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*Research Article*

# Effects of Acetoin on Growth Performance, Digestive Function, Antioxidant Status, and Immune Capacity of Largemouth Bass (*Micropterus salmoides*)

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The purpose of this study was to investigate the effects of dietary acetoin on Largemouth Bass (*Micropterus salmoides*). In 45 d of farming, the experimental diets were supplemented with 0%, 0.3%, 0.6%, and 0.9% of acetoin levels, respectively. Results showed that the activities of lipase (LPS), amylase (AMS), and trypsin in fish fed with acetoin at 0.6% displayed the most obvious improvement compared with the other levels of acetoin ($P < 0.05$). Furthermore, the incorporation of acetoin at 0.3% and 0.6% in *Micropterus salmoides* diet significantly enhanced the number of beneficial bacteria (such as *Cetobacterium*) and reduced the number of harmful bacteria (such as *Proteobacteria*) in the gut flora of *Micropterus salmoides*. Meanwhile, the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), and catalase (CAT) in the liver of the samples treated with acetoin at 0.6% were significantly higher than the control ($P < 0.05$), while the content of malondialdehyde (MDA) and the activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the liver notably decreased with the increase of the addition amount of acetoin ($P < 0.05$). Furthermore, the contents of IgA, IL-6, and TNF-$\alpha$ and the activity of acid phosphatase (ACP) in the serum of the samples added with acetoin exhibited a concentration-dependent decline compared with the control ($P < 0.05$). In summary, acetoin had potential feeding attraction effects on *Micropterus salmoides*, and the most suitable dose of dietary application was 0.6% acetoin, which can effectively improve the physiological characteristics and immune activity of *Micropterus salmoides*.

## 1. Introduction

Largemouth Bass (*Micropterus salmoides*) is a kind of precious carnivorous fish, which has the advantages of delicious meat, strong disease resistance, rapid growth, being easy to catch, and a wide temperature range. In recent years, the breeding scale of *Micropterus salmoides* has increased year by year. The health problems are very prominent due to unbalanced feed nutrition [1], deteriorating breeding environments [2], hypoxia, and high temperature stress [3]. The most common difficulties in aquaculture include the abuse of antibiotics [4], ichthyosis, and climate change, which all lead to loss of appetite in aquatic animals [5]. With regard to the loss of appetite in aquatic animals, feeding attractants can be added to daily feed to stimulate the animals to eat, thus improving the animal's starting decline due to various conditions. Food attractants can improve animal appetite under the condition of poor appetite caused by body discomfort and environmental changes [6]. How to maintain the physiological health, improve the appetite, and enhance the immune defense ability of farmed fish through feed nutrition is an important technical problem. It has become an inevitable trend in aquaculture production to develop and utilize new feed additives that can enhance the

appetite of aquatic organisms, improve the resistance of animal bodies, prevent animal diseases, and promote growth.

Acetoin (AC), also known as 3-hydroxybutanone, is a kind of popular edible spice, which is commonly used around the world. Because of its special cream flavor, AC is often used in the production of cream, dairy, yogurt, and strawberry flavors [7], while it is also an essential variety in liquor flavoring [8, 9]. Notably, our recent work presents that acetoin can be used as an attractant for zebrafish, which is similar to the attractant betaine, a yeast nucleotide, and squid paste sold on the market [10]. Its mechanism of action is that it stimulates the olfactory receptor cells of fish by its own physical characteristics (such as fragrance and color), which are transmitted to the central nervous system through the olfactory receptor, and then the central nervous system sends stimulus signals and releases appetite-promoting factors [11, 12].

Besides, intestinal tract is the most important digestive and absorption organ in aquatic organisms, while digestive enzymes in the gut promote absorption by breaking down nutrients in food into small molecules that the body can absorb, which benefits development. There are also a large number of microorganisms with complex structures living in the intestine, which depend on and restrict each other to the host to form a unique intestinal micro ecosystem in the long-term evolution process [13]. The relationship between intestinal microorganisms and the host has attracted much attention because of its specific value in disease prevention. Different intestinal microbial structures and compositions affect many important physiological activities such as nutrient processing, energy balance, immune function, growth, and development of the host [14, 15]. Besides, the dynamic balance between biological antioxidant systems and reactive oxygen species is one of the important factors for organisms to adapt to the environment and ensure their health. A large number of studies have shown that aquatic organisms can produce excessive amounts of reactive oxygen species (ROS) under the stress of environmental factors such as temperature, oxygen, exogenous chemicals, salinity, and heavy metals [16–21]. The antioxidant enzyme system plays an important role in removing excess superoxide anion in organisms, protecting biological macromolecules such as nucleic acids and proteins from oxidative damage and improving the adaptability of the biological environment. At the same time, the antioxidant system is closely related to the body's immunity, and the antioxidant system can affect immunity to some extent. Immunization reflects the tolerance of aquatic organisms, disease resistance, and stress resistance. The body's immune system plays a decisive role in its ability to survive in a variety of environments, and low immunity is the weakened ability to defend against the disease.

However, little has been done to incorporate acetoin into aquatic animals. Thus, the present study was performed to investigate the effects of different volume fractions of acetoin on the growth performance, intestinal digestive capacity, antioxidant capacity, and immune response of *Micropterus salmoides*. This will provide a theoretical basis for the application of acetoin in aquaculture.

## 2. Materials and Methods

*2.1. Ethical Statement.* The feeding trial and subsequent handling and sampling of the experimental fish were carried out as per the guidelines of the Institute of Biology, Shandong Academy of Sciences, Qilu University of Technology, China.

*2.2. Experimental Preparation. Micropterus salmoides* used in this experiment were provided by the Shandong Freshwater Fisheries Research Institute. The acetoins used in this experiment were obtained from Shanghai Aladdin Biotech Co., Ltd (97%, Shanghai, China). The kits used in this experiment were all from the Nanjing Jiancheng Bioengineering Institute. The kits used in this experiment included the trypsin assay kit (UV colorimetric, A080-2), $\alpha$-Amylase (AMS) test box (starch-iodine colorization method, C016-1-1), Lipase (LPS) assay kit (microplate method, A054-1-1), the Total Superoxide Dismutase (SOD) Assay Kit (WST-1 method, A001-3), Glutathione peroxidase (GSH-PX) assay kit (colorimetric method, A005-1), Malondialdehyde (MDA) determination kit (TBA method, A003-1), Catalase (CAT) assay kit (visible light method) (Ammonium molybdate method, A007-1-1), Aspartate aminotransferase (grass transaminase/AST/GOT) test cassette (microplate method, C010-2-1), Alanine aminotransferase (alanine transaminase/ALT/GPT) test box (microplate method, C009-2-1), the Immunoglobulin A (IgA) test box (h108-1), Interleukin-6 (IL-6) test box (h007-1-1), TNF-(TNF-) test box (h052-1), and Acid Phosphatase (ACP) Assay Kit (microplate method, A060-2-2).

*2.3. Experimental Feed and Design.* Table 1 shows the diet formulation. All components in the feed were crushed and sieved into fine powder by a 300 $\mu$m mesh. All raw materials are thoroughly mixed with soybean oil and water and dried in a 60°C oven. The moisture content is about 9%. After drying, the feed was crushed and sieved into particles of appropriate size. The dried particles were placed in a plastic container with a cover and stored in a refrigerator at 4°C. All feeds were analyzed approximately in duplicate.

The *Micropterus salmoides* with an initial fish weight of $90.78 \pm 17.14$ g was selected as the experimental fish and domesticated with basic feed for two weeks before the experiment started. Four fish ponds were selected, and the volume and size of the fish ponds were the same. The size of the pond was 0.3 $m^3$. Each group contains 30 fish, respectively. The experiment was divided into four groups: control group and three experimental groups (0.3% AC, 0.6% AC, and 0.9% AC). Each treatment was tested randomly in three replicates. Four kinds of equal nitrogen and equal energy feeds were designed. As the acetoin used in the experiment was liquid, the corresponding volume fraction of acetoin was dissolved in water and sprayed into the corresponding feed, which was dried before feeding. The control group was fed with basic feed, and the experimental group was fed with experimental feed supplemented with 0.3%, 0.6%, and 0.9% AC in basic feed, respectively.

Table 1: Approximate composition of *Micropterus salmoides* diet during 45 d ($g \cdot kg^{-1}$).

| item | Control (%) | 0.3% AC (%) | 0.6% AC (%) | 0.9% AC (%) |
|---|---|---|---|---|
| Shrimp shell powder a (CP 65.36%) | 50 | 50 | 50 | 50 |
| Chicken porridge (CP 12.48%) | 10 | 10 | 10 | 10 |
| Soyabean protein (CP 44.62%) | 10 | 10 | 10 | 10 |
| Wheat flour b (CP 13.58%) | 5 | 5 | 5 | 5 |
| Yeast powder c (CP 12.43%) | 5 | 5 | 5 | 5 |
| Compound vitamin d | 5 | 5 | 5 | 5 |
| Mineral elements e | 5 | 5 | 5 | 5 |
| Peanut oil f | 8 | 8 | 8 | 8 |
| Aquatic feed adhesive g | 2 | 2 | 2 | 2 |
| Acetoin | 0 | 0.3 | 0.6 | 0.9 |

a: shrimp shell powder (Guangdong Zhongsi Industrial Co., Ltd., China); b: chicken porridge (Guangdong Zhongsi Industrial Co., Ltd., China); c: soyabean protein (Shandong Hengfeng Flour Co., Ltd., China); d: wheat flour (Shandong Hengfeng flour Co., Ltd., China); e: yeast powder (Qingdao Huiruiyuan yeast powder Co., Ltd., China); f: compound vitamin (retinol acetate 30 mg; alpha-tocopherol 60 mg; ascorbic acid 600 mg; vitamin K3 7 mg; riboflavin 20 mg; vitamin B12 0.05 mg; inositol 100 mg; pantothenic acid 50 mg; niacin acid 35 mg; folic acid 8 mg; and biotin 0.06 mg); g: mineral elements ($CuSO_4 \cdot 5H_2O$ 20 mg; $FeSO_4$ 200 mg; $ZnSO_4$ 200 mg; $KH_2PO_4$ 100 mg; $K_2HPO_4$ 100 mg; and $MnSO_4$ 200 mg); h: peanut oil (Shandong Luhua Group Co., Ltd., China); i: aquatic feed adhesive (Qingdao Haixingyuan Biotechnology Co., Ltd., China); j: acetoin (Shanghai Aladdin Biochemical Technology Co., Ltd., China).

*2.4. Micropterus salmoides and Experimental Steps.* Before the beginning of the experiment, adaptive feeding was conducted under experimental conditions for two weeks. At the beginning of the experiment, 480 numbers of *Micropterus salmoides* with the uniform size and active behavior were randomly divided into four fish ponds with different treatments. Each treatment was tested randomly in three replicates. The stocking density of each fish pond was 30 numbers of fish. The same amount of feed is fed every day.

During the experiment (45 d), *Micropterus salmoides* was fed with 3%–5% of the body weight of *Micropterus salmoides* daily for 8:00 a.m. and 17:00 p.m., respectively. During the feeding period, dissolved oxygen was maintained at >6 mg/L, and the water temperature was 27~29°C. After the feeding experiment, the samples were fasted for 24 h before collection.

The total number, individual body length, and weight of fish from each tank were measured to calculate the survival, growth performance, and feed utilization of fish fed test diets according to the following formulae:

Survival (%) = 100 × (final no. of fish/initial no. of fish).

Weight gain rate (WGR, %) = (final body weight – initial body weight) × 100/initial body weight.

Specific growth rate (SGR % d − 1) = 100 × [(Ln (final body weight) – Ln (initial body weight))/duration (45 days)].

Feed efficiency rate (FER) = (final body weight – initial body weight)/feed intake.

*2.5. Sample Collection and Analysis*

*2.5.1. Sample Collection.* After the feeding experiment, *Micropterus salmoides* was placed on the anatomical plate, and the eyes of *Micropterus salmoides* were covered with wet gauze to prevent the penetration of the dorsal fin. A disposable needle tube was used to collect blood at the hip fin position of the tail fish. The blood samples were placed in the sterilized ep tube with antibiotics in advance. The supernatant was centrifuged at 2500 r/min for 20 min, and the serum was obtained. The serum was stored at −80°C.

The Micropterus salmoides that has been bloodied is killed and dissected from the anus along the abdomen. Then, the fish are made to lie laterally, left upward. From the opening before the anus to the back cut, a cut is made along the spine to the posterior edge of the gill cover, and a cut is made along the posterior edge of the gill cover to the pectoral fin. Removing the left body wall, it can be observed *Micropterus salmoides,* viscera, liver, intestines, and other organs, with a 4°C saline cleaning surface and the dry surface of the blood paper wipe water, after weighing and storing at −80°C.

*2.5.2. Determination of Intestinal Digestive Enzyme Activities.* 0.1 g of the preserved intestine was weighed and added to 0.9 ml of normal saline at 4°C to prepare 10% intestinal homogenate. The intestinal homogenate was centrifuged at 2500 r/min for 10 min in a freezing centrifuge. The supernatant was taken to obtain the supernatant of the intestinal homogenate and stored at −80°C for further use.

The prepared intestinal homogenate supernatant was diluted with corresponding multiples and then operated according to the instructions of the relevant kits. The activities of lipase (LPS), amylase (AMS), and trypsin in the intestinal tract of *Micropterus salmoides* were determined by a microplate reader and a spectrophotometer. Trypsin can catalyze the hydrolysis of the ester chain of arginine ethyl ester and increase its absorbance at 253 nm. The enzyme activity can be calculated according to the change of absorbance. AMS activity was determined by starch-iodine colorimetry. AMS in 100 ml serum (pulp) was hydrolyzed to 10 mg starch as one AMS unit at 37°C for 30 min. LPS activity was determined by the colorimetric method. The LPS activity unit was defined as the amount of 1 $\mu$mol substrate consumed per liter of serum (plasma) in the reaction system at 37°C for 1 min.

*2.5.3. Analysis of Intestinal Flora.* After the breeding experiment, five numbers of *Micropterus Salmoides* were randomly selected from each group and disinfected with

75% alcohol. The complete intestinal tract was dissected and taken out under sterile operation conditions and sent to Shenzhen Microscience Union Technology Group Co., Ltd. for sequencing analysis of 16S rDNA34.

*2.5.4. Determination of Liver Antioxidant Activity.* 0.1 g of the preserved liver was weighed and added to 0.9 ml of normal saline at 4°C to prepare 10% liver homogenate. The liver homogenate was centrifuged at 2500 r/min for 10 min in a refrigerated centrifuge. The supernatant was taken to obtain the supernatant of liver homogenate and stored at −80°C for further use.

The prepared liver homogenate supernatant was taken and diluted to the corresponding multiples according to the relevant kits. The content of malondialdehyde (MDA) and the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-PX), catalase (CAT), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) in the liver of *Micropterus salmoides* were determined by a microplate reader and a spectrophotometer. The activity of SOD was determined by the WST-1 method. SOD of a unit of activity (U) is defined as the amount of enzyme corresponding to the inhibition rate of SOD reaching 50%. Each 4 $\mu$l whole blood was required to react at 37°C for 5 minutes, excluding the effect of the enzymatic reaction, so that the concentration of GSH in the reaction system decreased by 1 mol as a GSH-PX enzyme activity unit. The content of MDA was determined by the TBA method. The activity of CAT was determined by the ammonium molybdate method. The CAT activity unit is defined as the amount of 1 $\mu$mol $H_2O_2$ per milliliter of serum or plasma per second. The activities of AST and ALT were determined by the microplate method.

*2.5.5. Determination of Serum Immune Activity.* The prepared serum dilution is taken in corresponding multiples according to the relevant kits. The contents of IgA, IL-6, and TNF-$\alpha$ and the activity of acid phosphatase (ACP) in serum were measured by ELISA. The contents of IgA, IL-6, and TNF-$\alpha$ in the samples was detected by the competition method. The samples were added to the precoated enzyme-labeled holes, and then the biotin labeled recognition antigen was added. After incubation at 37°C for 30 min, the two were competitively combined with the solid-phase antibody to form an immune complex. After washing with PBST, the unbound biotin antigen was removed, and then the affinity HRp was added. After incubation at 37°C for 30 min, the affinity HRp was combined with the biotin antigen. After washing, the combined HRP catalyzed tetramethylbenzidine (TMB) to become blue and then converted to yellow under the action of acid. There was an absorption peak at 450 nm. The absorbance value was negatively correlated with the concentration of the antigen in the sample. ACP can decompose disodium phenyl phosphate to produce free phenol and phosphoric acid. Phenol reacts with 4-aminoantipyrine in alkaline solution to form red quinone derivatives by potassium ferricyanide oxidation. The enzyme activity can be measured according to the red depth. A unit of ACP activity was defined as the amount of enzyme in 100 ml serum required to produce 1 mg nitrophenol at 37°C for 30 min.

*2.6. Statistics Analysis.* The one-way ANOVA was performed on the results using SPSS 25.0, followed by the LSD multiple comparison test. The data results were expressed as average ± standard deviation, and the significant difference was $P < 0.05$.

## 3. Results

*3.1. Growth Performance.* Growth performance and feed utilization data of *Micropterus salmoides* are shown in Table 2. The experiment results show that the supplementation of different concentrations of acetoin improved the growth performance and feed utilization in GIFT tilapia. The final body weight, weight gain rate, specific growth rate, and feed efficiency rate of fish fed diets supplemented with different concentrations of acetoin were significantly higher than those of fish fed control diet after 45 days of feeding ($P < 0.05$). There were no significant differences between 0.3% AC and 0.9% AC ($P > 0.05$).

*3.2. Application of Acetoin Increases the Intestinal Digestive Enzyme Activity in Micropterus salmoides.* Figure 1 represents intestinal digestive enzyme activities in *Micropterus salmoides* after 45 days of feeding. Adding different volume fractions of acetoin had significant effects on AMS and LPS activities ($P < 0.05$), and 0.6% AC increased the most. For trypsin, the activity of trypsin in 0.6% AC was significantly different from that of control ($P < 0.05$), and 0.3% and 0.9% AC was not ($P > 0.05$). The results showed that acetoin could increase the activities of the intestinal digestive enzyme of *Micropterus salmoides*, promote intestinal peristalsis, and accelerate the absorption of nutrients.

### *3.3. Application of Acetoin Alters the Intestinal Flora Structure in Micropterus salmoides*

*3.3.1. Application of Acetoin Increases the Number of OTUs in Micropterus salmoides.* As shown in Figure 2, there were 957 specific OUTs in the intestinal tract of *Micropterus salmoides* in the control, 2040 specific OTUs in the intestinal tract of 0.3% AC, 3103 specific OTUs in the intestinal tract of 0.6% AC, and 1074 specific OTUs in the intestinal tract of 0.9% AC. It can be seen that the number of specific OTUs in the intestinal tract of *Micropterus salmoides* was significantly increased after the addition of acetoin.

*3.3.2. Application of Acetoin Upregulates the Relative Abundance of Genus Level Species in Micropterus salmoides.* The annotation results were sorted according to the abundance at different classification levels, and the top 20 species with the largest abundance in each group at the genus classification level were selected to generate the

Table 2: Growth performance and feed utilization in *Micropterus salmoidesfed* test diets for 45 days*.

| Item | Control | 0.3% AC | 0.6% AC | 0.9% AC |
|---|---|---|---|---|
| Initial body weight (g) | $90.72 \pm 1.23^{a}$ | $90.53 \pm 0.35^{a}$ | $90.67 \pm 1.54^{a}$ | $90.81 \pm 0.22^{a}$ |
| Final body weight (g) | $115.62 \pm 2.9^{a}$ | $179.92 \pm 3.63^{b}$ | $294.72 \pm 3.15^{c}$ | $190.48 \pm 3.12^{d}$ |
| Weight gain rate (%) | $27.44 \pm 0.95^{a}$ | $98.32 \pm 8.10^{b}$ | $224.86 \pm 3.07^{c}$ | $109.96 \pm 5.49^{b}$ |
| Specific growth rate (% $day^{-1}$) | $0.53 \pm 0.09^{a}$ | $1.45 \pm 0.06^{b}$ | $2.61 \pm 0.05^{c}$ | $1.63 \pm 0.07^{b}$ |
| Feed efficiency rate | $1.12 \pm 0.45^{a}$ | $2.11 \pm 0.30^{b}$ | $5.06 \pm 0.25^{c}$ | $2.54 \pm 0.02^{b}$ |
| Survival (%) | 92 | 98 | 100 | 100 |

*All results were expressed as mean values and standard errors of 5 numbers of *Micropterus salmoides*. Different lowercase letters in the same line indicate significant difference between different mass concentrations ($P < 0.05$).

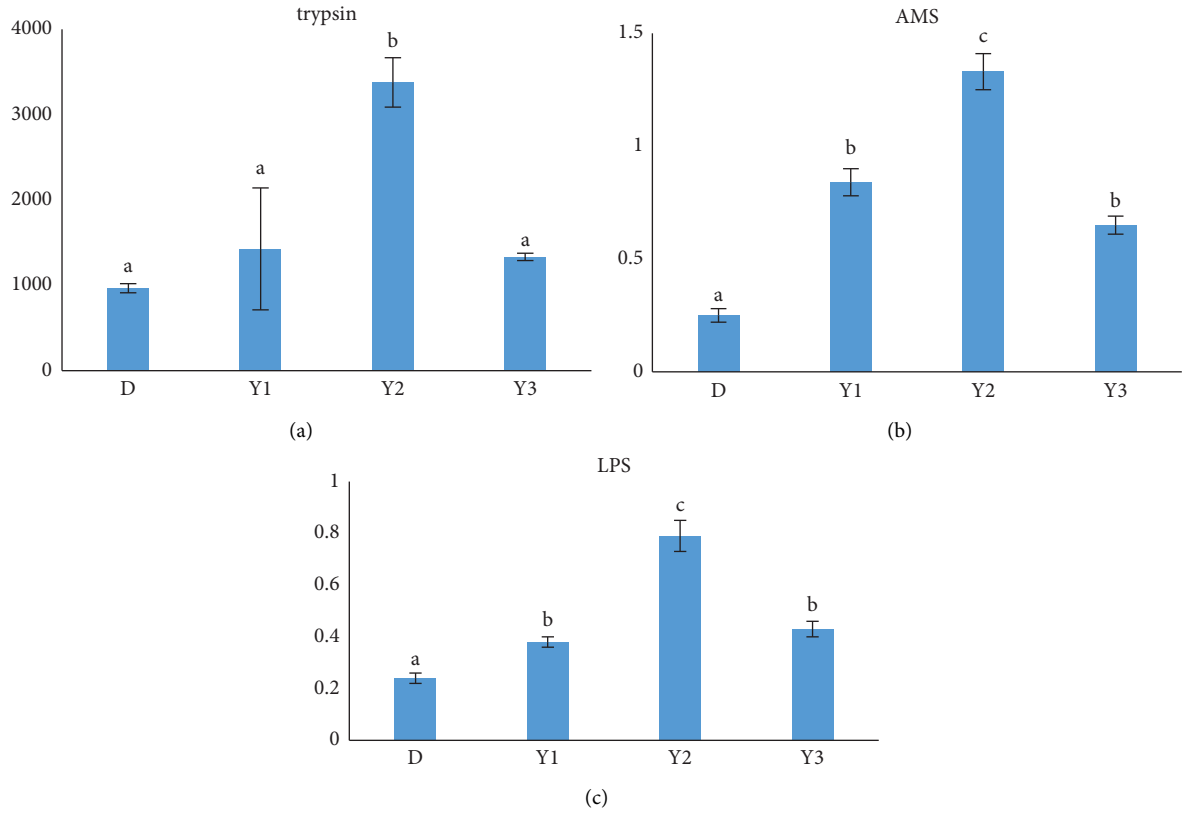


Figure 1: Effects of different concentrations of acetoin on intestinal digestive enzyme activities of *Micropterus salmoides* cultured for 45 days. Trypsin (a), AMS (b), and LPS (c) in the intestine of *Micropterus salmoides* were determined. All results were expressed as mean values and standard errors of 5 numbers of *Micropterus salmoides*. Different lowercase letters indicate significant differences between different mass concentrations ($P < 0.05$).

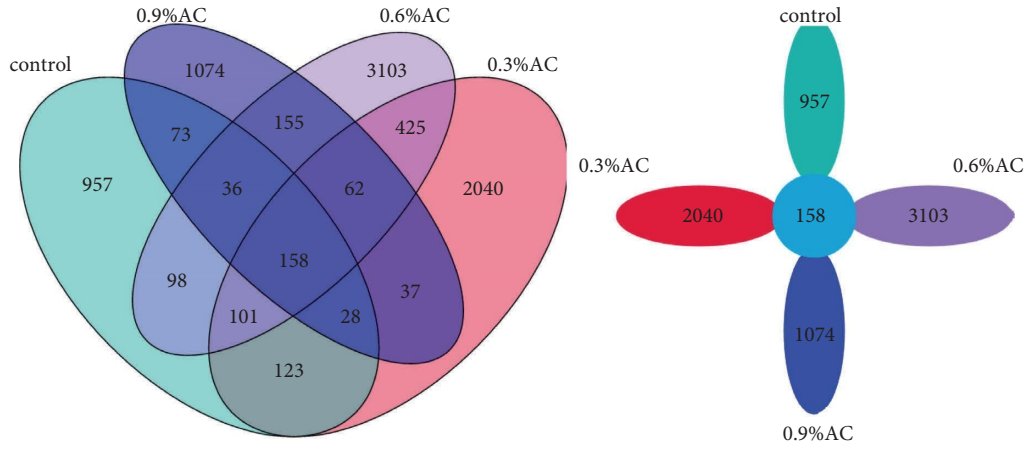


Figure 2: Effects of different concentrations of acetoin on the wayne diagram and the flower bud diagram of intestinal flora of *Micropterus salmoides* for 45 days.

relative abundance histogram of species. The results of their relative abundance are shown in Figure 3. At the level of affiliation, the eight species with the highest relative abundance were *Cetobacterium*, *Ralstonia*, *unclassified*, *Pseudomonadaceae_Pseudomonas*, *Mycoplasma*, *Sphingomonas*, *Agrobacterium*, and *Massilia*. The results showed that after 45 days, the relative abundance of *Cetobacterium* in groups supplemented with 0.3% AC and 0.6% AC was significantly higher than that in the control group ($P < 0.05$). However, the relative abundance of other genera, such as *Pseudomonadaceae_Pseudomonas, unclassified* was lower ($P < 0.05$).

*3.3.3. Application of Acetoin Raises the Diversity of Intestinal Flora α in Micropterus salmoides.* The results of α diversity of intestinal microbiota are shown in Figure 4. Compared with the control group, the Chao1, Observed-Otus, Shannon, Simpson, and faith-pd indexes of intestinal flora in 0.3% and 0.6% groups were increased to different degrees, and the differences were significant ($P < 0.05$). The α diversity of intestinal microbiota in the 0.9% group was lower than that in the control group.

*3.3.4. Application of Acetoin Increases the Relative Abundance of Phyla Level Species in Micropterus salmoides.* Figure 5 shows that the intestinal flora of each group is mainly composed of *Fusobacteria, Proteobacteria, Firmicutes, Tenericutes,* and *Bacteroidetes.* The relative abundance of *Fusobacteria* in 0.3% and 0.6% AC was significantly higher than that in the control. The relative abundance of *Fusobacteria* in 0.9% AC was slightly lower than that in controls. *Proteobacteria* and *Firmicutes* in 0.3% and 0.6% AC were slightly lower than those in control, and *Proteobacteria* and *Firmicutes* in 0.9% AC were higher than those in control.

*3.4. Application of Acetoin Enhances the Liver Antioxidant Activities in Micropterus salmoides.* The supplementation of 0.3% AC and 0.6% AC significantly improved the activities of SOD, GSH-Px, and CAT (Figure 6). Conversely, 0.3% AC and 0.6% AC inclusion generally decreased the activities of AST, ALT, and MDA, which were significantly lower in fish fed 0.3% AC and 0.6% AC diets compared to fish fed control diet ($P < 0.05$).

*3.5. Application of Acetoin Reduces the Immune Activity in Serum in Micropterus salmoides.* The activity of ACP and the contents of Ig A, IL-6, and TNF-α in *Micropterus salmoides* are shown in Figure 7. The results showed that the supplementation of different volume fractions of acetoin decreased the activity of ACP and the contents of Ig A, IL-6, and TNF-α in *Micropterus salmoides*. The activity of ACP and the contents of Ig A, IL-6, and TNF-α of fish fed diets supplemented with 1 0.3% AC and 0.6% AC were significantly lower than those of fish fed control diet after 45 days of feeding ($P < 0.05$).

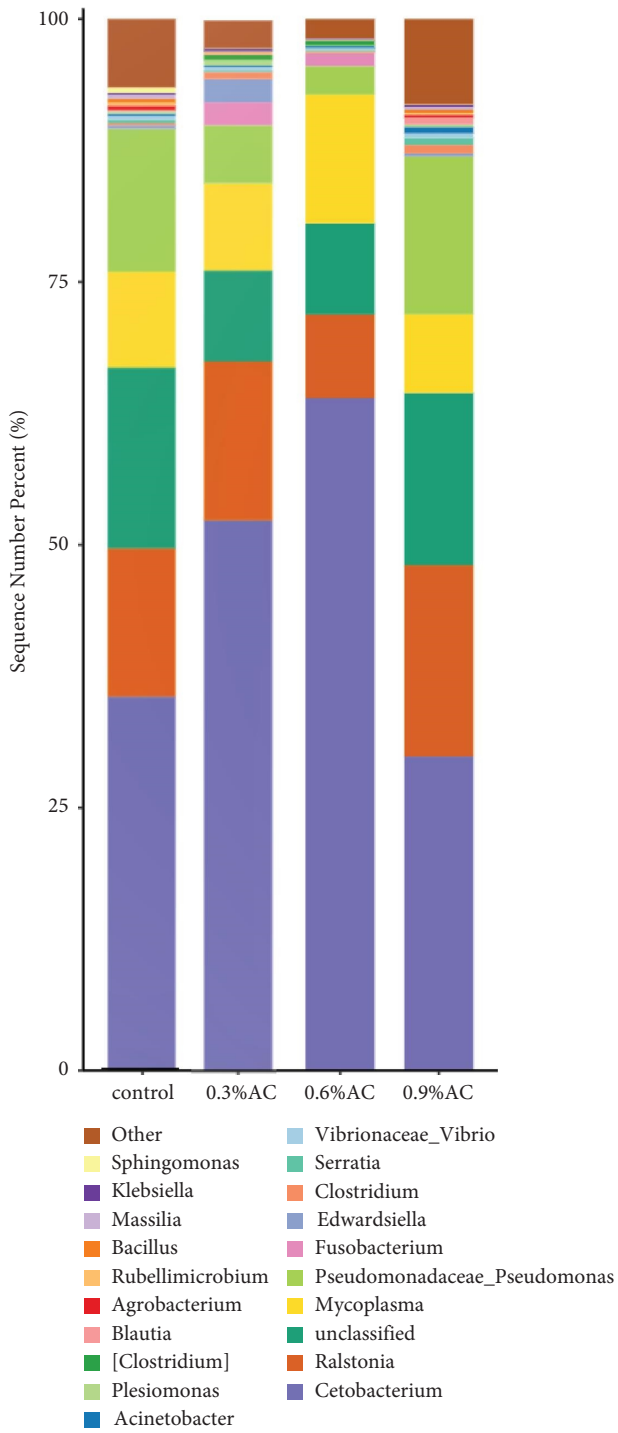


Figure 3: Effects of different concentrations of acetoin on the column diagram of relative abundance of species above the genus level in intestinal flora of *Micropterus salmoides* for 45 days.

## 4. Discussion

In this study, *Micropterus salmoides* was used as the experimental object to study the role of acetoin in inducing feeding and improving physiological function and further speculated the application effect and possible mechanism of acetoin in aquaculture.

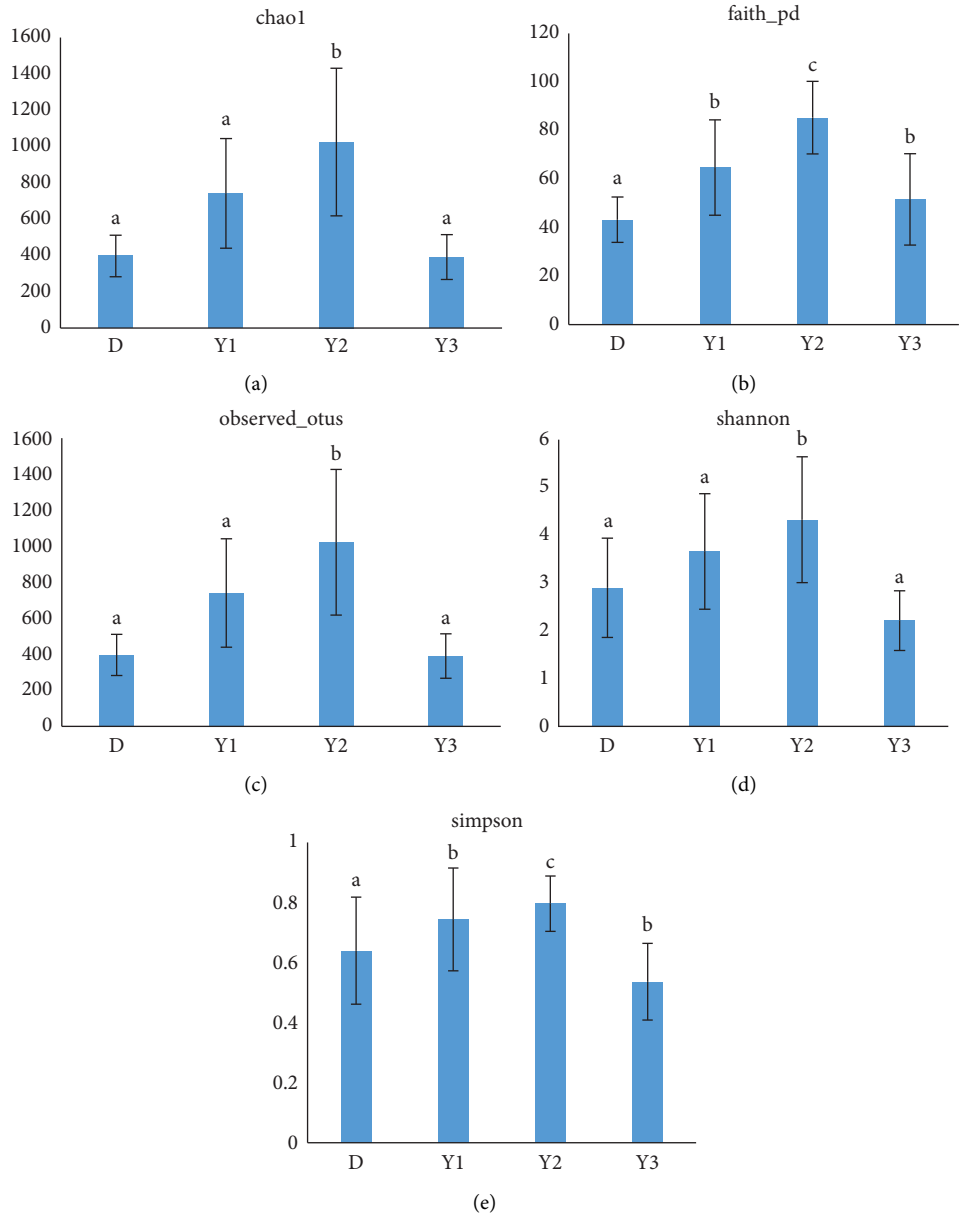


FIGURE 4: Effects of different concentrations of acetoin on $\alpha$ diversity of intestinal flora for 45 days of *Micropterus salmoides*. Chao1 index (a), faith-pd index (b), observed-otus index (c), Shannon index (d), and Simpson index (e) were measured. All results were expressed as mean values and standard errors of 5 numbers of *Micropterus salmoides*. Different lowercase letters indicate significant differences between different mass concentrations ($P < 0.05$).

Acetoin is an important metabolite of microorganisms and plants and has a strong cream, fat, and white off-sample aroma due to its special chemical structure [7]. In addition, acetoin can promote plant growth [22] and act as an insect attractant [23] to control pests. In terms of plant growth, acetoin promotes root development, making it easier for plants to obtain water and nutrients and thus promoting faster growth and development of the plants they host [22]. And in terms of pest control, acetoin is sprayed on a specific crop with an olfactory measuring device to attract insects with its unique fragrance, which not only avoids insect damage to crops but also prevents excessive chemical pesticides and environmental pollution caused by pesticides, thus protecting other untreated crops in the same land [23]. Furthermore, aquatic food attractants mostly induce fish to eat through their physical properties such as color and fragrance, and acetoin might have potential effects as attractants because of its special chemical structure and strong aroma.

Betaine has the function of enhancing fish metabolism [24], promoting fat metabolism, inhibiting liver fat deposition, alleviating stress, regulating osmotic pressure balance, improving intestinal digestive enzyme activity [25], and improving the feed utilization rate [26]. Studies have shown that acetoin and betaine have almost the same feeding effect [10]. Therefore, the results of this experiment also

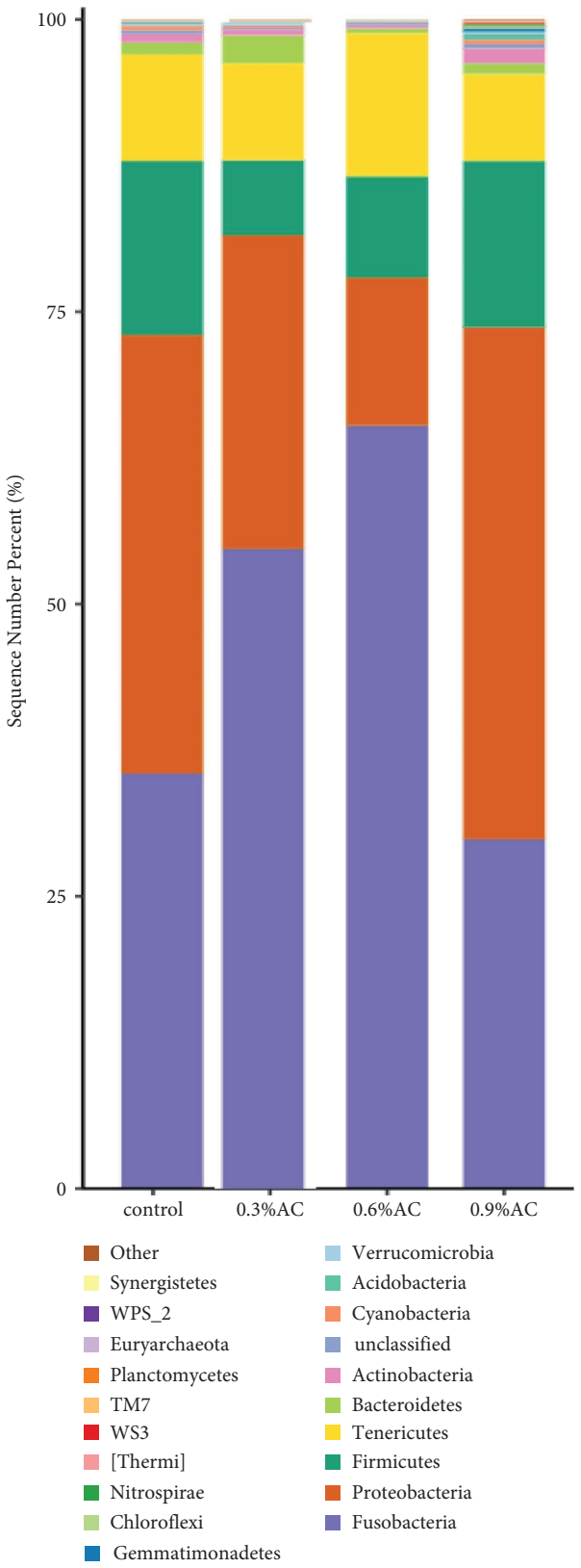


FIGURE 5: Effects of different concentrations of acetoin on columns of relative abundance of species above the phylum level in intestinal flora of *Micropterus salmoides* for 45 days.

verify the effects of acetoin on survival, growth performance, feed utilization of fish fed diets, and intestinal digestive enzyme activity. The experiment results show that the final body weight, weight gain rate, specific growth rate, and feed efficiency rate of fish fed diets supplemented with different concentrations of acetoin were significantly higher than those of fish fed control diet after 45 days of feeding ($P < 0.05$) (Table 2). In this experiment, the intestinal digestive enzyme activities of *Micropterus salmoides* increased with the increase of dietary acetoin level. The maximum value was reached when the acetoin was 0.6% (Figure 1). This indicated that the addition of acetoin in the diet could improve the feed intake and feed utilization rate of *Micropterus salmoides,* increase intestinal digestive enzyme activity, promote intestinal peristalsis, and accelerate the absorption of nutrients. The increase of digestive enzyme activity can improve the intestinal microenvironment and change the number of various bacteria in the intestine. Intestinal microorganisms can participate in the absorption and metabolism of nutrients such as carbohydrates and fats, the fermentation of indigestible dietary fiber, and the synthesis of certain vitamins in the host body [27]. At the same time, intestinal microorganisms can also regulate immune function by interacting with the inner wall of the host gastrointestinal tract [27, 28]. Studies have shown that a large number of *Cetobacterium* colonized in fish intestines can produce vitamin B12, which is closely related to body metabolism [29]. The relative abundance of *Cetobacterium* in the intestinal flora of *Micropterus salmoides* was significantly increased after adding acetoin (Figure 3), which may further promote the production of vitamin B12 in *Micropterus salmoides*. Therefore, because the body has to provide adequate nutrition, it presents a healthier state. Furthermore, the higher the relative abundance of *Proteobacteria* in fish, the lower the expression of tight junction proteins, resulting in intestinal damage [30]. At the phylum level, the relative abundance of *Proteobacteria* in the intestinal flora decreased after the addition of acetoin to the diet (Figure 5). This suggests that acetoin may increase the content of probiotics, reduce the number of pathogenic intestinal flora in the body, and increase the disease resistance of the body. Diversity $\alpha$ is usually used to evaluate the richness and diversity of microbial communities, which is often measured by observed otus, faith-pd, chao1, Shannon, and Simpson indexes [31]. Observed-otus, faith-pd, and chao1 indices are often used to indicate the number of species contained in the sample. The higher the value, the higher the species richness of the sample, and Shannon and Simpson indexes are mainly used to estimate the microbial diversity in the sample. The higher the value, the higher the community diversity [32]. The results of this study showed that acetoin could significantly increase the chao1, observed otus, Shannon, Simpson, and faith-pd indexes in intestinal flora (Figure 4). The indexes of chao1, observed-otus, and faith-pd in the experimental group were significantly different from those in the control group ($P < 0.05$). It indicated that acetoin could increase the richness and diversity of intestinal species in

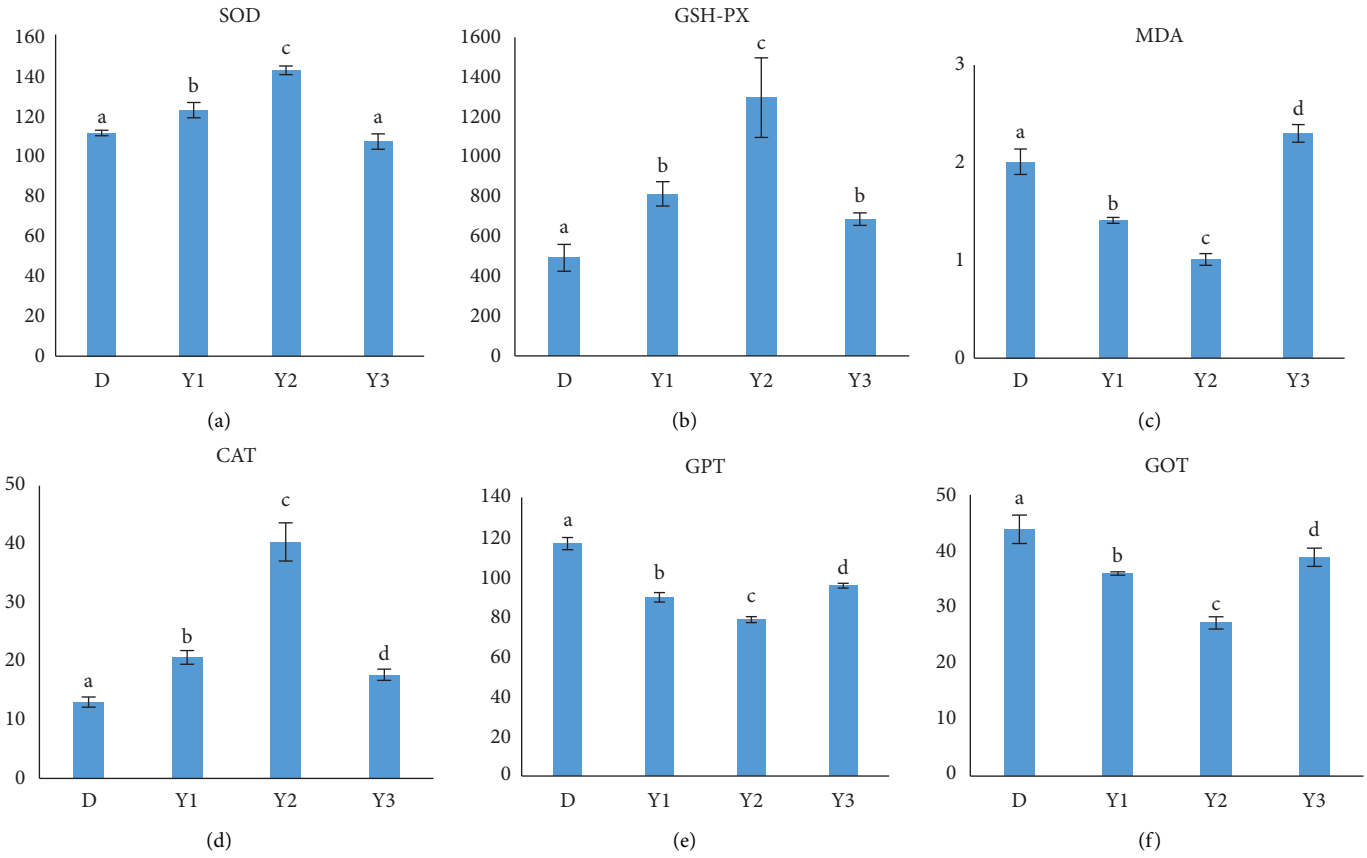


Figure 6: Effects of different concentrations of acetoin on antioxidant levels of the liver for 45 days of *Micropterus salmoides*. SOD (a), GSH-PX (b), MDA (c), CAT (d), AST (e), and ALT (f) in the liver of *Micropterus salmoides* were determined. All results were expressed as mean values and standard errors of 5 numbers of *Micropterus salmoides*. Different lowercase letters indicate significant differences between different mass concentrations ($P < 0.05$).

*Micropterus salmoides*. The body's balance is adjusted so that the body is in a more stable state.

The host provides a habitat for intestinal flora, and intestinal microorganisms have an irreplaceable impact on the nutrition, health, and immunity of the host [33, 34]. Intestinal microorganisms and antioxidant enzymes in vivo promote and complement each other and jointly act in the body. In this study, the activities of SOD, GSH-PX, and CAT in the liver of *Micropterus salmoides* supplemented with acetoin were increased, and the 0.6% acetoin concentration was the most significant (Figures 6(a), 6(b), and 6(d)). This may be related to the function of antioxidant indicators. CAT and SOD are important antioxidant enzymes in the body's antioxidant system. CAT plays a key role in maintaining the balance of hydrogen peroxide by catalyzing the decomposition of hydrogen peroxide in the body, so it is an essential enzyme to protect cells from oxidative damage [35]. SOD exists in aerobic respiratory organisms, which converts superoxide into hydrogen peroxide and removes multiple biological reactive oxygen species intermediates to avoid adverse conditions such as oxidative damage [36]. The activities of SOD, GSH-PX, and CAT in *Micropterus salmoides* supplemented with acetoin were significantly increased, which could effectively scavenge free radicals and reduce cell damage. Besides, MDA can directly reflect the level of lipid peroxidation and the degree of endogenous oxidative damage and reflect the degree of free radical damage to the body [37]. ALT is involved in the protein metabolism of organisms and widely exists in animal mitochondria. Damage or necrosis of hepatocytes or some tissues will increase the ALT in blood. Changes in AST activity are closely associated with inflammation, degeneration, and necrosis of liver cells [38]. Normally, the activity of AST was low, but when the corresponding cells were damaged, the membrane permeability increased, and the AST release concentration in the cytoplasm increased. These indicators directly or indirectly represent the degree of free radical damage to the body, reflecting the health of the body. The experimental results are consistent with this. The experimental results showed that the content of MDA and the activities of ALT and AST in each experimental group and the control group were significantly different ($P < 0.05$), and 0.6% AC was the best (Figures 6(c), 6(e), and 6(f)). MDA, ALT, and AST of the diet supplemented with 0.9% AC were slightly higher than those of the control group, which may be because some chemicals in the body could not be metabolized and accumulated in the body after the high concentration of acetoin entered the body. With increased oxidative damage to the liver, the body's antioxidant capacity decreased.

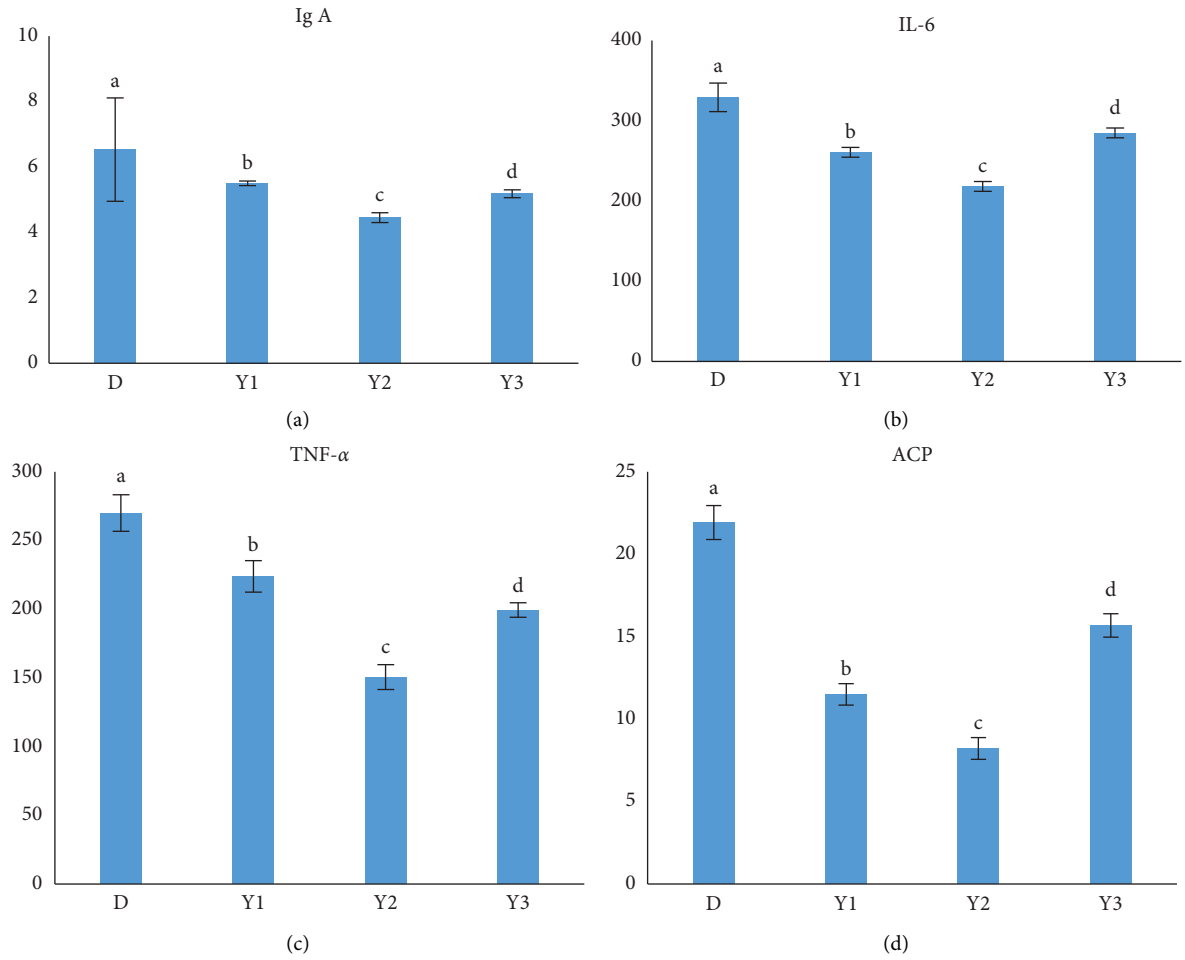


Figure 7: Effects of different concentrations of acetoin on serum indexes for 45 days of *Micropterus salmoides*. IgA (a), IL-6 (b), TNF-$\alpha$ (c), and ACP (d) in serum of *Micropterus salmoides* were determined. All results were expressed as mean values and standard errors of 5 numbers of *Micropterus salmoides*. Different lowercase letters indicate significant differences between different mass concentrations ($P < 0.05$).

Inflammatory factors can hinder the synthesis of nutrients provided by parenteral and enteral nutrition support in human tissues, resulting in malnutrition that cannot be significantly improved. Oxidative damage is closely related to inflammation. On the one hand, oxidative damage can promote the expression of inflammatory mediators such as TNF-$\alpha$ and IL-6. On the other hand, activated inflammatory cells produce more reactive oxygen species, resulting in increased levels of oxidative stress after inflammatory lesions [39]. Studies have reported that in animal models of liver fibrosis, serum contents of the inflammatory cytokines TNF-$\alpha$ and IL-6 increased with the aggravation of fibrosis and were positively correlated [40]. In this experiment, compared with the control group, the contents of IL-6, TNF-$\alpha$, and other inflammatory factors in the serum of *Micropterus salmoides* fed with a diet supplemented with acetoin were reduced to varying degrees (Figure 7). This corresponds to the report. This indicated that the addition of an appropriate amount of acetoin in the feed could improve the immunity of *Micropterus salmoides* and enhance the resistance to diseases.

This experiment showed that acetoin had an attractive effect on *Micropterus salmoides*, and the attractive effect gradually increased with the increase in concentration, which was the best at 0.6%, and then decreased with the increase in acetoin concentration. According to the antioxidant activity index in the liver, 0.3% and 0.6% of acetoin may be more effective in scavenging free radicals in the body, protecting the liver from damage, and even may have a certain ability to make the liver more healthy. The antioxidant index in the 0.9% AC liver was low, which might be due to the excessive accumulation of chemical organic substances in the body and the high concentration of acetoin, resulting in liver damage and affecting its activity and immunity.

## 5. Conclusion

To sum up, acetoin has a good lure effect on *Micropterus salmoides*, and 0.6% is the best lure concentration of acetoin. Its mechanism may be related to antioxidation and anti-inflammation. The unique chemical structure of acetoin

endows it with a pleasant aroma to induce fish to eat, which accelerates intestinal peristalsis of *Micropterus salmoides* and enhances intestinal digestive enzyme activities, thus leading to changes in the living environment of intestinal microorganisms. The increased activity of intestinal digestive enzymes leads to changes in the living environment of intestinal microbes, resulting in an increase in the number of beneficial bacteria and a decrease in the number of harmful bacteria. The activity of antioxidant enzymes in the liver were further enhanced to reduce the damage of free radicals. Finally, the immunity of *Micropterus salmoides* was enhanced. This experiment only made a preliminary discussion on this aspect, and the specific mechanism is not clear. Whether B-marriage has antioxidant and anti-inflammatory abilities needs further study.

## Data Availability

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

## Ethical Approval

The experiments conducted at ishwere in accordance with the current animal welfare laws of China. The fish, i.e., *Micropterus salmoides,* is not an endangered species. Herewith, the authors declare that all guidelines were followed for the care and use of fishFsh in the present study.

## Consent

All authors agreed for publication.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

## Authors' Contributions

Chunjing Cai, Junhua Lu, and Ying Wang determined the sample and processed the data. Zhe Li corrected the manuscript. Chen Wang and Pengfei Li collected the sample. Hui Wang provided breeding grounds. Jie Chu designed and supervised the research work and guided the experiments. Wu Meng provided project and technical support. All authors read and approved the manuscript.

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