ± 1.57^{ab}	2.47 ± 0.04^b	14.44 ± 0.85^a	0.61 ± 0.06^b
OEC+P	83.67 ± 1.19^a	91.74 ± 1.57	44.34 ± 0.75^{bc}	2.43 ± 0.03^b	14.42 ± 1.20^a	0.54 ± 0.06^b
MC+OEC+P	83.51 ± 1.00^a	91.44 ± 0.87	43.66 ± 0.68^c	2.46 ± 0.03^b	12.03 ± 1.46^{abc}	0.59 ± 0.08^b

MDA content reduced significantly, and serum lysozyme activity increased in all except for the MC and MC+P groups ($P < 0.05$), when compared to the NC group. In serum T-NOS, the PC and MC groups showed significantly lower, and the OEC, P, MC+OEC, and MC+OEC+P groups showed significantly higher activities than the NC group ($P < 0.05$) (Table 6).

3.5. Intestinal Histology. Compared to the NC group, the supplementation of P, MC+OEC, and OEC+P increased

TABLE 6: Effects of multienzyme complex, organic acid-essential oil complex, and prebiotic on serum indexes of rainbow trout.

Groups	SOD (U/mL)	ALT (U/mL)	AST (U/mL)	AKP (U/mL)	MDA (nmol/mL)	LZM (ug/mL)	T-NOS (U/mL)
PC	100.7 ± 6.5^e	1.70 ± 0.05^c	2.63 ± 0.07^b	103.1 ± 3.2^{ab}	4.08 ± 0.39^{cd}	5.36 ± 0.36^b	29.31 ± 1.48^e
NC	99.3 ± 2.6^e	1.97 ± 0.05^a	2.77 ± 0.04^a	91.9 ± 3.9^d	5.31 ± 0.33^b	4.05 ± 0.55^c	32.56 ± 0.37^d
MC	100.7 ± 6.6^e	1.66 ± 0.03^c	2.51 ± 0.19^b	94.4 ± 1.7^{cd}	6.14 ± 0.42^a	4.40 ± 0.21^c	29.59 ± 0.79^e
OEC	146.2 ± 5.6^{ab}	1.21 ± 0.05^g	2.29 ± 0.07^c	102.0 ± 6.0^{abc}	3.29 ± 0.39^e	5.71 ± 0.36^{ab}	37.19 ± 1.10^b
P	135.5 ± 8.4^{bc}	1.56 ± 0.03^d	1.76 ± 0.07^e	101.8 ± 2.1^{abc}	3.73 ± 0.08^{ed}	5.48 ± 0.21^b	36.67 ± 1.72^b
MC+OEC	151.3 ± 4.6^a	1.47 ± 0.04^e	2.58 ± 0.05^b	101.9 ± 4.2^{abc}	4.12 ± 0.27^{cd}	6.31 ± 0.21^a	41.15 ± 1.34^a
MC+P	137.1 ± 7.4^{bc}	1.87 ± 0.04^b	1.96 ± 0.05^d	106.0 ± 3.5^a	3.20 ± 0.27^e	4.29 ± 0.36^c	33.55 ± 0.88^{cd}
OEC+P	127.6 ± 5.2^{cd}	1.85 ± 0.08^b	2.15 ± 0.07^c	105.3 ± 5.5^a	4.61 ± 0.19^c	5.24 ± 0.21^b	33.36 ± 1.59^{cd}
MC+OEC+P	124.4 ± 5.3^d	1.38 ± 0.04^f	2.24 ± 0.05^c	95.8 ± 5.5^{bcd}	4.65 ± 0.46^c	5.48 ± 0.55^b	35.02 ± 1.59^{bc}

TABLE 7: Effects of multienzyme complex, organic acid-essential oil complex, and prebiotic on intestinal structure of rainbow trout.

Groups	Villus height/ μm	Villus width/ μm	Muscle thickness/ μm
PC	706.4 ± 38.5^{ab}	111.8 ± 11.6^b	158.0 ± 17.0^{abc}
NC	623.7 ± 4.2^b	114.2 ± 9.3^b	131.9 ± 10.8^{cd}
MC	711.3 ± 51.1^{ab}	142.0 ± 1.2^a	166.6 ± 12.8^{ab}
OEC	688.2 ± 40.4^{ab}	117.5 ± 15.0^b	118.4 ± 13.8^d
P	731.4 ± 59.6^a	114.5 ± 9.4^b	144.1 ± 16.1^{bcd}
MC+OEC	752.1 ± 41.4^a	126.9 ± 14.4^{ab}	176.2 ± 18.2^a
MC+P	698.2 ± 45.0^{ab}	116.5 ± 16.7^b	139.0 ± 15.3^{cd}
OEC+P	748.1 ± 56.3^a	105.4 ± 6.1^b	146.9 ± 11.4^{bc}
MC+OEC+P	711.1 ± 60.6^{ab}	121.5 ± 8.7^b	182.8 ± 12.1^a

the villi height ($P < 0.05$), and the supplementation of MC increased the villi width ($P < 0.05$). The muscle thickness of MC, MC+OEC, and MC+OEC+P groups was significantly higher than that of the NC group ($P < 0.05$) (Table 7). The histological pictures are shown in Figure 1.

3.6. Liver Histology. The liver tissue sections are shown in Figure 2. The nucleus and cell structure of liver cells in all groups were normal, and there was no obvious difference in the morphology and structure of liver tissue in all the groups.

3.7. Intestinal Microbiota. In Table 8, the coverage value of each group is close to 1, indicating that the bacterial community has been adequately sampled. Compared to the PC group, the abundance and diversity of intestinal microbes in the NC group were significantly reduced ($P < 0.05$). Compared to the NC group, the supplementation of MC, OEC, and P alone or in combination did not change the abundance and diversity of intestinal microbes ($P > 0.05$).

At the phylum and genus levels, the rainbow trout intestinal bacterial was dominated by *Firmicutes* and *Mycoplasma*, which accounted for 82.44% and 81.72% of the total in the PC group. Compared to the PC group, the proportions of *Firmicutes* and *Mycoplasma* in the NC group and all the additive-supplemented groups were significantly

increased ($P < 0.05$), but no differences among these groups were found ($P > 0.05$) (Table 9).

3.8. Principal Component Analysis (PCA). The PCA of WG, FCR, ADCDM, PER, and PR of each group were shown in Figure 3. Principal component 1 and principal component 2 were explained 71.09% and 20.87%, respectively. The additive-supplemented groups were concentrated in the middle, and the PC in the blue circle and NC in the red circle were distributed on both sides. The additive-supplemented groups overlapped each other, indicating no differences among these groups. There were no overlaps among PC, NC, and the additive-supplemented groups, indicating that they were different from each other.

4. Discussion

4.1. The Effect of Replacing Dietary Fish Meal with Soybean Meal on Rainbow Trout. Although soybean meal has been widely used in aquatic feeds, the high replacement of fish meal with soybean meal may inhibit the growth of fishes. When dietary fish meal was decreased from 490 g/kg to 200 g/kg by soybean meal, the growth performance and feed utilization of rainbow trout were significantly reduced [24]. Kumar et al. [2] reported that the replacement of fish meal (160 g/kg) with soybean meal in a diet containing 250 g/kg fish meal reduced the nutrient availability of rainbow trout. Similar results were also found in the present study. The NC diet containing 100 g/kg fish meal showed lower WG, ADCDM, PER, PR, and higher FCR than the PC diet containing 200 g/kg fish meal. Soybean meal has a low nutrient digestibility than fish meal [25], and the ANFs contained in soybean meal can adversely affect the intestinal absorption of nutrients. In this study, the low fish meal (100 g/kg) diet reduced the species richness and diversity of the intestinal microbiota, increasing the abundance of *Firmicutes* from 82.44% to 99.96% and *Mycoplasma* from 81.72% to 99.95%. Desai et al. [26] also found that plant-based feed was beneficial to the propagation of *Firmicutes* in the intestine of rainbow trout. Some studies have reported that mycoplasma dominates the gut of Atlantic salmon (*Salmo salar*), but the role of mycoplasma in fish is still unclear [27, 28].
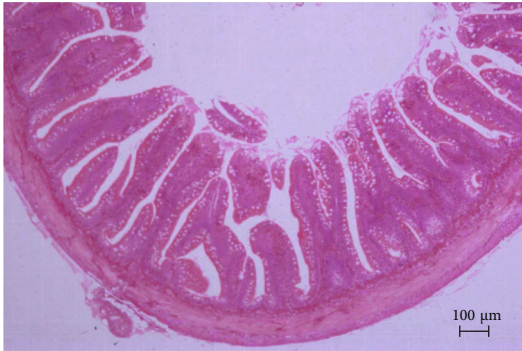
SOD is the first defense line against oxidative stress in the body, and it can catalyze the conversion of superoxide
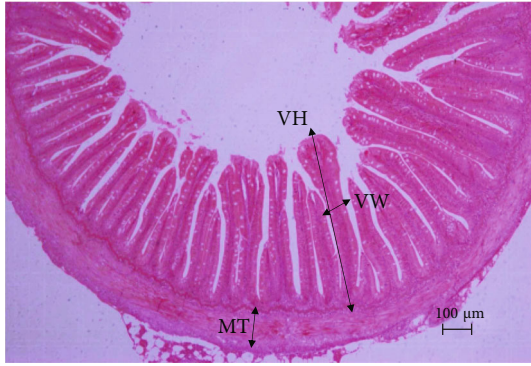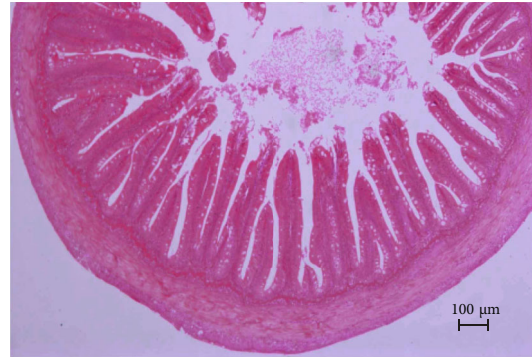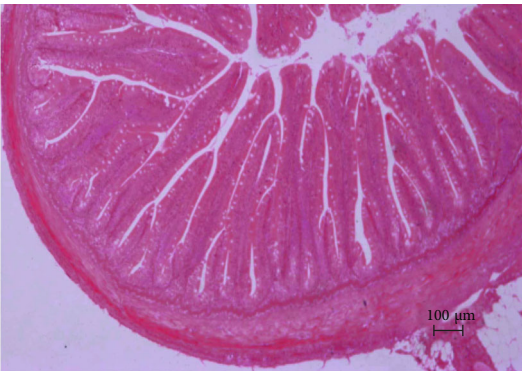
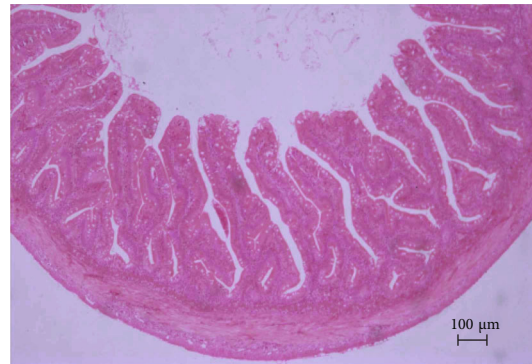(a) PC; MT: muscular thickness; VH: villus height; VW: villus width
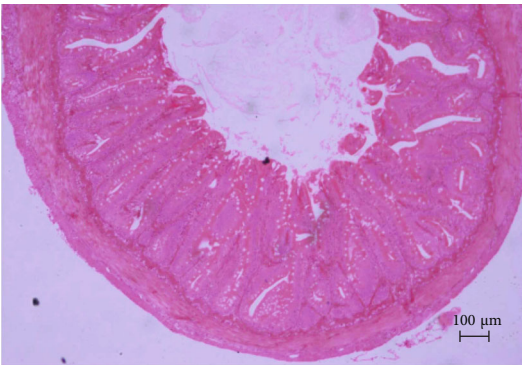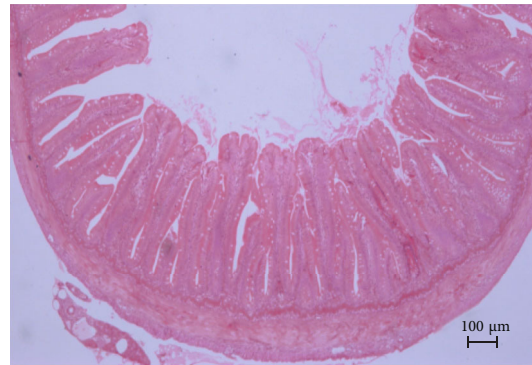
(b) NC

(c) MC
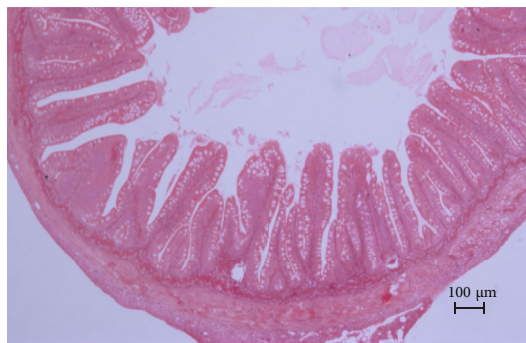
(d) OEC

(e) P
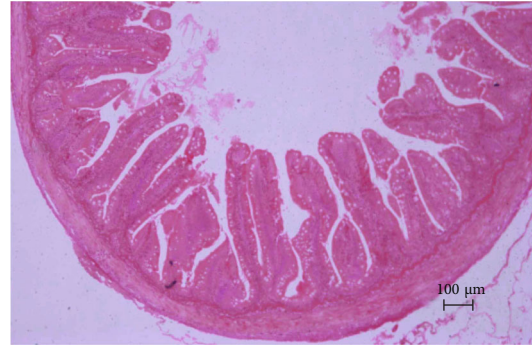
(f) MC+OEC

(g) MC+P

(h) OEC+P

Figure 1: Continued.

(i) MC+OEC+P

FIGURE 1: Effects of multienzyme complex, organic acid-essential oil complex, and prebiotic on intestinal histology of rainbow trout.

anion radicals into O_2 and H_2O_2 [29]. The activity changes of AST and ALT in serum can reflect the health status of liver [30]. AKP and LZM are important indicators reflecting the state of immune function [31]. NOS can catalyze the synthesis of NO, which plays an important role in immune defense [32]. MDA is an index reflecting the body's peroxidative damage [33]. Soy bean meal contains many ANFs such as trypsin inhibitor, soybean agglutinin, saponins, and phytic acid. Thus, the high inclusion of soybean meal may damage the organs and tissues, thereby reducing the immune function and growth. In this study, the fish fed NC diet containing low fish meal and high soybean meal presented significantly higher serum activities of ALT, AST, T-NOS, higher MDA content, and lower activities of AKP and LZM than the PC fish, indicating that the immune function of rainbow trout might be damaged. The NC group also showed significantly higher T-NOS activity than the PC group, which might result from the stimulation by the damaged liver and other tissues, as NOS could catalyze the production of NO, which has special functions in stabilizing liver circulation and protecting liver cells.

4.2. The Effect of Supplementing Multienzyme Complex on Rainbow Trout. Previous studies have reported the positive effects of dietary supplementation of enzymes in aquatic feeds. Shi et al. [34] found that supplementing 0.15 g/kg and 0.175 g/kg protease in pelleted feed increased the WG and PER and reduced the FCR of gibel carp (*Carassius auratus gibelio*). The supplementation of 0.5 g/kg protease in diet also improved the growth performance and intestinal digestive enzymes activity of grass carp (*Ctenopharyngodon idella*) [35]. Drew et al. [10] reported that dietary protease (250 mg/kg) improved the nutrients availability of rainbow trout. The supplementation of protease in diets not only compensates the deficiency of animal's own protease but also promotes the secretion of endogenous proteases, thus improves the nutrients utilization and growth performance of animals [11, 36, 37]. Soybean meal contains a large amount of nonstarch polysaccharides (NSP), which could increase the viscosity of chyme, reducing the contact area between enzymes and substrates. The existence of NSP is an important reason for the low digestibility of plant materials, as animals lack the corresponding enzymes to degrade

NSP. Carbohydrase is a group of enzymes capable of degrading carbohydrate polymers. Zhou et al. [38] observed that dietary cellulase improved the growth performance of grass carp. In broiler, dietary xylanase increased the height of ileal villi [39] and promoted the growth of intestinal lactic acid bacteria [40]. Xylanase is able to degrade NSP to form oligosaccharides, which can promote the proliferation of lactic acid bacteria and other beneficial bacteria, thereby improving intestinal health.

In the present study, the supplementation of 0.125 g/kg MC improved the growth performance and promoted ADCDM, PR, PER, intestinal villus width and muscle thickness of rainbow trout. The MC used in this study is composed of protease and carbohydrase. Protease and carbohydrase can degrade or reduce the ANFs and NSP in plant materials, thereby improving intestinal health. The increase of intestinal muscle thickness will contribute to the mechanical digestion of the intestine, and the increase of intestinal villi will increase the contact area between the intestine and feed, thereby promoting the digestion and absorption of nutrients. This may be the main reason for the promoted growth by dietary MC in this study. In addition, the supplementation of MC reduced serum ALT, AST, and T-NOS activities. The reason might be that the enzymes degrade the ANFs, thereby protecting the liver and other tissues from damage. Zheng et al. [35] once reported that the supplementation of protease in diets reduced the MDA content and increase the activity of LZM of grass carp serum. However, the present serum MDA content was found to be increased by dietary MC. MDA was produced by lipid peroxidation, and its content could reflect the degree of lipid peroxidation in the body. However, it is unknown how much MDA content represents the damage to the body's antioxidant function. Therefore, the increase of MDA content in this experiment means the decrease of antioxidant capacity or not, which needs further research.

4.3. The Effect of Supplementing Organic Acid and Essential Oil Complex on Rainbow Trout. In rainbow trout, organic acids as citric acid [13] and essential oils as thymol [41, 42] have been confirmed the positive effects on the growth performance. Plant essential oil has hydrophobicity, which

(a) PC

(b) NC

(c) MC

(d) OEC
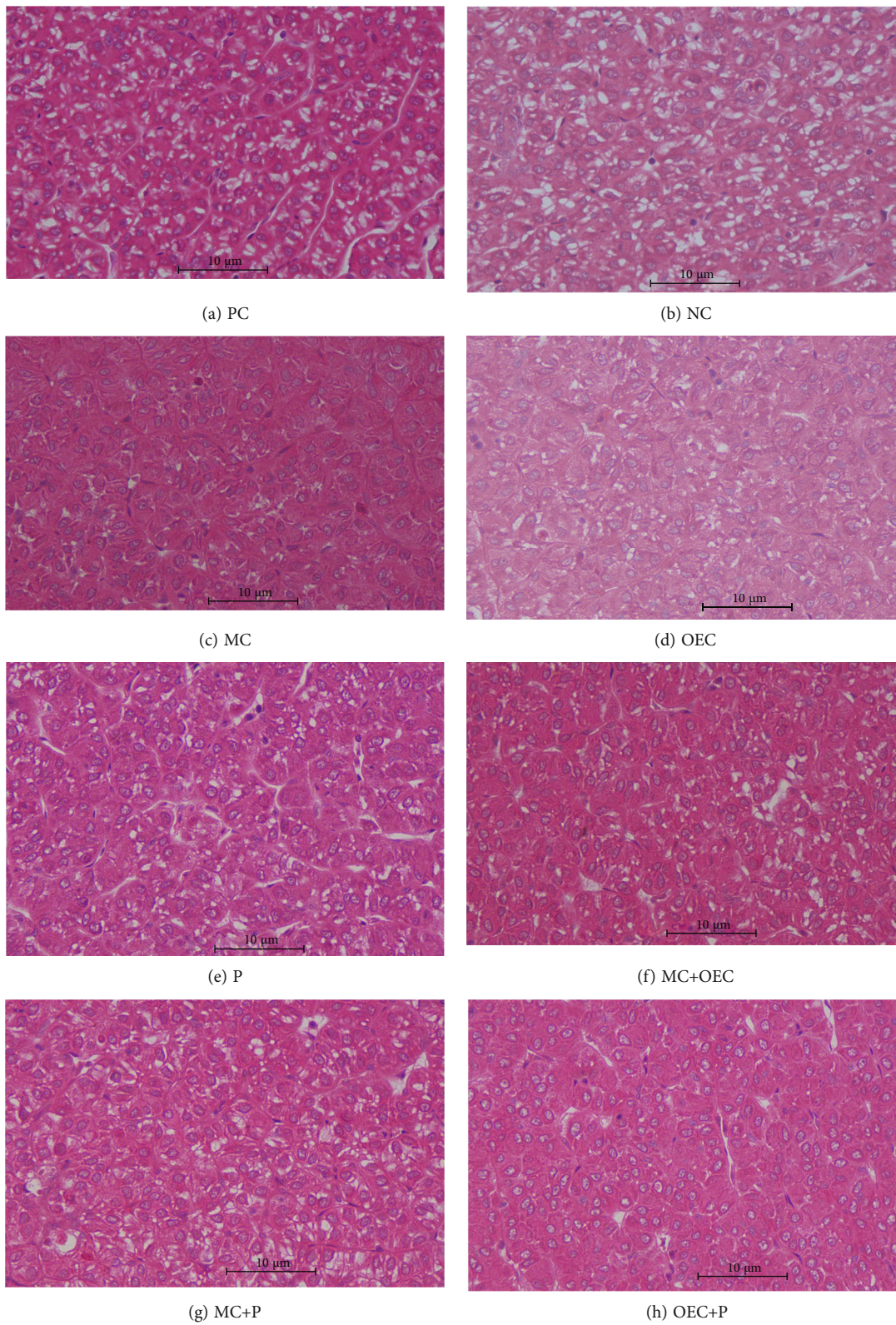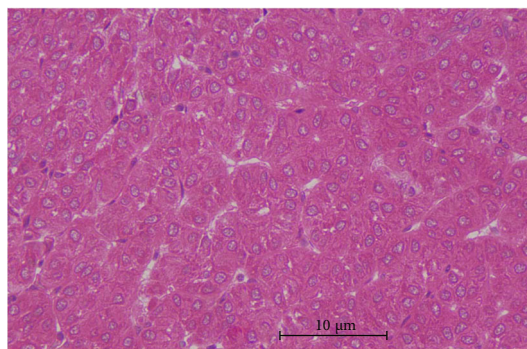
(e) P

(f) MC+OEC

(g) MC+P

(h) OEC+P

Figure 2: Continued.

(i) MC+OEC+P

Figure 2: Effects of multienzyme complex, organic acid-essential oil complex, and prebiotic on liver histology of rainbow trout.

Table 8: Intestinal microbial diversity index of rainbow trout.

Groups	Coverage	Sobs	Chao	Shannon	Simpson
PC	0.9990	1336.0 ± 42.4[a]	1418.0 ± 20.3[a]	1.61 ± 0.19[a]	0.67 ± 0.04[b]
NC	0.9997	18.33 ± 8.50[b]	51.33 ± 46.80[b]	0.01 ± 0.00[b]	1.00 ± 0.00[a]
MC	0.9990	54.50 ± 10.61[b]	84.28 ± 6.76[b]	0.04 ± 0.01[b]	0.99 ± 0.00[a]
OEC	0.9998	15.00 ± 1.41[b]	27.25 ± 15.20[b]	0.01 ± 0.00[b]	1.00 ± 0.00[a]
P	0.9995	45.67 ± 12.74[b]	72.84 ± 29.08[b]	0.02 ± 0.01[b]	1.00 ± 0.00[a]
MC+OEC	0.9997	16.50 ± 6.36[b]	49.33 ± 12.87[b]	0.01 ± 0.00[b]	1.00 ± 0.00[a]
MC+P	0.9996	50.00 ± 39.60[b]	77.69 ± 36.33[b]	0.01 ± 0.01[b]	0.99 ± 0.01[a]
OEC+P	0.9997	51.50 ± 3.54[b]	61.84 ± 9.58[b]	0.04 ± 0.01[b]	0.99 ± 0.00[a]
MC+OEC+P	0.9996	37.00 ± 22.63[b]	78.50 ± 3.54[b]	0.03 ± 0.01[b]	0.99 ± 0.00[a]

Table 9: Intestinal microbiota community abundance of rainbow trout at phylum and genus level (%).

Items	Phylum		Genus	
	Firmicutes	Others	Mycoplasma	Others
PC	82.44 ± 2.16[b]	17.56 ± 2.16[a]	81.72 ± 2.43[b]	18.28 ± 5.03[a]
NC	99.96 ± 0.02[a]	0.04 ± 0.002[b]	99.95 ± 0.02[a]	0.05 ± 0.02[b]
MC	99.72 ± 0.13[a]	0.28 ± 0.13[b]	99.65 ± 0.13[a]	0.35 ± 0.13[b]
OEC	99.96 ± 0.01[a]	0.04 ± 0.01[b]	99.95 ± 0.01[a]	0.05 ± 0.01[b]
P	99.82 ± 0.09[a]	0.18 ± 0.09[b]	99.77 ± 0.10[a]	0.23 ± 0.10[b]
MC+OEC	99.84 ± 0.07[a]	0.16 ± 0.07[b]	99.79 ± 0.06[a]	0.21 ± 0.06[b]
MC+P	99.76 ± 0.11[a]	0.24 ± 0.11[b]	99.68 ± 0.16[a]	0.32 ± 0.16[b]
OEC+P	99.77 ± 0.11[a]	0.23 ± 0.11[b]	99.71 ± 0.08[a]	0.29 ± 0.08[b]
MC+OEC+P	99.81 ± 0.04[a]	0.19 ± 0.04[b]	99.80 ± 0.08[a]	0.20 ± 0.08[b]

could be enhanced in a low pH environment; thus, its antibacterial ability would be promoted. Organic acids can reduce the pH of chyme and digestive tract, so the combined supplementation of organic acids and plant essential oils might produce a synergistic effect. Previous studies in broilers have shown the synergistic effect of organic acids and essential oils [43, 44]. Pelusio et al. [45] once reported that the specific growth rate of rainbow trout was increased, and FCR was decreased by dietary supplementation of organic acid and essential oil complex (citric acid, sorbic acid, thymol, vanillin). In *Litopenaeus vannamei*, He et al. [46] also found that the supplementation of organic acids

and essential oil complex (citric acid, sorbic acid, thymol, and vanillin) improved the abundance of lactic acid bacteria and other beneficial bacteria and promoted the resistance ability against *Vibrio parahaemolyticus*. The improvement of growth performance and immune function by essential oil and organic acid complex may be attributed to the improved intestinal health. Recently, Huyben et al. [47] reported that dietary supplementation of essential oil and organic acid complex increased intestinal villus height and reduced the richness of Aeromonas, a common pathogen in fish. The inhibited growth of pathogenic bacteria will provide a suitable environment for the reproduction of
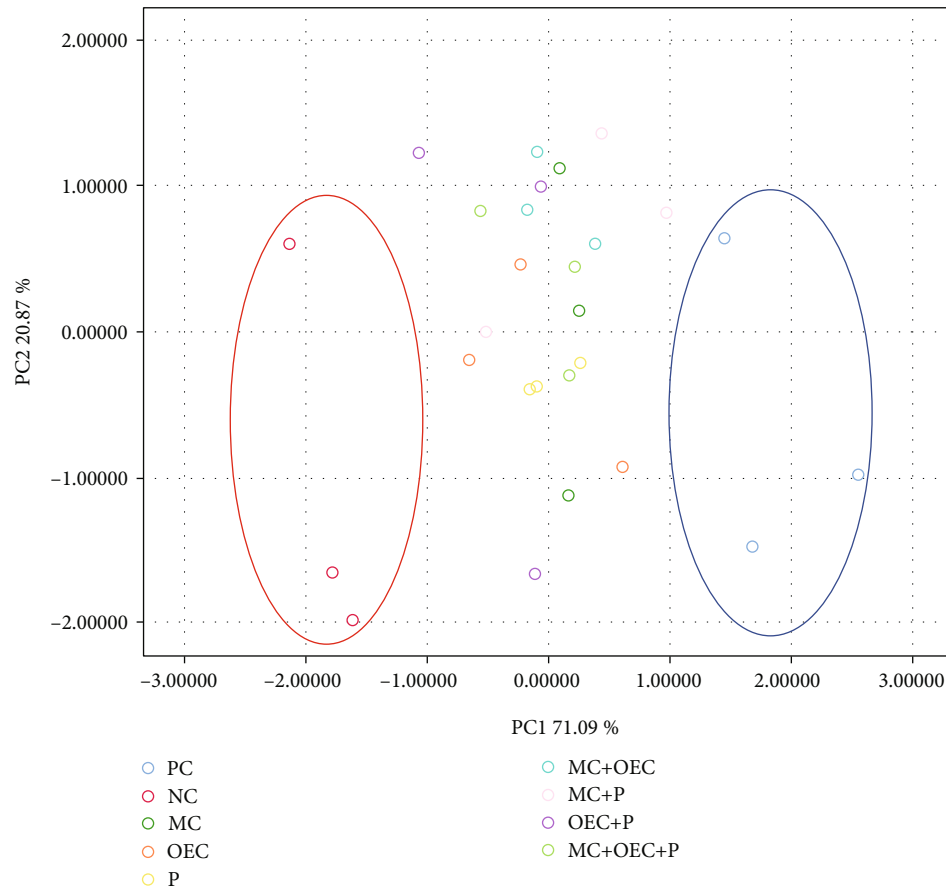
FIGURE 3: The scatter plot of main components of WG, FCR, ADCDM, PER, and PR of rainbow trout in different treatments.

beneficial bacteria, thereby improving the immune function of the body. In this study, dietary organic acid-essential oil complex also significantly improved the growth performance, nutrient availability, and immune function of rainbow trout. However, it is unclear that such positive results came from the synergistic effect or not, due to no individual supplementation of organic acid and essential oil designed in the present study.

4.4. The Effect of Supplementing Prebiotic on Rainbow Trout. Prebiotic is the energy source for intestinal microbes [48], which can promote the proliferation of beneficial bacteria, thereby improving fish growth performance and flora structure [49]. It was reported that dietary fructo-oligosaccharide (10 g/kg) increased the growth performance and immune function and promoted the proliferation of lactic acid bacteria in juvenile stellate sturgeon (*Acipenser stellatus*) [50]. In tilapia (*Oreochromis niloticus* × *O. aureus*), the supplementation of xylo-oligosaccharide in diet increased the growth performance and the amounts of lactic acid bacteria and *Bacillus* [49]. The prebiotic used in this study was composed of fructo-oligosaccharide and xylo-oligosaccharide, and the prebiotic inclusion improved the growth performance, nutrient availability, immune function, and villi height of rainbow trout. It is generally believed that prebiotic can improve the intestinal flora composition by inhibiting harmful bacteria and promoting the proliferation of beneficial

bacteria as lactic acid bacteria, thereby promoting the growth performance of farmed animals. However, in this study, the prebiotic did not affect the intestinal flora composition, which may be related to the special parasitic relationship between *Mycoplasma* and rainbow trout intestine. The present result showed that *Mycoplasma* occupied an absolute advantage in the intestine of rainbow trout, accounting for more than 99% of the total in the NC and additive-supplemented groups. In both wild and farmed salmon (*Oncorhynchus*), *Mycoplasma* showed an absolute predominance in the intestine [51]. However, the role of *Mycoplasma* in the intestine of salmonids is still unclear, and further study is needed in the future.

4.5. The Synergistic Effect of MC, OEC, and P on Rainbow Trout. The present results showed that the combined supplementation of MC, OEC, and P improved the growth performance, ADCDM, PR, and PER, and immune function of rainbow trout, but no synergistic effects were observed when compared to the individual supplementation. In the study of Yao et al. [37], the combined supplementation of protease, carbohydrase, and microencapsulated organic acid salt showed a synergistic effect on the WG of *Litopenaeus vannamei*. In tilapia, the combined supplementation of protease complex and organic acid salts (calcium propionate, calcium formate, and sodium acetate) also improved the apparent digestibility of dry matter when compared to the individual

supplementation [52]. The three additives used in the present study are composed of two or more substances, and the interaction among them is sophisticated, which needs more studies in the future.

5. Conclusion

In the present study, when dietary fish meal inclusion was decreased from 200 g/kg to 100 g/kg, the growth performance and feed utilization of rainbow trout were significantly reduced. In a low fish meal diet containing 100 g/kg fish meal, the supplementation of a multienzyme complex, an organic acid-essential oil complex, and prebiotic P alone or in combination improved the growth performance and feed utilization of rainbow trout. No synergistic effects were observed in the combination of the three supplements.

Data Availability

All data generated or analyzed during this study are included in this article.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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