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Research Article

Studies on the growth performance, physiological and biochemical indicators and intestinal flora of largemouth bass (*Micropterus salmoides*) in different growth stages

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Abstract

This paper studies the differential changes in growth performance, physiological and biochemical indicators, and intestinal flora of Largemouth Bass in different growth stages, and explores and explains the reasons for the differences, so that breeders can take effective measures to improve the breeding efficiency in the necessary growth stages, and add a complete fish production file to the local and similar areas. The aquaculture pond was regularly monitored and sampled for about five months, with a total of 153 days and an interval of 30 days. After sampling the fish tissue in the test base, put the sample into a foam box with ice bags, and take it back to the laboratory to measure the changes in growth indicators, liver function indicators, antioxidant indicators, and Digestive enzyme indicators of Largemouth Bass in each group according to the national monitoring standards. In addition, the high-throughput sequencing method was used for the intestinal microorganisms of each group, and 16s rRNA analysis was carried out. During the breeding process of Largemouth Bass, the average range of weight gain rate is 35.33-71.33 ($p<0.05$), of hepatosmatic index is 1.61-2.71 ($p<0.05$), of total bilirubin is 1.00-4.12 ($p<0.05$), of cereal grass Transaminase is 3.24-9.53 ($p<0.05$), of Alanine transaminase is 10.45-38.80 ($p<0.05$), of Superoxide dismutase is 2.02-3.14 ($p<0.05$), of Catalase is 4.36-6.91 ($p<0.05$), and of Pepsin is 52.92-81.84 ($p<0.05$). The most obvious is that Largemouth Bass has a rapid growth period from June to July. In August, when parasites are widespread, the antioxidant defense system in the body of the Bass is most vulnerable to damage. In October, when the climate gradually cools, the Bass begins to accumulate fat. In addition, among the intestinal flora, the dominant bacteria indifferent growth stages are mainly Mycoplasma and Cetobacterium, and the difference in intestinal flora in different stages is obvious. With the change in growth months, there are obvious differences in the growth performance, physiology and biochemistry, and intestinal flora of Largemouth Bass in different growth stages. In the necessary stages of the breeding process, measures should be taken to improve the health status of the fish, feed nutrition, and pond water quality, and some microecological reagents can be accurately used to improve breeding efficiency. This experiment provides data support for breeding technology improvement, probiotics screening, disease prevention, and intestinal microecology research of Largemouth Bass during its growth.

Introduction

Largemouth Bass (*Micropterus salmoides*) has the laudatory name of freshwater grouper and belongs to Actinopteri, Perciformes, Centrarchidae and Micropterus. The body is Fusiform shaped, and the optimal water temperature is 25-30°C (Hiroshi and Yoshito, 2018; Harimana *et al.*, 2019) This fish is native to Canada and the United States and was introduced to China in the 1970s. With strong adaptability, fast growth, short breeding cycle, easy fishing, fresh and tender meat as well as beautiful appearance, it is deeply loved by farmers and consumers (Wu *et al*., 2019; Da-Silva *et al*., 2020). The global cultivation of Largemouth Bass has shown an upward trend in recent years. Therefore, in the different growth stages of Largemouth Bass, it is crucial to adopt appropriate scientific cultivation management. Nowadays, in many pond aquaculture areas, farmers lack systematic monitoring and statistics on the growth and health changes of Bass during the aquaculture process, so they are unable to take effective measures to improve aquaculture efficiency in specific stages and aspects, and even cause losses (Hiroshi and Yoshito, 2018; Nurmahendra *et al*., 2021). Therefore, this test aims to study the differential changes in growth performance, physiological and biochemical indicators, and intestinal flora of Largemouth Bass in different growth stages and explores and explains the reasons for the differences, so that breeders can take effective measures to improve the breeding efficiency in the necessary growth stages and add a complete fish production file to the local and similar areas.

At present, the research on the cultivation of Largemouth Bass mainly focuses on the differential changes under different environments, different feeding modes, and disease conditions. For example, some researchers explored the differences in physiological and biochemical indicators of Largemouth Bass under different temperatures (Aguilar *et al*., 2023). Some researchers explored the changes in growth indicators of Largemouth Bass under different feeding frequencies (Liu Q. *et al*., 2022). Some researchers explored the changes in the liver function indicators of Largemouth Bass when yeast and starch components were mixed in different proportions under different nutritional components of feed (Feng *et al*., 2021). Some researchers tested the difference in antioxidant index changes of Largemouth Bass when the concentration of dissolved oxygen in water is different (He *et al*., 2022). To sum up, there are few experiments to explore the physiological differences of Largemouth Bass at different growth stages. Therefore, the entry point of this study is to track the whole process of Largemouth Bass from entering the pond to selling out of the pond. In a total of 153 days, the physiological and biochemical indicators of Largemouth Bass in different growth stages were sampled and tested, in order to explore the differences in health indicators of Largemouth Bass in different growth stages. The experiment provided data support and theoretical basis for the improvement of breeding technology, probiotics screening, disease prevention, and intestinal microecology research during the growth of Largemouth Bass, enabling breeders to take measures during the necessary stages of Largemouth Bass rearing and improve the culture defects.

Materials and methods

Experimental Conditions

The Largemouth Bass used in this experiment was obtained from Tianjin Jiahe Tianyuan Ornamental Fish Culture Co. Ltd., and the ponds were also provided by this company. Three ponds were selected for regular sampling. The fish were released on 10 May 2021 and sold on 8 October 2021. Pond 1 covers an area of 5,086.7 m^2, with a depth of 2.0 m; Pond 2 covers an area of 4,826.7 m^2, with a depth of 2.3 m; Pond 3 covers an area of 4,786.7 m2, with a depth of 2.3 m. During the breeding of Largemouth Bass, the water temperature of the ponds ranged between 21.75 ˚C and 28.94 ˚C. Each pond was equipped with three aerators, with an engine power of 3kw. The feed used was the extruded feed for Bass produced by Shandong Haibo Agricultural and Animal Husbandry Technology Co. Ltd. According to the labels, the types and proportions of raw materials in the feed used at each stage were the same, despite the differences in particle size. Among the main components, crude protein, fat, ash, and fiber were accounted at 48%, 9%, 12%, 12%, and 4% in dry matter, repectively. The main raw materials were fish oil, soybean meal, and flour. Other inputs included chlorine dioxide, citric acid, and medicine that protects the liver and cultivates the intestines and stomach.

The initial data on the water quality of the three ponds are shown below. The sampling time was 9 May 2021, the sampling points were the centers of the ponds at one-meter depth. In Table 1, the pH value, nitrite, ammonia nitrogen, and dissolved oxygen were measured according to national standards. The glass electrode method, the diazo coupling method, Nessler's reagent method, and the iodine method were used to measure the pH value, nitrite, ammonia nitrogen, and dissolved oxygen, respectively (Lei, 2006).

Table 1: The initial state of the main indicators of the pond.

Project	1	2	3
pH (mg/L)	8.32	8.28	8.29
NIT (mg/L)	0.05	0.03	0.04
NH3-N (mg/L)	0.18	0.22	0.2
DO (mg/L)	8.39	8.6	8.19

Notes: nitrite (NIT), ammonia nitrogen (NH3-N), dissolved oxygen (DO).

Content of the experiment

Three ponds were selected and sampled regularly for five months, 153 days in total. The interval between every two samples was 30 days. The changing trends of growth, liver function, antioxidant capacity, digestive enzymes, and gut microbiota of Largemouth Bass at each stage were investigated, and the influences of various factors such as the growth characteristics of the fish, environmental factors, and nutritional factors under the conditions of traditional pond farming on the growth and health of Largemouth Bass were analyzed.

The fish were released on 10 May 2021, with 7,540 in Pond 1，9,330 in Pond 2, and 11,960 in Pond 3. The initial weight of the fish was (54.00±2.30) g, and the sizes were similar. After 153 days, the fish were caught and sold on 8 October 2021. The dates of the monthly sampling during this period were 10 June, 10 July, 10 August, 10 September, and 10 October. For simplicity, we named each date as Q1, Q2, Q3, Q4, and Q5.

The aerators were turned on every sunny morning for two hours and in the second half of the night on cloudy days. In addition, the aerators were turned on to maintain dissolved oxygen above 4 mg/L when the pond water lacked oxygen. The fish were fed at 6 a.m. and 4 p.m. with a feeding rate of 4%-5% body weight.

On cloudy or rainy days, the feeding amount was halved or not feeding. During the breeding period, citric acid (2.5L of citric acid diluted in 12L of water) was sprayed over the ponds every 25 days to supplement carbon sources (Pang *et al*.,2023). In the last two months of the breeding period, medicine that protects the liver and cultivates the intestines and stomach were mixed into the feed to improve the health of the fish. The medicine accounted for 1.5%-2% of the total feed each day, and this mixture was fed once a month, each time lasting three days. During the period of a high incidence of lernaeosis among Largemouth Bass, a chlorine dioxide liquid agent was added to a small amount of water, diluted to 0.1-0.2mg/L, and sprayed in the ponds for three consecutive days (Agnihotri *et al*., 2020). The drug was used once at around 9 to 10 a.m. When the water quality of the ponds was unstable and the transparency was low, some pond water would be pumped out, with some fresh water added. Before adding water, one-third of the original water was pumped out. A large rubber skin hose was used to pump water from the main reservoir, and the water inlet was filtered with a dense mesh to prevent debris from entering the fish pond.

Methods of measuring growth performance

Before the fish were released, the weight of the fish was measured, and the average value was used as the initial value. After the fish were released into the ponds, they were sampled every 30 days. To measure the weight of the fish, 30 fish were sampled from each pond in each stage (5 stages), and their livers were weighed. The equations of indexes including the growth rate of fish weight, hepatosomatic index, specific growth rate, and feed coefficient rate are as follows (Chen *et al*., 2023). Here, it needs to be explained that as the growth indicators of this experiment are measured periodically every other month, the final weight value in the original formula will be replaced by the current weight value, and the initial weight value will be replaced by the previous stage value. However, the original meaning of the formula has not changed at all:

Hepatosomatic index (HSI, 100%) = 100 × liver weight/fish weight Growth rate of fish weight (WGR, 100%) = 100 × (final weight - initial weight)/initial weight

Specific growth rate(SGR,%/d)=100 × (ln final weight – ln initial weight)/number of feeding days

Methods of measuring liver function

For the liver samples obtained from each sampling, liver function indexes, including total bilirubin (TBIL), total cholesterol (T-CHO), glutamic oxalacetic transaminase (GOT), glutamic alanine transaminase (GPT), and albumin (ALB), were measured. Before the measurement, pre-cooled normal saline was added at a mass-to-volume ratio of 1:9 (mg/mL), and 10% homogenate was made by a homogenizer, centrifuged at 1788.8×g for 10 min at 4˚C. The supernatant was taken for the measurement of liver function (Che *et al*., 2023). The TBIL, T-CHO, GOT, GPT, and ALB kits used to measure liver function indexes were purchased from Nanjing Jiancheng Institute of Bioengineering Institute. The vanadate oxidation method, the GPO-PAP enzyme method, the UV colorimetric method, the Reitman-Frankel colorimetric method, and the bromocresol green method were used to measure TBIL, T-CHO, GOT, GPT, and ALB, respectively (Ni *et al*., 2021; Jing *et al*., 2022).

Methods of measuring antioxidant capacity

For the liver samples obtained from each sampling, the indexes of antioxidant capacity, including superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA), and total antioxidant capacity (T-AOC), were measured. Before the measurement, pre-cooled normal saline was added at a mass-to-volume ratio of 1: 9 (mg/mL), and 10% homogenate was made by a homogenizer, centrifuged at 1788.8×g for 10 min at 4℃. The supernatant was taken for the measurement of antioxidant capacity (Che *et al*., 2023). The SOD, CAT, MDA, and T-AOC kits used to measure the indexes of antioxidant capacity were purchased from Nanjing Jiancheng Institute of BioengineeringInstitute. The chemiluminescence colorimetric method, the ultraviolet absorption method, the thiobarbituric acid test, and the iron ion reduction method were used to measure SOD, CAT, MDA, and T-AOC, respectively (Feng *et al*., 2021).

Methods of measuring digestive Enzymes

For the stomach samples obtained from each sampling, the indexes of digestive enzymes, including amylase (AMS), lipase (LPS), and pepsin (PEP), were measured. Before the measurement, pre-cooled normal saline was added at a mass-to-volume ratio of 1:9 (mg/mL), and 10% homogenate was made by a homogenizer, centrifuged at 1788.8×g for 10 min at 4℃. The supernatant was taken for the measurement of digestive enzymes. The AMS, LPS, and PEP kits used to measure the indexes of digestive enzymes were purchased from Nanjing Jiancheng Institute of Bioengineering Institute. AMS was measured through the starch-iodine method, and LPS and PEP were measured through chemical colorimetric methods (Zhou P. *et al*., 2022).

Methods of measuring gut microbiota

After the fish were released into the ponds, they were sampled every 30 days. During the process, one fish was selected from each pond (three in total) for dissection, and the samples of intestinal contents were reserved. The contents of the three fish at each stage were preserved in sample vials, and the five vials (for stage Q1 to stage Q5) were stored at -80°C for measurement. The 16SrRNA samples of the gut microbiota of Largemouth Bass preserved in the above five vials were sent to Shanghai Majorbio Bio-Pharm Technology Co., Ltd. for high-throughput sequencing (Liu Q. *et al*., 2022).

Data analysis and processing

All data to be analyzed were collated by Excel 2019, and SPSS 22.0 was used for statistical analysis. After using Excel software to collect data, we compared the inter-group differences between samples using SPSS software, and the screening condition was 5% level.

Results

Changes in the growth performance of Largemouth Bass in different growth stages

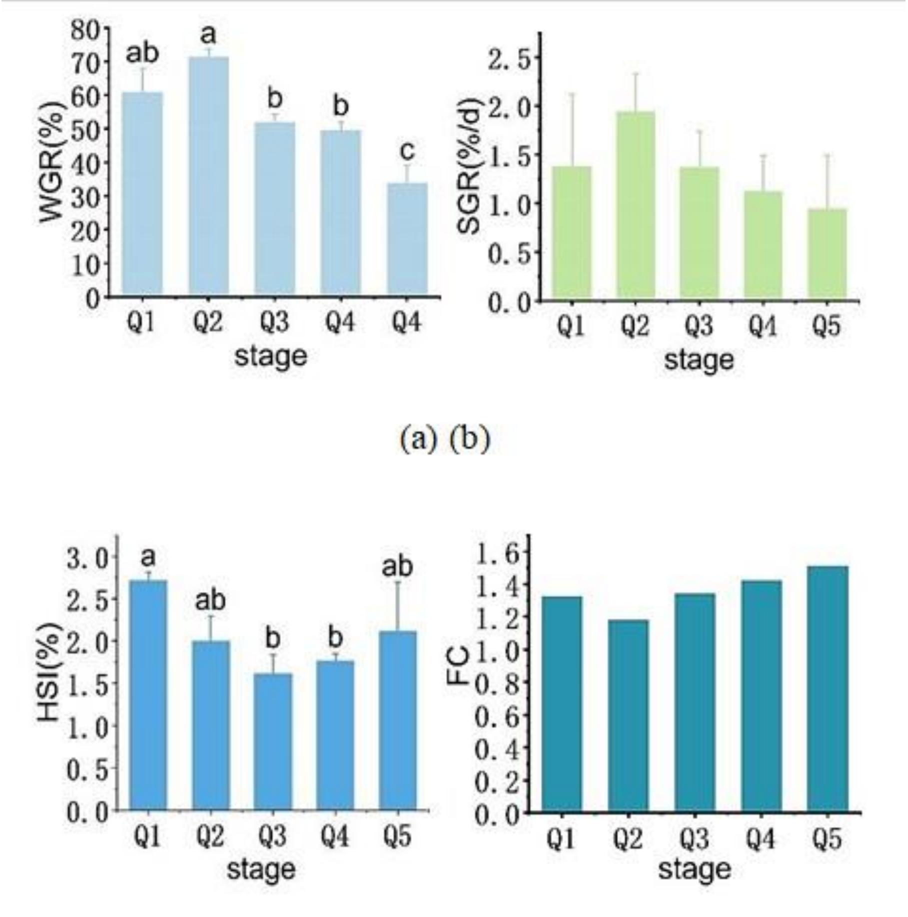
WGR and SGR can explicitly reflect the growth of the fish, and HSI is also a critical index that is sensitive to long-term nutrition, The liver, as the main organ of intermediate metabolism for fish, plays an important role in the metabolism of lipids, carbohydrates, and other nutrients. Therefore, the changes in HSI can help researchers grasp the nutrition and health status of the fish. In addition, the measurement of FC can reflect the changing trend of the feed conversion ratio and the effects of the feed on the farmed fish.

The specific sampling dates were June 10, July 10, August 10, September 10, and October 8, 2022, which were abbreviated as Q1, Q2, Q3, Q4, and Q5, respectively. Therefore, the group Q1 in the chart represented May 10 to June 10, the group Q2 represented June 11 to July 10, the group Q3 represented July 11 to August 10, the group Q4 represented August 11 to September 10, and the Group Q5 represented September 11 to October 8. Figure 1 showed the changes in the growth performance of Largemouth Bass in different growth stages with data, and Figure 1 (a to d) showed these changes with images. It can be clearly seen from Figure 1 (a to d) that the fish weight gain rate (WGR) showed a trend of first increasing and then decreasing, with the peak appearing in Group Q2. There were significant differences between Q2 and Q3 groups, Q4 and Q5 groups (p<0.05). Group Q1, group Q3, and group Q4 showed significant differences compared to group Q5 (p<0.05). The hepatosmatic index (HSI) showed a trend of first decreasing and then increasing, with the peak appearing in Group Q3. Group Q1 showed significant differences compared to group Q3 and group Q4 (p<0.05). The specific growth rate (SGR) showed a trend of first increasing and then decreasing, with the peak appearing in group Q2. There was no significant difference in each stage

(p>0.05). The feed coefficient (FC) showed a trend of first decreasing and then increasing, with the minimum appearing in group Q2.

Changes in the liverfunction of largemouth bass in different growth stages

TBIL is an important index of liver function, and a value higher than the normal range can cause hepatitis, fatty liver, cirrhosis, and other serious liver diseases.



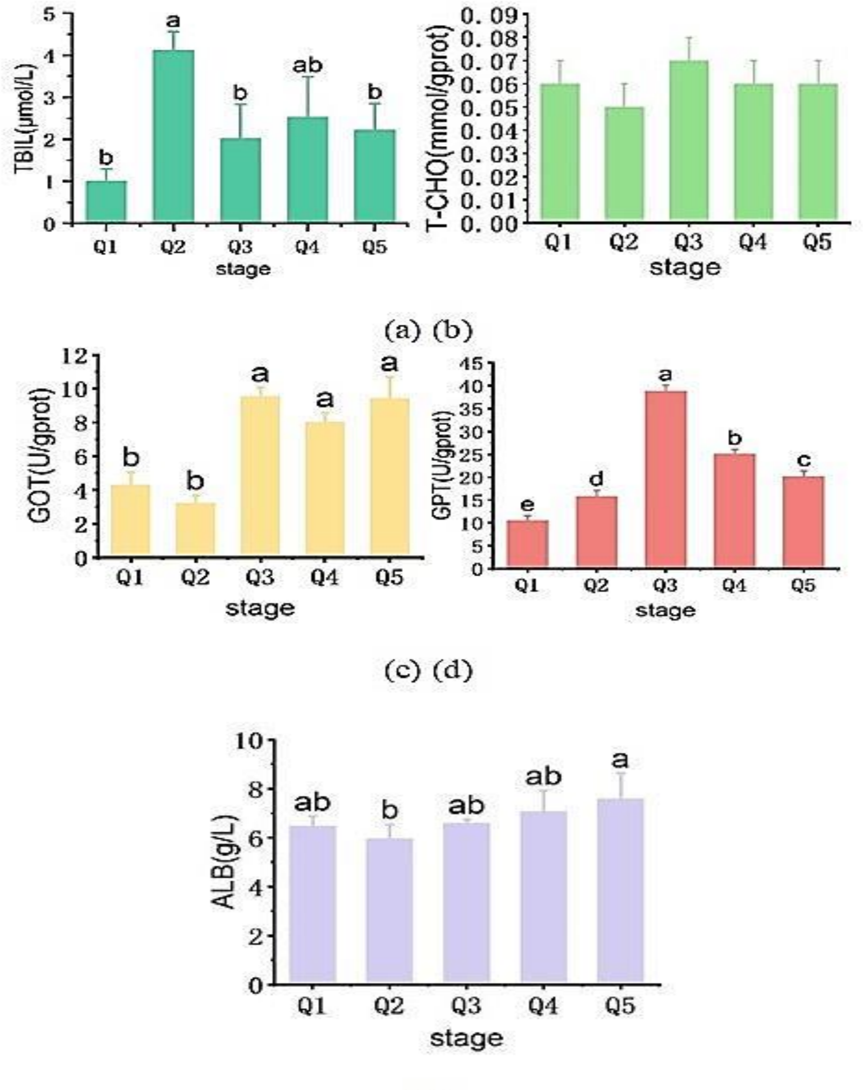
(a) (b)

(c) (d)

Figure 1: Changes in the growth performance of Largemouth Bass in different growth stages (5% level). Notes: Different letters in the superscripts of figures in the same row indicate a significant difference (p<0.05); the same letter or no letter indicates an insignificant difference (p >0.05). The same applies to the subsequent tables. growth rate of fish weight (WGR), specific growth rate (SGR), hepatosomatic index (HSI), feed coefficient rate (FC).

T-CHO is a main index that reflects the lipid metabolism of fish, and its high level can reflect fatty liver in fish (Du , 2014). In addition, a concentration that is too high or too low is likely to cause many diseases. The levels of GOT and GPT can suggest the degree of liver cell damage, and ALB, as a sensitive index of liver function, can help assess the degree of liver damage. Therefore, liver function is an important measure of hepatocyte damage, and some indexes of liver function, such as GOT and

GPT, can be used as biomarkers when individuals are exposed to environmental stress (Mcgill , 2016). Figure 2 showed the changes in the liver function of Largemouth Bass in different growth stages with data, and Figure 2 (a to e) used images to show these changes. From Figure 2 (a to e), it can be clearly seen that the total bilirubin (TBIL) in the liver of Largemouth Bass showed an overall trend of first rising and then declining. Q2 reached the peak, and Q4 slightly recovered. There were significant differences between Q2 and Q1, Q3 and Q5 groups (p<0.05).



(a) (b)

(c) (d)

(e)

Figure 2:Changes in the liver function of Largemouth Bass in different growth stages (5% level). Notes: total bilirubin (TBIL), total cholesterol (T-CHO), glutamic oxalacetic transaminase (GOT), glutamic alanine transaminase (GPT), albumin (ALB).

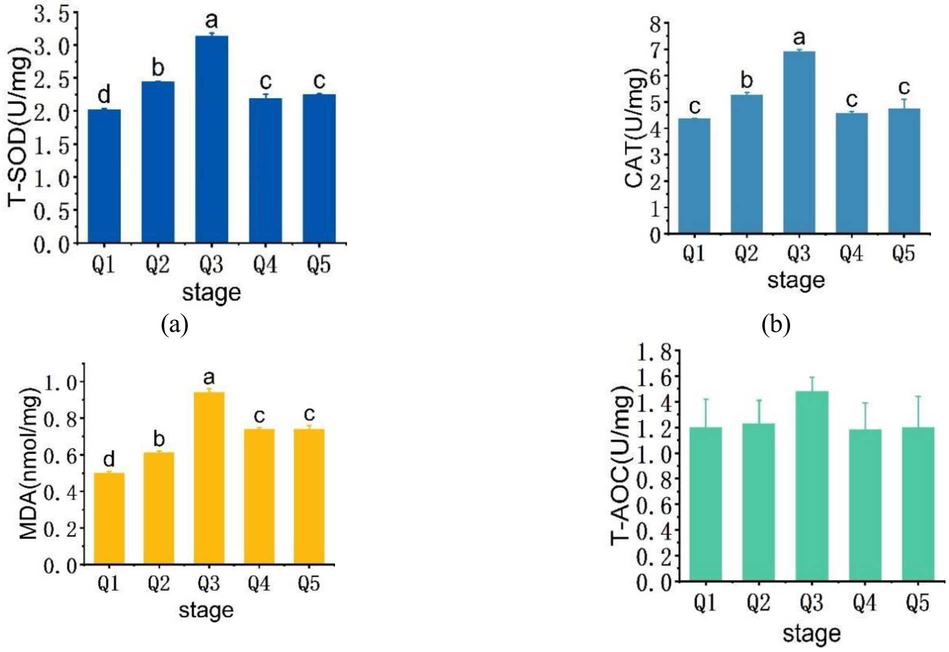
The total cholesterol (T-CHO) of the liver showed no significant changes overall, and there was no significant difference in each stage (p>0.05). Gluten grass Transaminase (GOT) in the liver showed an overall upward trend and reached the peak in Group Q3. There was a significant difference in Q3, Q4, Q5, and Q1 groups (*p*<0.05), while there was a significant difference in Q3, Q4, Q5, and Q2 stages (*p*<0.05). The alanine transaminase of the liver showed an overall trend of first rising and then falling, and there were significant differences in Q1 to Q5 (*p*>0.05), with the peak value appearing in group Q3. The albumin (ALB) of the liver showed an overall upward trend, reaching its peak in group Q5, and slightly decreasing in group Q2. Except for group Q2, there was no significant difference between the groups (*p*>0.05).

Changes in the antioxidant capacity of largemouth bass in different growth stages

Oxidative damage is an important mechanism of liver injury. The liver, as an important metabolic organ, is susceptible to free radical attack. Hepatocytes are the bearers of liver function, and mitochondria, microsomes, and peroxisomes in hepatocytes can produce a large number of free radicals, causing oxidative stress damage in hepatocytes and then even liver dysfunction (Yu *et al*., 2021). The antioxidant defense system of hepatocytes is composed of related antioxidant enzymes and cytokines, among which SOD is the most important enzyme in the system (Dosumu *et al*., 2021). CAT mainly exists in livers and erythrocytes of animals, with a higher content in the liver. Its enzymatic activity provides the main antioxidant defense for the body. MDA is the end product of lipid peroxidation that can indirectly reflect the severity of the free radical attack (Akgun *et al*., 2021). T-AOC can reflect the sum of the antioxidant capacities of the system and, to some extent, the total capacity of the body to scavenge reactive oxygen species.

Figure 3 showed the changes in the antioxidant capacity of Largemouth Bass in different growth stages with data, and Figure 3 (a to d) showed these changes with images. It can be clearly seen from and Figure 3 (a to d) that the superoxide dismutase (SOD) of the fish liver showed a trend of first increasing and then decreasing, and reached the peak in group Q3. There were significant differences (*p*<0.05) among groups Q1 to Q4 groups, while there were no significant differences (*p*>0.05) between Q4 and Q5 groups. The Catalase (CAT) showed a trend of increasing first and then decreasing.

There was a significant difference between Q1 to Q3 groups (*p*<0.05), between Q2 group and other groups (*p*<0.05), between Q3 group and other groups (*p*<0.05), and there was no significant difference between Q4 and Q5 groups (*p*>0.05). Malondialdehyde (MDA) showed an overall trend of first rising and then falling with a significant difference in the first three groups (*p*<0.05), and no significant difference between Q4 and Q5 groups (*p*>0.05). The total antioxidant capacity (T-AOC) of the liver showed a less significant trend of increasing first and then decreasing, but there was no significant difference between the groups (*p*>0.05).



(a) (b)

(c) (d)

Figure 3:Changes in the antioxidant capacity of Largemouth Bass in different growth stages (5% level). Notes: superoxide dismutase (T-SOD), catalase (CAT), malondialdehyde (MDA), and total antioxidant capacity (T-AOC).

Changes in the digestive enzyme activities of largemouth bass in different growth stages

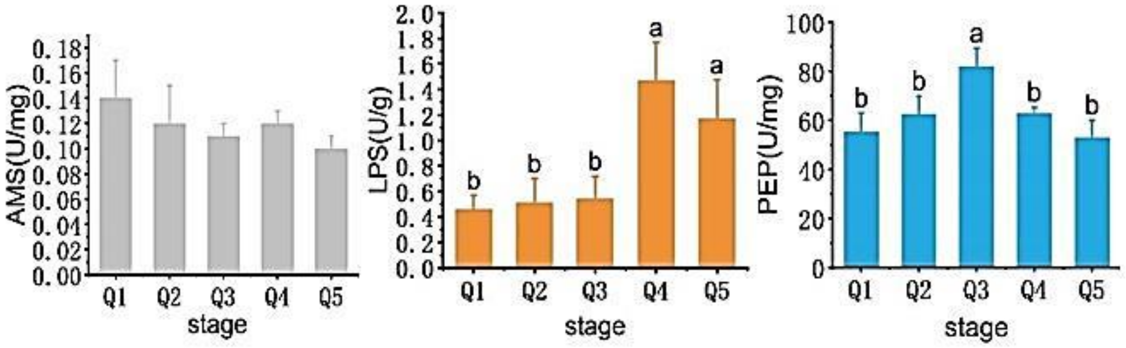
Digestive enzymes play a key role in the digestion and absorption of nutrients, and the activities of these enzymes directly affect the degree of absorption and utilization of nutrients and the growth and development of animals. For fish, digestive enzymes are indexes that reflect the ability to digest and absorb nutrients, the characteristics of whose activities can help better understand the mechanisms of ingestion, digestion, and absorption of fish as well as the health status.

Figure 4 showed the changes in the digestive enzyme activities of Largemouth Bass in different growth stages with data, and Figures 4 (a to c) showed these changes with images. It can be clearly seen from and Figure 4 (a to c) that there was no significant difference between the stages of gastric amylase ($p>0.05$).

The overall trend of gastric lipase showed an increase, reaching a peak in group Q4 with a slight and insignificant decrease in group Q5. There were significant differences between Q1, Q4, and Q5 groups ($p<0.05$), between Q2, Q3 and Q4 groups ($p<0.05$) and between Q3 and Q5 groups ($p<0.05$), while there were no significant differences between Q4 and Q5 groups ($p>0.05$). Pepsin generally showed a trend of first rising and then falling, reaching the peak in Q3 group, and there was a significant difference between Q3 group and each group ($p<0.05$).

Changes in the intestinal flora of largemouth bass in different growth stages

As shown in Table 2, the coverage rate of all samples was above 99%, indicating the authenticity and reliability of the sample community.



(a) (b) (c)

Figure 4: Changes in the digestive enzyme activities of Largemouth Bass in different growth stages (5% level). Notes: amylase (AMS), lipase (LPS), and pepsin (PEP).

The Sobs index, ACE index, and Chao index of group Q3 samples were all the highest, indicating that group Q3 has the highest number and richness of live bacterial species in the fish richness, followed by group Q4. The ace index of groups Q2 and Q5 was relatively close. In addition, the Simpson index of group Q1 was the highest, indicating that the community diversity of this group was the worst. Group Q4 has the highest Shannon index and the lowest Simpson index, suggesting the best community diversity in this group (Table 2).

Table 2: Alpha diversity index of intestinal flora of Largemouth Bass in different growth stages.

Sample	Ace	Chao	Coverage	Shannon	Simpson	Sobs
Q1	220.731587	172.75	0.999145	0.486601	0.787213	129
Q2	164.827573	120.869565	0.999372	1.226793	0.357005	95
Q3	314.970424	313.27907	0.999364	1.505619	0.423904	301
Q4	288.436207	285.552632	0.999469	1.688225	0.342841	277
Q5	188.188302	189	0.999476	0.787564	0.633213	175

As shown in Figure 5, we can see that group Q1 obtained 129 OTUs, group Q2 obtained 95 OTUs, group Q3 obtained 301 OTUs, group Q4 obtained 277 OTUs, and group Q5 obtained 175 OTUs. By comparison, we can see that Group Q3 had the maximum microorganisms, while Group Q2 had the minimum microorganisms. There were a total of 23 OTUs in Q1, Q2, Q3, Q4, and Q5 groups, including 19 OTUs in group Q1, 7 OTUs in group Q2, 113 OTUs in group Q3, 117 OTUs in group Q4, and 67 OTUs in group Q5.

The community heat map can be used to calculate the biological richness of various samples at different Taxonomy levels, and the community composition of sample organisms can be clearly understood by means of column charts, heat maps, and other graphical methods. As shown in Figure 6, the red patches meant that this group had rich bacteria, and the blue patches meant that this group had few bacteria. In group Q3, Mycoplasma, Vibrio, Plesiomonas, and Bacteria were in light red color with rich flora. In groups Q1 to Q3 and (p>0.05).

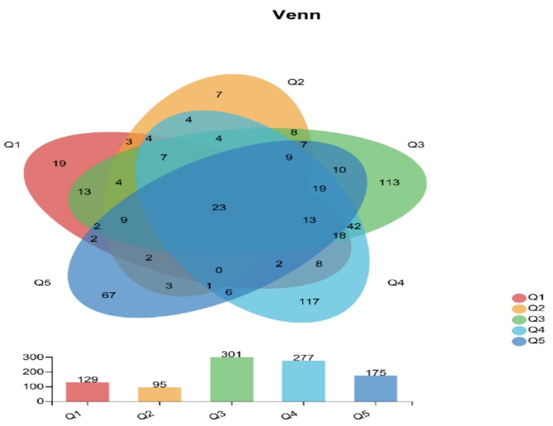


Figure 5: OTU venn diagram of intestinal flora of Largemouth Bass in different growth stages.

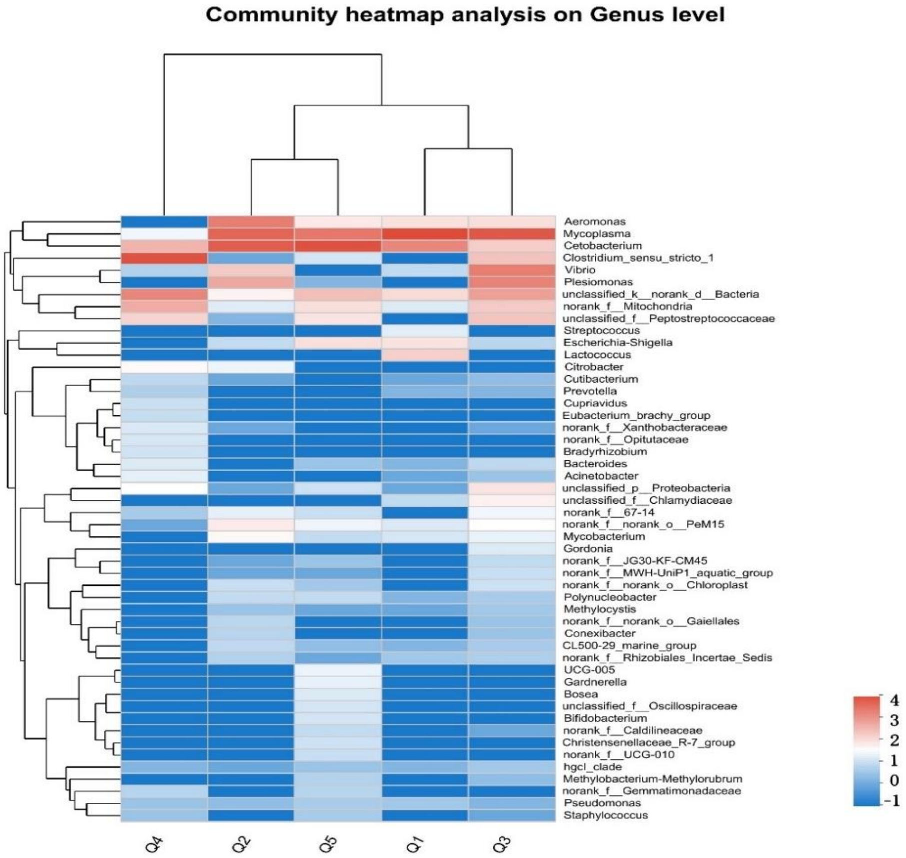


Figure 6: Community heat map of the intestinal flora of Largemouth Bass in different growth stages.

Cupriavidus, Eubacterium, Opitutaceace and Bradyrhizobium were in blue color, with less richness. In addition, in groups Q1 to Q4, Gradnerella, Oscillospiraceae, and Bifidobacterium were in blue color, with less richness. The first dominant genus shared by group Q1 and Q3 was Mycoplasma in a dark red color, accounting for a high proportion. It is a very important phylum of bacteria. It is named because of the extremely diverse shapes of bacteria, including at least 75 species of bacteria. The second was Aeromonas, accounting for a high proportion.

Aeromonas belongs to Aeromonadaceae, was widely distributed in nature, and can be separated from water, soils, and feces and can cause various diseases such as enteritis and sepsis in humans and animals. The first dominant genus shared by groups Q2 and Q5 was Cetobacterium, accounting for a large proportion. According to color depth, the second dominant genus was Mycoplasma. The dominant genus in Group Q4 was Clostridium. In addition, as shown in Figure 6, among the five groups Q1 to Q5, the common dominant strains in these five stages were mainly Mycoplasma, Cetobacterium, and Bacteria. To sum up, the richness of intestinal flora of Largemouth Bass in different growth stages was significantly different.

Discussion

In this experiment, WGR increased in the first two stages because the water temperature increased in the seasons in Q1 and Q2. In these two stages, the water temperature was suitable, and the fish had strong growth and metabolism, coupled with a good feed conversion ratio. Therefore, it was a good time for the fish to grow. The reason for the decline in the Q3 may be the inappropriate addition of plant-based protein sources to the feed, which may inhibit lipoprotein synthesis in the liver of fish, resulting in liver lipid accumulation and metabolic imbalance, thus inhibiting the growth of fish (Wang A. *et al*., 2022). As a result, the WGR of fish stagnated. The index kept declining in Q4 and Q5 because the outside temperature decreased under natural conditions in the later stages of breeding, which drove the fish to increase fat accumulation to withstand the low temperature. As a result, nutrient conversions occurred in the fish body, and the original metabolic rate gradually slowed down under the influence. The growth was hindered (Zhang *et al*., 2021). In addition, another reason may be that in the middle and later stages of breeding, the carrying capacity per square meter of Largemouth Bass farming increased, and the inappropriate feeding method generated excessively large amounts of residues in the ponds, causing the accumulation of organic oxygen-demanding substances in the environment. Therefore, the greater burden on the environment resulted in negative feedback in terms of dissolved oxygen level and organic oxygen consumption, affecting the growth of the fish. The HSI of Largemouth Bass first decreased and then increased in this experiment. The index declined in Q2 because, according to the above analysis of WGR and SGR, the fish had good growth and normal metabolism in vivo at this stage when there was almost no lipid accumulation. HSI, as a sensitive index of fish metabolism, showed a decreasing trend at this stage (Ti *et al*., 2019). HIS increased in Q4 and Q5 because fat accumulation increased in the fish body when the outside temperature decreased, which may cause fat accumulation and slow down the metabolic rate, thus driving up HSI. Another possible reason was that in the artificial formula feed for the Largemouth Bass, the components did not have a fatty acid balance, and there were anti-nutritional factors in the ingredients, which caused a disorder of lipid metabolism and fat accumulation in the liver (Wang S. *et al*., 2022). As a result, HSI rose, which might also be a reason for the lower feed conversion rates in the later stages. In addition, the starch added to the feed was also a possible reason for the lower feed conversion rates. The Largemouth Bass is a carnivorous fish that has poor tolerance to starch. Therefore, the starch added to the feed might inhibit part of the pathway of nutrient metabolism of Largemouth Bass. A long time of feeding caused anorexia and slow growth and metabolism of the fish. In addition, studies have found that the inappropriate addition of plant-based protein sources to the feed causes fat accumulation in the liver tissues as the lipoprotein synthesis in the liver of fish is prone to be inhibited due to an excessive intake of plant-based protein sources, which affects the normal physiological function of the liver (Martinez-Llorens *et al*., 2012). As a result, the feed conversion rate declines, and the feed coefficient increases.

In this experiment, TBIL increased in Q2. As TBIL is a metabolic waste product for animals, a sudden increase conveys a message that the liver is suffering from lesions or damage at this stage (Georgina *et al*., 2022). The reason for the damage to the liver in Q2 was the rapid growth of the fish. According to Table 1, the growth rate of Largemouth Bass peaked in Q2. The size of the fish suddenly grew, and the burdens on the ponds rose sharply at this stage, with oxygen consumption increasing. In addition, the excrement of the fish was not removed in time, resulting in more organic matter in the water. The decomposition of organic matter consumed more oxygen, making the oxygen level in the water lower than the normal range. Furthermore, the resulting large amount of residual feeds and excrement caused an increase in the nutrient salts in the sediment and water and the instability and deterioration of the water (Masood *et al*., 2022). The problems of the water indirectly increased the burden on the liver. As a result, the autoregulation of Largemouth Bass stagnated, which placed burdens on the liver, and therefore TBIL increased in Q2. After the fish adapted themselves to this resistance, TBIL gradually went down. The reason for the slight increase in Q4 may be that some Largemouth Bass were contaminanted with lernaeosis.

As the fragility of erythrocytes increased in the diseased fish, the life cycle of erythrocytes shortened, and more dead erythrocytes were released. The relatively large amount of hemoglobin was converted into TBIL in the end (Stolmeijer *et al*., 2015). T-CHO of the liver did not change significantly, and the differences between the five stages were not significant, indicating that the fish, the environment, and feeding did not have a significant impact on the T-CHO of the liver. GOT and GPT are directly involved in protein metabolism and transfer, and their activities are affected by the growth stage, environmental changes, nutritional status, and diseases. They are key indexes to assess liver function (Azizbeigi *et al*., 2014). Under normal conditions, the activities of GOT and GPT are low, and when the liver cells of animals are suffering from lesions or damage, the activities of these enzymes increase significantly (Kurnia *et al*., 2022). According to Figure 2 (c and d), both GOT and GPT increase first, reaching a peak in Q3, indicating that the livers of the fish had been damaged in Q3, or even earlier. The main reason may be the rapid growth and metabolism of the fish from Q2 to Q3 and the inappropriate feeding methods during the breeding period, which led to excessively large amounts of residues in the ponds, with more ammonia nitrogen released. As a result, the intensity required for decomposition and nitrification in the ponds increased, and the ponds lacked carbon sources. In such a situation, the microbes and beneficial bacteria lacked energy and thus could not effectively decompose organic matter in the water, resulting in the instability of water. Therefore, the water quality affects the health of the fish and places burdens on the liver (Li *et al*., 2015). In Q4 and Q5, the trend of GOT remained unchanged, and GPT gradually dropped. The main reason for the decline in GPT was the slower growth of the fish, the improved feeding methods in the later stages, and the timely water change in the ponds. GOT did not decrease in Q4 and Q5 and remained almost the same as the level in Q3 because the outside temperature decreased under natural conditions in the later stages of breeding, which drove the fish to increase fat accumulation to withstand the low temperature. As a result, nutrient conversions occurred in the fish body, which caused the abnormal metabolic function of the livers of the fish (Yin *et al*., 2018). In addition, a small part of the reason may be that the breeder mixed functional liver-protective drugs into the feed for the Largemouth Bass at the later stage of breeding, but the concentration of the drugs was not precise and was not fully suitable for the health condition of the fish at that time, which may result in the decline in GPT and the unchanged GOT. ALB of the liver showed an overall increasing trend, reached a peak in Q5, and slightly decreased in Q2. The index peaked at Q5 may be because lipids in fish organs gradually increased as the fish grew in the later stage of breeding. Lipids were digested and absorbed in fish organs and involved in the material metabolism and at this time the fish needed to maintain the osmotic regulation during metabolism by synthesizing albumin (Zhang *et al*., 2007). ALB slightly declined in Q2 because the analysis of the above indexes shows that the burden on fish livers was huge in Q2, and the inflammation in the fish body was aggravated. The feeding rate dropped, and some Largemouth Bass were not in a satiated state. Relevant studies have suggested that the total protein, albumin, and globulin in fish blood are reduced after the fish suffer from starvation (Naghmeh *et al*., 2018).

In this experiment, the SOD and CAT in the bodies of Largemouth Bass first showed a rising trend, reaching a peak in Q3. SOD and CAT, as two important enzymes in the antioxidant defense system, suggested that the defense system of the fish liver was damaged at that time. The main reason for the liver damage of the fish at this stage should be the rapid growth of the fish in Q2. According to Figure 1, the growth rate of Largemouth Bass peaked in Q2. The size of the fish suddenly grew and the burdens on the ponds rose sharply at this stage with an increase in oxygen consumption rate.

In addition, the excrement of the fish was not removed in time, resulting in more organic matter in the water. The decomposition of organic matter consumed more oxygen. Furthermore, the resulting large amount of residual feeds and excrement caused an increase in the nutrient salts in the sediment and water and the instability and deterioration of the water (Masood *et al*., 2022). The autoregulation of Largemouth Bass stagnated, which placed burdens on the liver. Another reason for the liver damage of the fish at this stage may be the rapid growth and metabolism of the fish, coupled with the inappropriate feeding methods in the early stages of the farming, which led to excessively large amounts of residues in the ponds, with more ammonia nitrogen released. Furthermore, due to the lack of carbon sources, most of the algae-beneficial bacteria in the culture pond could not be effectively utilized, which resulted in excessive nitrite, leading to the instability of the water and placing burdens on the liver (Lin *et al*., 2018). All these problems were caused by the failure to treat the water quality in time. Studies have found that recirculating pond aquaculture systems (RPAS), compared to traditional pond farming methods, can greatly reduce these problems (Xu *et al*., 2023). In addition, part of the reason may be the nutritional components of the feed. Due to the high content of fresh fish oil in fish feeds, fish often do not sufficiently break down highly unsaturated fatty acids after a long time of feeding.

As a result, these fatty acids are stored in body tissues and peroxidized in the body (Du *et al*., 2008; Todorcevicˇ *et al*., 2009), generating many free radicals and thus increasing the peroxidative stress in the fish body (Du *et al*., 2013). Therefore, SOD and CAT, as biomarkers, are sensitive to stress at the molecular and cellular levels and can be rapidly activated (Xu *et al*., 2023), quickly forming a defense mode. It is also possible that the animal proteins in the feed did not meet the nutritional requirements of Largemouth Bass, resulting in a disorder of protein anabolism in the fish and therefore causing oxidative stress in the fish. Therefore, SOD and CAT showed an overall increasing trend from Q1 to Q3. The two indexes declined in Q4 mainly because the autoregulation of the fish had not been seriously damaged, and the breeders had changed the water in the ponds as much as possible to repair the antioxidant system of the fish to some extent. As a result, SOD and CAT gradually declined in Q4. MDA rose at the first three stages because of lipid peroxidation in living bodies when the generation of harmful substances such as reactive oxygen species and free radicals attacked the antioxidant system. MDA, as a sensitive index of lipid peroxidation (Devasena *et al*., 2001), increased in the first three stages. MDA declined in Q4 because the whole antioxidant system withstood the attack from the outside and itself due to the autoregulation of the fish body and was not severely damaged. In addition, the breeders timely changed the water and improved the feeding methods and the ingredients of the feed, which restored the antioxidant system of fish to some extent. Besides, the changing trend of T-AOC, the index reflecting the total antioxidant capacity of the system, was largely consistent with that of SOD and CAT.

In this study, the activity of AMS in the stomach of Largemouth Bass was not high and did not show significant changes as the fish grew. During the breeding of Largemouth Bass, the activity of AMS in the fish is generally related to its ability to absorb and utilize carbohydrates, and the changing trend of the activity of AMS in the fish body is the result of autoregulation. Therefore, the main reason should be the feeding habit of Largemouth Bass. Studies have found that the activity of AMS in the fish body is related to their feeding habits, and the activity of AMS in carnivorous fish is lower than that in herbivorous and omnivorous fish (Hidalgo *et al*., 1999). Another reason contributing to the changing trend of AMS in the stomach of Largemouth Bass may be the almost unchanged proportion of carbohydrates in the feed as the changing trend of AMS in fish bodies is related to the carbohydrates in the feed (Magouz *et al*., 2022). Due to the low AMS activity detected in the stomach of Largemouth Bass in this experiment, it is recommended that the amount of starch in the feed be appropriately adjusted or biological enzyme preparations be added during the breeding process to improve the efficiency of absorption and utilization of starchy substances in the feed by Largemouth Bass. For aquatic fish with a weak ability to utilize carbohydrates, the role of fat in supplying energy is crucial. Fat can be broken down into fatty acids and glyceraldehyde mixture under the action of LPS and transported to various body tissues to provide essential nutrients for fish growth (Venter Leonie *et al*., 2022). In this experiment, gastric lipase showed a rising trend in general, reached a peak in Q4, and slightly fell in Q5. The main reasons for the increase in LPS from Q1 to Q4 should be the growth characteristics of Largemouth Bass and the feed. First, Largemouth Bass accumulate more fat as they grow. Second, Largemouth Bass, as a carnivorous fish, has a greater demand for fat. Third, some additives in the feed enhance the ability of Largemouth Bass to ingest and absorb fatty substances (Kenji and Sadao, 1986).

It has been found that LPS exists in many tissues of the fish body, and the activity of LPS is generally positively correlated with the fat content of the fish (Oki , 1985). As a result, LPS increased from Q1 to Q4. The activity of gastric lipase in Largemouth Bass decreased in Q5 may be because the fish accumulated more fat to withstand cold temperatures when the outside temperature dropped at this stage, which, coupled with the fat accumulation in the previous stages, placed huge burdens on the fish body. As a result, the body was damaged after exceeding a certain range, and the activity of LPS was inhibited. PEP is an important digestive enzyme in gastric juice. In this experiment, PEP increased first and then decreased. The rise of PEP in the first three stages was related to the growth of Largemouth Bass. As shown in

Figure 1, the WGR, SGR, and FC of Largemouth Bass show good trends in the first three stages, during which the fish need to continuously increase the intake of nutrients from the feed to meet their demands for protein (Einarsson *et al*., 1996). Besides, the stomach is the main place for the digestion of protein in the body of carnivorous fish, so the pepsin of Largemouth Bass shows an increasing trend in the first three stages. Therefore, the PEP of Largemouth Bass showed a rising trend in the first three stages. In addition, the FC was low in the first three stages, and the fish with high intake absorbed more nutrients and grew faster than those with low intake, thus increasing the activity of PEP.

PEP declined in Q4 may be because animal ingredients only took up a part of the artificial formula feed, with plant ingredients accounting for the remaining part. Plant ingredients contain abundant starch and plant protein sources (Li *et al*., 2021), and an excessively high content of plant ingredients after a long-term intake would affect the normal nutritional requirements of carnivorous fish such as Largemouth Bass due to the protein structure and the composition and proportions of amino acids in the feed, which adversely affected the normal physiological and nutritional requirements of Largemouth Bass. In addition, the trend of rising first and then dropping of PEP may be correlated with different water temperatures in different seasons. Some researchers studied the impact of water temperature on the digestive enzymes activity of threespine stickleback and found that the water temperature in the pond had a positive impact on the protease in the fish's digestive system (Hani *et al*., 2018).

This study performed high-throughput sequencing of 16SrRNA from the intestinal bacteria of five samples obtained from the five growth stages of Largemouth Bass and plotted the Venn diagram and the table of alpha diversity indexes through platforms. The Venn diagram can be used to make statistics on common and unique species (*e.g*., OTU) in multiple groups of samples, and the table of alpha diversity indexes reflects the diversity within a specific region or ecosystem, including common indexes such as Sobs, Chao, ACE, coverage, Shannon, and Simpson. Shannon and Simpson reflect community diversity, while Sobs, Chao, and ACE reflect community richness. Figure 5 shows that the Q3 group had the largest number of microorganisms, with 301 OTUs. As shown in Table 2, the coverage of all samples exceeds 99%, indicating the authenticity and reliability of the sample communities. Samples in Q3 had the greatest values of Sobs, ACE, and Chao, suggesting the species of live bacteria in the intestine of the fish was the most abundant and had the largest number at this stage, as well as the best community richness. Samples in Q4 had the highest value of Shannon and the lowest value of Simpson, indicating the best community diversity. In addition, Q1 had the highest value of Simpson, indicating the worst community diversity. In summary, according to the analysis of the Venn diagram and the alpha diversity analysis, the community diversity of the gut microbiota in Q4 was the best, and the community richness of gut microbiota in Q3 was the best. Some researchers conducted a diversity analysis of gut microorganisms of tilapia in several breeding areas through high-throughput sequencing and found that the diversity of gut microbiota in the middle and later stages was better than that in the early stages (Wu *et al*., 2012), which is consistent with the results of the present study. The maximum values of the diversity of gut microorganisms in various fishes and different areas largely appeared in the middle or later stages, and some studies on channel catfish (Bledsoe *et al*., 2016) and zebrafish (Stephens *et al*., 2016) suggested that the communities of gut microorganisms constantly changed as the fish grew. The results of all these studies show that the diversity and richness of the gut microbiota of fish are highly correlated with the growth and development of fish. Besides, other studies have shown that such changes are related to the nutrients in the feed and water environment. For example, different viscous feeds have differential influences on the structure of the gut microbiota of Largemouth Bass (Liu Y. *et al*., 2022). Another example is the correlation between the gut microbiota of mandarin fish and the salinity stress in the water (Ouyang *et al*., 2023). Figure 6 shows that the genus *Mycoplasma* and the genus *Cetobacterium* were dominant in the gut of Largemouth Bass at all five stages. Cetobacterium is a large genus of bacteria, widely distributed in the environment, plants, and animals (Tamir *et al*., 2021; Mariana *et al*., 2022). Cetobacterium is the core dominant genus in the intestine of fish. Studies on other carnivorous fish such as rainbow trout (Merrifield *et al*., 2009) and mandarin fish (Liu *et al*., 2016) have suggested that the genus Cetobacterium is absolutely dominant in terms of abundance. Other researchers studied the mechanism of action of Cetobacterium on fish and the bacterial communities adhered to the intestine of fish and found that the genus Cetobacterium has better adherence and survival capabilities (Zhang *et al*., 2022). In addition, the genus Cetobacterium can produce vitamin B group and ferment polypeptides and carbohydrates, and the genus significantly increases in the later stages of culture. The above studies suggest that the genus Cetobacterium is an important part of the beneficial microbiota in the intestine of fish, and the abundance of the genus is related to the growth of fish. Other studies have shown that the addition of beneficial bacteria to the feed can increase the competitiveness of the beneficial intestinal microbiota (Chen *et al*., 2021). In terms of the genus Mycoplasma, a study found that the relative abundance of the genus was 96% in carnivorous wild salmon and 25% and 81% in the gut of farmed salmon, respectively (Holben *et al.,* 2022). Another study focused on the intestinal bacterial community of Largemouth bronze gudgeon in the upper reaches of the Yangtze River found that the genus Mycoplasma was dominant in the female fish (Li *et al*., 2016).

The above studies show that the genus Mycoplasma is widely distributed in the intestine of fish. The increase in the genus

Mycoplasma which belongs to the phylum Firmicutes means a close association with obesity and diabetes in mammals. A study on gnotobiotic zebrafish suggested that gut microbiota facilitated fat absorption and lipid droplet formation in the intestinal epithelium and liver, and the phylum Firmicutes, as a main member of beneficial intestinal microbiota, played a key role (Semova *et al*., 2012). In the present study, the proportion of the phylum Firmicutes increased in the final stage of growth of Largemouth Bass, which may suggest that fish farming is under greater pressure of diseases such as fat deposition at the later stage. According to the analysis of the heatmap of the gut microbiota, in Q4, the stage with the best community diversity (the highest value of the Shannon index and the lowest value of the Simpson index), the genus Clostridium was dominant. Some researchers used high-throughput sequencing to analyze the gut microbiota of blunt snout bream and found that the genus Clostridium was distributed in the gut microbiota and accounted for a large proportion (Wang *et al*., 2019).

Another study suggested that the genus Clostridium made up a large proportion of the bacterial communities in the intestine of grass carp (Wu *et al*., 2012). It can be seen that the genus Clostridium accounts for a large proportion of the gut microbes of many herbivorous fishes. Therefore, the proportion of the genus Clostridium in the gut of Largemouth Bass in Q4 suggested an accumulation of excessive cellulose that had not been consumed, which may be due to the inappropriate addition of plant ingredients in the feed.

Therefore, Clostridium, an important genus that breaks down carbohydrates and plant-based proteins (Zhao *et al*., 2017), sharply increased at this stage. The Clostridium genus is a beneficial bacterium in most cases, which can participate in the hydrolysis in the host of a variety of carbohydrates and proteins, producing fatty acids such as acetate, formate, and lactate that are more easily absorbed and utilized by the host. Furthermore, the Clostridium genus in the gut promotes the synthesis of vitamin B12 (Nayak , 2010). In this experiment, the genus Aeromonas existed in all the stages except Q4. This genus is a conditional pathogen that produces a variety of antibiotics and active small molecules with antibacterial and bacteriolytic effects in good conditions. However, in poor conditions, it can cause ulceration on the surface of the fish and internal swelling and congestion (Zhou X . *et al*, 2022).

Thus, this genus has both advantages and disadvantages. Some researchers found that the synergistic effect of Aeromonas worsened the condition and increased the mortality of tilapia with Tilapia Lake Virus disease (Nicholson *et al*., 2020). The above studies show that the genus Aeromonas is a threat to the host and needs to be controlled and regulated.

Conclusion

This experiment explored the differential changes in growth performance, physiological and biochemical indicators, and intestinal flora of Largemouth Bass in different growth stages through 153-day monitoring and regular sampling. The experiment concluded that with the change of growth months, there were obvious differences in the growth performance, physiology and biochemistry, and intestinal flora of Largemouth Bass in different growth stages. The most obvious was that Largemouth Bass had a rapid growth period from June to July. As a feeding habit of carnivorous fish, it is necessary to increase the content of animal protein and starch in the feed; In August, when parasites are widespread *Lernaea* as a high-incidence disease of perch, needs to be treated and controlled timely. If necessary, probiotics can be added to the pond for defense. During the cultivation of Largemouth Bass, the accumulation of organic matter in the pond will cause a series of chemical reactions leading to negative water quality problems. Therefore, it is necessary to regularly improve the sediment in the pond and enhance the water cycle mechanism of the aquaculture pond; In addition, the experiment found that for the intestinal flora of Largemouth Bass in different growth stages, beneficial bacteria can be added to the feed to improve the competitiveness of dominant flora in the intestinal tract of Bass and promote the healthy development of the fish. This experiment provides data support for the breeding technology improvement, probiotics screening, disease prevention, and intestinal microecology research of Largemouth Bass during its growth, which is conducive to producing high-quality and economically efficient aquatic products and enhancing the motivation of Bass farmers to improve their farming methods, to improve farming efficiency from specific stages and aspects.

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Conflicts of interest

The authors declare no competing interests.

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