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Effects of Artificial Compound Feed for Live Fish Feed on Growth Performance, Nutrient Composition, Digestive Performance, and Gut Microbiota of Giant Salamander (*Andrias davidianus*)

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Abstract: This study explored the effects of replacing live fish feed with artificial compound feed on the growth performance, nutritional composition, digestive performance, and gut microbiota of Chinese giant salamander (*Andrias davidianus*). The experiment was divided into three groups, each with three biological replicates. The salamander juveniles were fed artificial compound feed (S), live fish (H), and frozen fish (D) for 184 days. Results showed no significant differences in growth performance among the three groups ($p > 0.05$). The water content in the S group was significantly higher than that in the H group ($p < 0.05$), and the crude fat content was slightly higher, whereas other nutritional components showed no significant differences ($p > 0.05$). The analysis of amino acid composition found no significant differences among the three groups of Chinese giant salamanders ($p > 0.05$). The analysis of fatty acid composition revealed that the feed group had significantly lower levels of polyunsaturated fatty acids compared with the other two groups ($p < 0.05$), and significantly higher levels of saturated fatty acids ($p < 0.05$). There were no significant changes in digestive enzyme activity among the three salamander groups ($p > 0.05$). There were also no significant differences in the remaining antioxidant indicators in the intestine and liver among the three groups of salamanders ($p > 0.05$). The S group had significantly higher Pielou_e and Shannon indices ($p < 0.05$), thereby indicating a greater diversity and evenness of the gut microbiota. In conclusion, replacing live feed with artificial compound feed does not affect the growth performance, nutritional composition, antioxidant capacity, and digestive function of Chinese giant salamander juveniles. Moreover, it enhances the diversity and richness of the gut microbiota, providing effective data to support the development of artificial feeds for giant salamanders.

Keywords: feeding mode; Chinese giant salamander; nutritional quality; antioxidant; digestion; intestinal function

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Key Contribution: This study showed that feed substitution had no significant effect on the growth and digestion level of Chinese giant salamanders and, simultaneously, it could significantly improve the richness and diversity of intestinal microbial communities of giant salamanders, which provided theoretical support for the development of artificial feed for giant salamanders.

1. Introduction

The Chinese giant salamander (*Andrias davidianus*) belongs to the order *Urodela* and the family *Cryptobranchidae* and is the largest extant amphibian species, with its body length reaching nearly 2 m and its weight reaching 95 kg [1]. It is a national-level II protected animal [2] and has been listed as a living fossil [3]. Previous studies have shown that the Chinese giant salamander was once widely distributed in the Yellow River, Yangtze River and Pearl River basins [4]. The Chinese giant salamander is a rare amphibian species known for its delicate meat texture, delicious taste, and high nutritional value; its muscle protein content is between 12.07–17.15%, and the lipid content is lower than that of other aquatic animals [5–7]. It has high economic value in the fields of cuisine, health care, medicine, and ornamental use [8,9]. The cartilage of the giant salamander contains highly thermostable collagen, which makes it a superior source of biomedical materials [10]. Its muscle fat contains beneficial omega-3 and omega-6 polyunsaturated fatty acids [11]. In recent years, the wild giant salamander population has declined sharply owing to infectious diseases, habitat loss, environmental pollution, and overfishing [12,13]. However, the development and progress of artificial breeding technologies have provided an effective way to protect this species [14]. Since Aisheng et al. initially successfully developed a technology for the artificial breeding of giant salamanders in 1979 [15], this breeding has gradually matured in China, leading to continual large-scale expansion [16,17]. However, feed supply has become a key issue restricting industrial development [14,17]. Currently, giant salamanders are primarily fed live fish, shrimp, and frozen fish; however, this method is expensive, prone to carrying diseases, difficult to transport and store, and involves a complex feeding process [18]. There is a lack of specialized compound feed for giant salamanders in the market, and the research and development of feed are relatively slow, which limits further development of the breeding industry. Earlier studies indicated that the protein requirement for the larval stage of giant salamanders was 42.3–45.6%, while the protein requirement for adult giant salamanders was 43.8–48.9% [19,20]. These data provide a scientific basis for the development of a compound feed for Chinese giant salamanders. Although the protein content of Chinese giant salamander muscle is lower than that of some aquaculture animals [21], it is rich in essential amino acids, especially lysine and tryptophan [5], giving it a unique advantage in palatability and nutritional value. China artificially breeds approximately two million giant salamanders per year [22]. Solving the feed problem and improving breeding technology are urgent tasks to promote the sustainable development of the industry. This problem continues to exist, while related research is limited [23]. Therefore, developing a compound feed suitable for the nutritional needs of giant salamanders, improving breeding efficiency, reducing dependence on wild populations, protecting this rare species, and meeting the market demand for healthy foods have great significance.

In addition, the gut plays a key role in the health and survival of the Chinese giant salamander, which possesses numerous microbial flora and is a vital organ for digestion, absorption, and immune defense in aquatic animals [24]. Intestinal microorganisms play a vital role in the life activities of the body, such as nutrient absorption, immune response,

and metabolism [25]. These gut microbes are also closely related to the health status of the host [26]. The balance of the gut microbiota is essential for maintaining the overall health of the host [27] and contributes to the growth and development of the host by participating in the digestive process, providing essential enzyme resources [28], and regulating the host immune response [29]. In addition, certain microorganisms, such as *Cetobacterium*, can produce metabolites beneficial to the host like butyrate and acetate [30]. They help contribute to the immune response of the host and regulate its metabolic process [31]. The diversity and function of gut microbiota are essential for the health and survival of aquatic animals. Therefore, studying and maintaining the diversity and function of the gut microbiota in the Chinese giant salamander is equally important for its health and the success of its aquaculture.

This study conducted a comparative analysis of the growth, nutritional composition, digestive and antioxidant enzyme activity, and gut microbiota of Chinese giant salamanders raised in live prey groups (H), feed groups (S), and frozen fish groups (D) over a period of 184 days. The purpose of this research is to evaluate the impact of replacing traditional live feed with artificial feed on the growth performance, nutritional status, digestive capacity, and gut microbiota structure of the Chinese giant salamander. The aim is to provide scientific evidence and data support for the development of artificial compound feeds for the Chinese giant salamander.

2. Materials and Methods

2.1. Study Site

The experiment was carried out in the indoor breeding facility of Jinggangshan Huabao Rare Aquaculture Co., Ltd., Ji'an, China (114°4′35.677″ E, 26°39′24.646″ N) for 183 days.

2.2. Experimental Diet

The experimental live bait consists of low-cost live fish, and the frozen fish are freshly frozen fish; both types of fish are crushed using a pulverizer before feeding and prepared about an hour in advance each day. The artificial feed formula used in the experiment is based on the specific formula provided by Professor Luo Li's team at Southwest University, and it was written using Excel software [32], as shown in Table 1. All feed ingredients were sifted through a 40-mesh screen, mixed evenly in a mixer, extruded into feed pellets with a diameter of about 2.0 mm, air-dried, packaged, and stored at −20 °C for later use. They are taken out to thaw 30 min before each feeding.

Table 1. Formula of Artificial Compound Feed.

	Compound Feed Group	Live Fish Group	Frozen Fish Group	Unit Price, Yuan/ton
Ingredient (%, DM)				
Fish Meal [1]	50			14,300
Gluten Meal [1]	31			11,400
Amylopectin Starch [1]	11			9000
Microcrystalline Cellulose [1]	2.71			18,000
Soybean Oil	2			12,300
Calcium Dihydrogen Phosphate	2			3350
Premix [2]	0.75			26,000
Antioxidants	0.01			20,000
Calcium Propionate	0.03			12,000
Choline Chloride [2]	0.5			20,000
Crucian carp meat [2]		100	100	60,000

Table 1. *Cont.*

	Compound Feed Group	Live Fish Group	Frozen Fish Group	Unit Price, Yuan/ton
Nutrients (%, DM)				
Moisture	11.37	72.57	70.10	
Crude Protein	58.41	15.23	18.65	
Crude Lipid	6.82	6.11	3.97	
Crude ash	10.02	3.76	3.63	
Gross energy (GE) (kcal/g) [3]	3.94	4.15	4.05	

Note: [1]. Fish Meal, Gluten Meal, Amylopectin Starch and Microcrystalline Cellulose were provided by Jiangxi Aohua Industrial (Nanchang, Jiangxi, China). [2]. Premix and choline chloride provided by MGO Ter Bio-Tech (Qingdao, Shandong, China). Premix composition (mg/kg diet): KCl, 200; KI (1%), 60; $CoCl_2$_$6H_2O$ (1%), 50; $CuSO_4$_$5H_2O$, 30; $FeSO_4$ H_2O, 400; $ZnSO_4$ H_2O, 400; $MnSO_4$_H_2O, 150; Na_2SeO_3_$5H_2O$ (1%), 65; $MgSO_4$_H_2O, 2000; zeolite power, 3645.85; VB1, 12; riboflavin, 12; VB6, 8; VB12, 0.05; VK3, 8; inositol, 100; pantothenic acid, 40; nicotinic acid, 50; folic acid, 5; biotin, 0.8; VA, 25; VD3, 5; VE, 50; VC, 100; ethoxyquin, 150; wheat meal, 2434.15. [3]. Gross energy (GE) (kcal/g) was calculated by the difference [100 − (crude protein + crude lipid + ash)] and 4 kcal/g for crude protein and carbohydrate, and 9 kcal/g for lipid [33].

2.3. Experimental Animals and Husbandry Management

The salamanders used in the experiments were sourced from Jinggangshan Huabao Rare Aquaculture Co., Ltd., Ji'an, China where juvenile salamanders were also raised. The experiment was divided into three groups, with each group using live and frozen fish to feed the salamanders, with three biological replicates for each group. The experiment lasted for 184 days. Due to the long digestion time of the Chinese giant salamander, feeding is performed once every three days and weighed every two weeks, while adjusting the amount of feed based on the weight of the salamanders (0.3–1% of body weight).

2.4. Sample Collection

At the end of the aquaculture experiment, after a 3-day fasting period, 10 individuals were randomly harvested from each replicate for statistical analysis of growth data. Subsequently, three salamanders were randomly selected from each replicate, anesthetized with 0.01% MS-222, and dissected on an ice tray to separate the intestines, livers, dorsal muscles, and intestinal contents, which were then quickly frozen in liquid nitrogen for subsequent experimental analyses.

2.5. Indicator Measurement

2.5.1. Growth and Morphological Indicator Measurement

After completion of the aquaculture experiment and a 3-day fasting period, the body weight and feed weight of the salamanders in each group were accurately measured to calculate their growth indicators. The calculation formulae are as follows:

$$\text{Weight growth rate (WGR, \%)} = (Wt - W0)/W0 \times 100;$$

$$\text{Specific growth rate (SGR, \%/day)} = 100 \times (\ln Wt - \ln W0)/t;$$

$$\text{Feed conversion rate (FCR)} = Wf/(Wt - W0);$$

$$\text{Survival rate (SR, \%)} = Nt/N0 \times 100$$

where Wt is the final average weight (g), W0 is the initial average weight (g), Wf is the dry weight of the ingested feed, t is the number of days of the aquaculture trial, N is the number of fish, N0 is the initial number of individuals (tails), and Nt is the final number of individuals (tails).

2.5.2. Routine Nutritional Component Measurement

The routine nutritional components of the experimental salamander feed and muscle were measured using AOAC (1995) methods [34]. Crude protein was measured using a protein analyzer (GB/T 6432-1994) [32], crude fat was determined using a Soxhlet extractor (GB/T 6433-1994), moisture was measured using the 105 °C constant temperature drying method (GB/T 6435-2006), and crude ash was determined using a muffle furnace (GB/T 6438-1992) at 550 °C by the ashing method.

2.5.3. Amino Acid Analysis

The composition of muscle amino acids was measured using an acid hydrolysis method with liquid chromatography-mass spectrometry. Approximately 30 mg of the sample was accurately weighed, added to 2 mL of 6 mol/L hydrochloric acid solution, hydrolyzed at 110 °C for 22 h, filtered, and 30 μL of the filtrate was dried. Subsequently, 600 μL of water was used to redissolve the dried sample. Twenty microliters of the redissolved solution was placed in a 1.5 mL centrifuge tube, to which 5 μL of internal standard and 40 μL of isopropanol (0.1% formic acid) were added and vortexed for 5 min. The centrifuge tube was then placed in a low-temperature centrifuge at 4 °C and 12,000 rpm for 10 min. Ten microliters of the supernatant was placed in a 1.5 mL centrifuge tube, and 70 μL of boric acid buffer and 20 μL of AccQ Tag derivatization reagent (Kairos amino acid reagent kit, Milford, MA, USA) were added and shaken immediately for 10 s. After 1 min, the excess derivatization agent was hydrolyzed, and the derivatization reaction was completed; the centrifuge tube was then heated at 55 °C for 10 min; afterwards, 400 μL of water was added for dilution before measurement. Chromatographic separation was performed using a Waters ACQUITY UPLCI-CLASS ultra-high-performance liquid chromatograph, and mass spectrometry analysis was conducted using a Waters XEVO TQ-S Micro triple quadrupole mass spectrometer. Peak area calculations were performed using MassLynx V4.1 quantification software with a retention time tolerance of 15 s, and quantitative results were obtained using the standard curve method.

2.5.4. Fatty Acid Analysis

Muscle fatty acid composition was determined by high-performance gas chromatography-mass spectrometry. Samples were thawed at 4 °C. Approximately 50 mg of the sample was accurately weighed, homogenized, and mixed with 3 mL of hexane, shaken at 50 °C for 30 min. Then, 3 mL of KOH methanol solution (0.4 mol/L) was added and shaken at 50 °C for 30 min to derivatize. Once the temperature returned to room temperature, 1 mL of water was added and mixed. After standing and phase separation, 90 μL of the upper clear liquid was taken, 10 μL of internal standard (methyl tridecanoate 125 μg/mL) was added, and then detected by gas chromatography-mass spectrometry. Target data peak area calculations were performed using MassHunter10.2 quantification software, and concentrations were determined using the standard curve method.

2.5.5. Physiological and Biochemical Indicator Analysis

Giant salamanders' hepatopancreas and intestinal tissue Lipase (LPS), α-amylase (AMS), Na^+K^+-ATPase, trypsin, superoxide dismutase (SOD), acid phosphatase (ACP), alkaline phosphatase, and malondialdehyde (MDA) were all measured using assay kits and methods produced by the Nanjing Jincheng Bioengineering Research Institute. The activity of LPS is determined by the rate of formation of ester red products in the tissue, the activity of AMS is calculated based on the depth of the blue complex formed by the combination of starch hydrolysis products with iodine solution, the activity of Na^+K^+-ATPase is measured by the concentration of inorganic phosphorus decomposed by it, the activity of trypsin

is determined by the rate of change of arginine catalyzed by it, the activity of SOD is measured by the depth of color after the color reaction of nitrites produced by its oxidation, the activity of ACP is determined by the depth of the red color generated by the reaction of phenols produced by ACP decomposition with potassium ferricyanide, and the content of MDA is measured by the depth of the red product formed by its combination with thiobarbituric acid.

2.6. DNA Extraction and PCR Amplification

Samples of the intestinal contents of giant salamanders were sent to Novogene Technology Co., Ltd. (Beijing, China) for testing. Total genomic DNA was extracted from the samples using the CTAB method. DNA concentration and purity were monitored using 1% agarose gel electrophoresis. Based on this concentration, the DNA was diluted to 1 ng/μL with sterile water. PCR reactions were performed using 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA), 2 μM each of forward and reverse primers, and approximately 10 ng of template DNA. The thermal cycling included an initial denaturation at 98 °C for 1 min, denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and extension at 72 °C for 30 s, for a total of 30 cycles. A final extension was performed at 72 °C for 5 min. The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified using universal primer pairs (341F: 5′-CCTAYGGGRBGCASCAG-3′; 806R: 5′-GGACTACNMGGGTATCTAAT-3′).

2.7. Library Construction and Sequencing

The PCR products were purified using magnetic beads, and equal amounts of PCR products were mixed according to the concentration. After thorough mixing, the PCR products were inspected, and the target bands were recovered. Sequencing libraries were generated using the TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) following the manufacturer's recommendations, with the addition of index codes. Library quality was assessed using a Qubit@2.0-fluorometer (Thermo Scientific, Waltham, MA, USA) and an Agilent Bioanalyzer 2100 system. Finally, libraries were sequenced on the Illumina NovaSeq platform and generated 250 bp paired-end reads.

2.8. Bioinformatics Analysis

The 16S rRNA gene amplicon sequencing and data processing were performed by Novogene Technology Co., Ltd. in Beijing, China. The raw data of each sample were obtained by splitting according to the barcode, and the barcode and primers were removed, and then the R1 and R2 sequence data were spliced by FLASH1.2.11 software. The spliced Tags were QC to obtain Clean Tags, and then the chimera was filtered to obtain Effective Tags that could be used for subsequent analysis. Finally, the software output a table representing the read counts and ASV abundance. The representative reads for each ASV were selected using the QIIME2 package. All representative reads were annotated and blasted against the Silva database version 138 using the q2-feature-classifier with default parameters. Alpha and beta diversities were analyzed using QIIME2 202202 software. The microbial richness and diversity of the salamander gut samples were estimated using the Chao1, observed species, Shannon, and Simpson indices. Principal component analysis (PCA) was used to reduce the dimensionality of the original variables using the ade4 and ggplot2 software packages (V4.0.3) in R4.0.3 software. Non-metric multidimensional scaling (NMDS) was used to reduce the data dimensions. Similar to PCoA, NMDS also uses a distance matrix, but emphasizes numerical ranks. The distances between the sample points on the graph reflect only rank information and not numerical differences. NMDS analysis was performed using R software with ade4 and ggplot2 software packages. A series of statistical analyses, including Anosim, Adonis, MRPP, Simper, *t*-tests, MetagenomeSeq, and

LEfSe, were used to reveal community structure differentiation. Functional prediction of the gut microbiota was performed using the Tax4Fun (V0.3.1) software. Two-dimensional and three-dimensional network graphs were visualized to explore the symbiotic relationships between species and reveal the impact of environmental factors on the community structure.

2.9. Data Analysis

All experimental data were analyzed by SPSS 27.0 software, and the Duncan test was used for multiple comparisons after one-way ANOVA, $p < 0.05$ indicated significant differences, and the data were expressed as mean ± standard deviation (Mean ± SD). Data were plotted using the GraphPad Prism V8.0 software.

3. Results

3.1. Effects of Feed Instead of Live Feed on the Growth of Giant Salamander

As shown in Table 2, the final body weight of giant salamanders in the bait group was significantly higher than that in the feed group ($p < 0.05$). The weight gain and specific growth rate of the feed group were not significantly lower than those of the other two groups.

Table 2. Growth performance data of three groups of giant salamanders after culture test.

Group	H	S	D	*p*-Vaule
IW (g)	45.63 ± 3.59	39.25 ± 3.16	43.42 ± 1.83	>0.139
FW (g)	95.14 ± 6.38 a	78.94 ± 5.94 b	81.72 ± 3.53 ab	0.011
WGR (%)	108.51 ± 13.98	101.12 ± 15.12	88.19 ± 8.14	>0.266
SGR (%)	0.25 ± 0.02	0.23 ± 0.03	0.22 ± 0.02	>0.428
FCR (%)	1.64 ± 0.14 a	2.05 ± 0.06 b	2.18 ± 0.03 b	0.001
SR (%)	100	100	100	>0.732

Note: Different letters in the table represent significant differences ($p < 0.05$), and the same letters indicate no significant differences ($p > 0.05$). IW, initial weight; FW, final weight; WGR, weight gain rate (%); SGR, specific growth rate; FCR, feed conversion rate; SR, survival rate.

3.2. Effects of Feed Instead of Live Feed on Nutrient Composition of Giant Salamanders

The crude nutrient, amino acid composition, and fatty acid composition of the muscles of different groups of giant salamanders after 180 days of feeding are shown in Tables 3–5.

Table 3. Muscle crude nutrients of giant salamanders.

Group	Moisture (%)	Crude Lipid (%) *	Crude Protein (%) *	Crude Ash (%) *
H	83.24 ± 0.53 b	5.13 ± 0.44	13.53 ± 0.29	0.99 ± 0.02
S	85.89 ± 1.07 a	6.05 ± 0.27	12.11 ± 0.50	1.03 ± 0.03
D	83.91 ± 0.36 ab	5.49 ± 0.47	12.43 ± 0.55	0.97 ± 0.04
p-Value	0.040	>0.162	>0.066	>0.137

Note: * indicates the percentage of dry weight in muscle. Different letters in the table represent significant differences ($p < 0.05$), whereas the same letters indicate no significant differences ($p > 0.05$).

The water content of the feed group was significantly higher than that of the live-bait group ($p < 0.05$), and the crude fat content was slightly higher than that of the other two groups; however, the difference was not significant ($p > 0.05$). The cysteine and tyrosine contents in the muscles of the giant salamanders in the feed group were significantly lower than those in the frozen fish group ($p < 0.05$). There were no significant differences in the composition of other amino acids, total essential amino acids, total non-essential amino acids, or total amino acid content among the three groups ($p > 0.05$).

Table 4. Amino acid composition of muscle of three groups of giant salamanders.

Amino Acid Species	H (mg/g)	S (mg/g)	D (mg/g)	*p*-Value
EAA				
His	4.32 ± 0.10	4.13 ± 0.26	4.70 ± 0.20	>0.083
Arg	12.66 ± 0.41	12.47 ± 0.68	13.23 ± 0.36	>0.326
Met	3.34 ± 0.08	2.81 ± 0.53	3.87 ± 0.32	>0.081
Val	8.42 ± 0.23	8.04 ± 0.43	9.01 ± 0.40	>0.109
Ile	8.31 ± 0.16	8.41 ± 0.51	9.18 ± 0.22	>0.112
Leu	15.52 ± 0.42	15.07 ± 0.73	16.79 ± 0.47	>0.072
Phe	7.97 ± 0.29	7.64 ± 0.35	8.50 ± 0.29	>0.100
Thr	9.04 ± 0.36	8.70 ± 0.48	9.51 ± 0.35	>0.207
Lys	18.34 ± 0.81	17.34 ± 0.61	19.52 ± 0.72	>0.076
NEAA				
Ala	11.42 ± 0.45	10.81 ± 0.49	11.91 ± 0.35	>0.122
Pro	7.16 ± 0.54	6.92 ± 0.27	7.56 ± 0.32	>0.289
Cys	2.16 ± 0.12 ab	1.81 ± 0.12 a	2.23 ± 0.06 b	0.029
Tyr	6.72 ± 0.29 ab	6.31 ± 0.18 a	7.11 ± 0.17 b	0.044
Ser	8.99 ± 0.53	8.48 ± 0.30	9.51 ± 0.36	>0.124
Gly	9.57 ± 0.92	9.05 ± 0.51	9.52 ± 0.36	>0.584
Asp	14.46 ± 0.71	13.42 ± 0.68	15.19 ± 0.68	>0.121
Glu	30.01 ± 1.23	29.75 ± 1.04	32.07 ± 1.18	>0.205
∑EAA	78.88 ± 4.19	75.91 ± 5.58	84.80 ± 4.92	>0.069
∑NEAA	90.49 ± 8.07	86.54 ± 5.71	95.09 ± 5.81	>0.165
∑AA	169.37 ± 12.26	162.45 ± 11.28	179.89 ± 10.66	>0.111

Note: Different letters in the table represent significant differences ($p < 0.05$); same letters represent no significant differences ($p > 0.05$). EAA stands for essential amino acids, and NEAA stands for non-essential amino acids.

Table 5. Composition of muscle fatty acid of three groups of giant salamanders.

Types of Fatty Acids	D (μg/g)	S (μg/g)	H (μg/g)	*p*-Value
C10:0	0.06 ± 0.02	0.03 ± 0.01	0.26 ± 0.24	>0.128
C12:0	0.14 ± 0.06	0.08 ± 0.03	0.3 ± 0.22	>0.190
C14:0	1.96 ± 0.32 a	2.81 ± 0.35 ab	3.69 ± 0.68 b	0.005
C15:0	3.5 ± 1.16 a	1.58 ± 0.5 a	9.84 ± 2.43 b	<0.001
C16:0	412.67 ± 65.88 ab	346.72 ± 74.08 a	496.25 ± 66.5 b	0.038
C17:0	14.38 ± 2.35 a	6.08 ± 1.59 b	16.67 ± 3.69 a	0.003
C18:0	213.43 ± 27.43 ab	151.64 ± 27.64 b	215.79 ± 34.43 a	0.045
C20:0	1.24 ± 0.13	1.18 ± 0.13	1.34 ± 0.07	>0.135
C21:0	2.09 ± 0.1	2.14 ± 0.16	1.99 ± 0.06	>0.158
∑SFA	649.47 ± 88.72 ab	512.26 ± 102.25 a	746.13 ± 105.79 b	0.028
C16:1	38.29 ± 9.99 ab	29.1 ± 5.29 a	50.88 ± 12.39 b	0.033
C18:1n9c	276.71 ± 37.22 a	207.47 ± 28.3 b	323.32 ± 64.28 a	0.021
C20:1	3.72 ± 0.99	4.52 ± 1.51	4.77 ± 0.86	>0.309
∑MUFA	318.72 ± 47.12 ab	241.09 ± 29.1 a	378.98 ± 76.95 b	0.022
C18:2n6c	111.77 ± 16.81 a	218.76 ± 71.91 b	178.82 ± 21.54 ab	0.026
C20:2n6	3.96 ± 1.3	4.11 ± 1.29	6.58 ± 2.07	>0.092
C20:3n6	21.44 ± 3.36 a	14.26 ± 2.92 b	25.08 ± 4.29 a	0.010
C20:4n6	351.82 ± 32.11 a	247.25 ± 60.72 b	374.99 ± 61.65 a	0.026
∑n-6PUFA	489 ± 51.55	484.38 ± 76.86	585.47 ± 88.38	>0.145
C18:3n3	24.85 ± 4.89 a	12.19 ± 2.06 b	39.89 ± 8.47 c	0.001
C20:3n3	3.54 ± 1.21 a	0.79 ± 0.6 b	5.55 ± 2.24 a	0.008
C20:5n3	92.41 ± 12.76	97.13 ± 22.59	120.05 ± 24.41	>0.151
C22:6n3	226.54 ± 27.26 a	143.31 ± 19.18 b	245.05 ± 49.15 a	0.011
∑n-3PUFA	347.35 ± 42.13 ab	253.41 ± 42.51 a	410.54 ± 83.93 b	0.018
∑PUFA	836.35 ± 92.39 ab	737.79 ± 112.29 a	996.01 ± 169.08 b	0.049
n-3/n-6	0.71 ± 0.03 a	0.52 ± 0.06 b	0.7 ± 0.06 a	0.006

Note: Different letters in the table represent significant differences ($p < 0.05$); same letters indicate no significant differences ($p > 0.05$).

It can be seen from the fatty acid content table that the highest contents of palmitic acid and stearic acid in the live bait group were 496.25 ± 66.5 μg/g and 215.79 ± 34.43 μg/g, respectively, which were significantly higher than those in the other two groups ($p < 0.05$). The contents of total saturated fatty acids (∑SFA), total monounsaturated fatty acids

(∑MUFA), total polyunsaturated fatty acids (∑n-3PUFA), and total n-6 polyunsaturated fatty acids (∑n-6PUFA) in the feed group were significantly lower than those in the live bait group ($p < 0.05$).

3.3. Effects of Feed Instead of Live Food on Liver and Intestinal Digestive Enzymes of Giant Salamanders

The effects of feeding on the digestive enzymes of giant salamanders are shown in Figure 1.

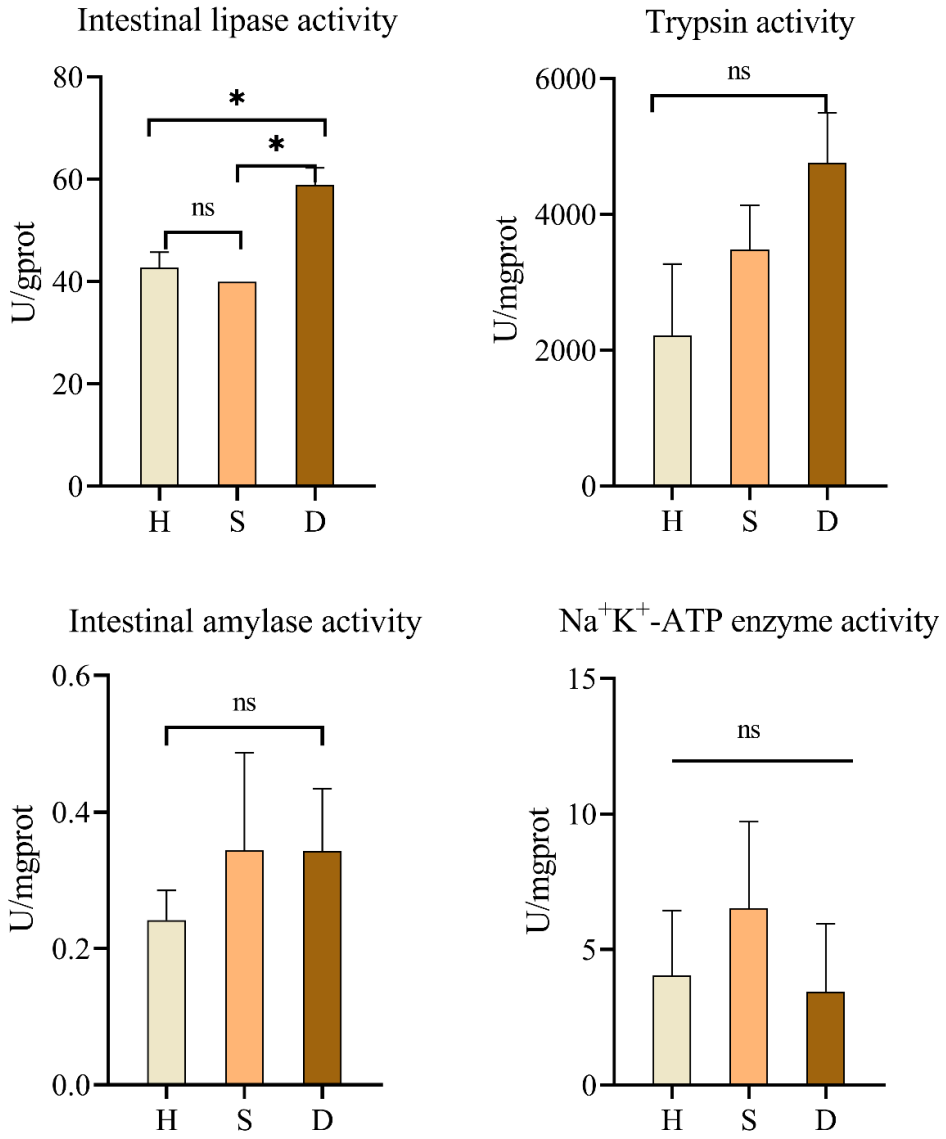


Figure 1. Digestive enzyme activities in intestinal tissue of the three groups of giant salamanders after the experiment (* in the figure indicates significant differences between the three groups and ns indicates no significant difference between the three groups).

As shown in the figure, the intestinal lipase activity of the frozen fish group was significantly higher than that of the other two groups ($p < 0.05$), whereas the activities of amylase, trypsin, and Na^+K^+-ATPase in the intestinal tissues of giant salamanders in the feed group were slightly higher than those in the live bait group, and there was no significant difference in digestive enzyme activities among the three groups ($p > 0.05$).

3.4. Effects of Feed Instead of Live Feed on Antioxidant Enzymes in Liver and Intestine of Giant Salamanders

The effects of different diets on antioxidant enzyme activity in giant salamander tissues are shown in Figures 2 and 3.

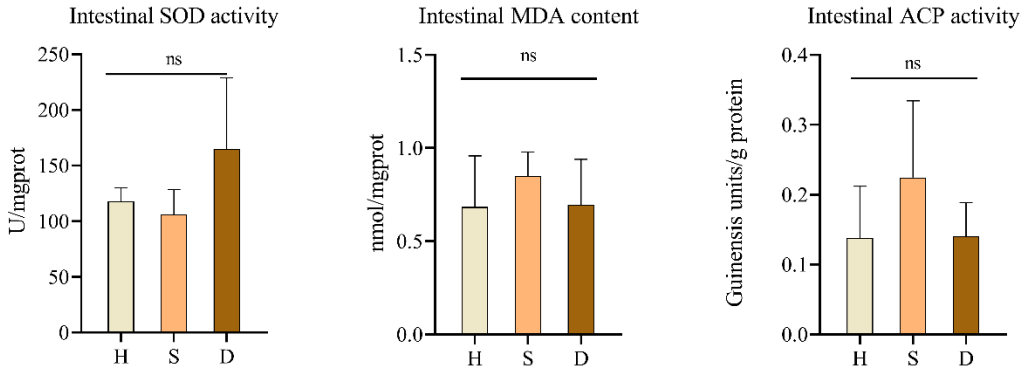


Figure 2. Antioxidant enzyme activity in intestinal tissue of the three groups of giant salamanders after the experiment.

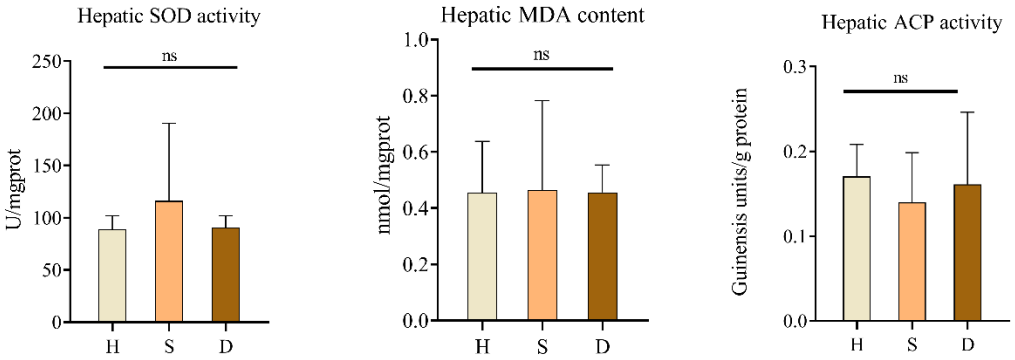


Figure 3. Activity of antioxidant enzymes in liver tissues of the three groups of giant salamanders after the experiment.

There were no significant differences in intestinal antioxidant enzyme levels among the three groups ($p > 0.05$). The ACP activity in the feed group was slightly higher than that in the other two groups. In terms of liver antioxidant activity, there were no significant differences among the three groups ($p > 0.05$), and the SOD activity of the feed group was slightly higher than that of the other two groups ($p > 0.05$).

3.5. Effects of Feed Instead of Live Feed on Intestinal Microbial Community of Giant Salamanders

High-throughput sequencing data were obtained based on 16s sequencing. To eliminate errors and suspicious sequence influences, the measured sequences were screened. Table 6 presents the results. A total of 1,883,470 high-quality sequences were obtained from all samples, with an average of 104,637 sequences per sample, average length of 409 bp, and average efficiency of 79.81%. This value was much higher than 70%, indicating the high reliability of the sequencing results.

After the experiment, the α diversity index of intestinal microbial community of the three groups of giant salamanders was shown in Figure 4.

As shown in the figure, the dominance index of the feed group was significantly higher than that of the live bait group on the pielou_e, Shannon, and Simpson indices ($p < 0.05$), while the dominance index was significantly lower than that of the other two groups ($p < 0.05$). There were no significant differences in the chao1 and observed_features indices among the three groups ($p > 0.05$).

Table 6. Statistics of sequences in each sample.

Sample	RawPE	Clean Tags (#)	Effective Tags (#)	Avglen (nt)	Effective (%)
NH1	105,575	104,022	85,453	407.51	80.94%
NH2	102,715	101,439	84,395	408.66	82.16%
NH3	106,672	104,897	89,804	408.81	84.19%
NH4	107,765	106,375	86,483	408.11	80.25%
NH5	115,009	113,479	86,678	407.53	75.37%
NH6	104,509	103,290	84,755	408.28	81.10%
NS1	116,981	115,087	86,354	410.05	73.82%
NS2	105,213	103,642	84,039	412.87	79.88%
NS3	105,398	104,121	79,380	408.02	75.31%
NS4	104,523	103,196	87,122	410.93	83.35%
NS5	103,464	102,024	81,708	411.97	78.97%
NS6	105,699	104,019	91,482	410.03	86.55%
ND1	106,625	105,176	89,802	408.26	84.22%
ND2	102,084	100,260	80,809	410.88	79.16%
ND3	103,788	102,416	70,742	408.89	68.16%
ND4	104,173	102,893	75,389	407.71	72.37%
ND5	106,149	104,727	88,608	407.03	83.48%
ND6	104,177	102,407	90,991	406.9	87.34%

Note: # represents the number of sequences and nt represents the length of the sequence.

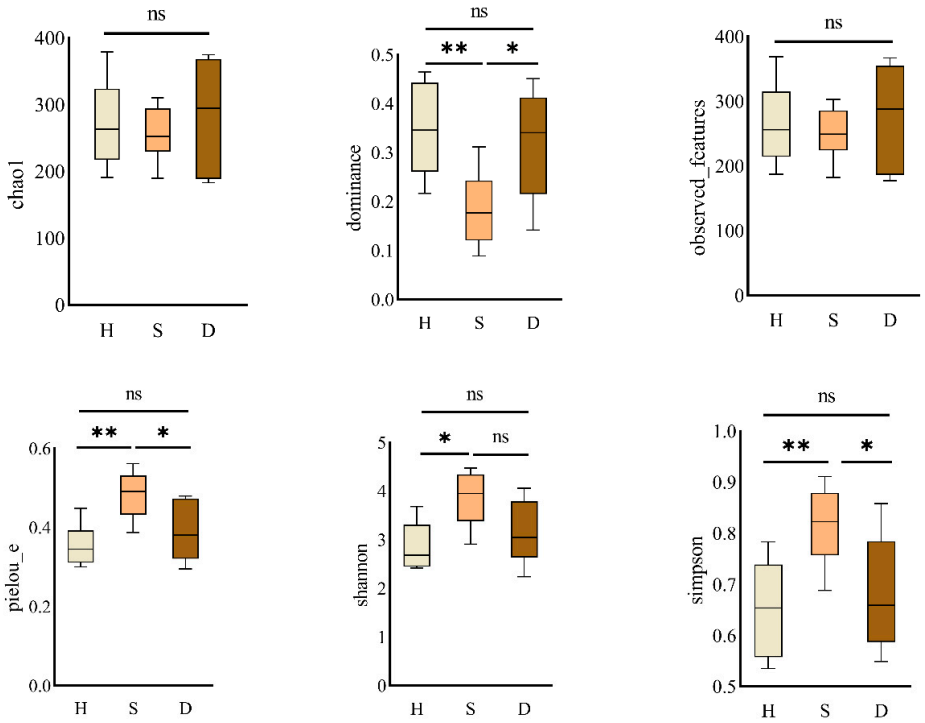


Figure 4. α diversity of intestinal microorganisms of the three groups of giant salamanders after the experiment (Note: * in the figure indicates significant differences between the three groups, $p < 0.05$, ** in the figure indicates very significant differences between the three groups, $p < 0.01$ and ns in the figure indicates no significant difference between the three groups. chao1: To estimate the total number of species contained in the community sample, the more low-abundance species in the community, the greater the chao1 index. pielou_e: Evenness index, the more uniform the species, the larger the pielou_e. Shannon: Total number of categories in the sample and their proportion. The higher the community diversity, the more uniform the species distribution and the larger the Shannon index. Simpson: represents the diversity and evenness of species distribution in a community. The better the evenness of species, the larger the Simpson index. dominance: The probability of taking two random sequences from the same sample. The greater the homogeneity of the community, the smaller the dominance index. observed_features: Number of visually observed species. The larger the index, the more species observed.

Sequences were annotated to study the relationship between the intestinal microbial community compositions of the three groups of giant salamanders. Based on the annotation results of species at different classification levels, we selected the top 10 species with the maximum relative abundance in each sample or group at each classification level (Phylum, Class, Order, Family, Genus, Species) and set the remaining species as others. A relative abundance histogram of the species annotation results for each group at different taxonomic levels was drawn.

As can be seen from Figure 5, the top five phyla of intestinal microbiota in the live feeding group were Fusobacteriota (61.63%), Firmicutes (24.62%), Verrucomicrobiota (7.95%), Bacteroidota (3.66%), and Actinobacteriota (0.74%); the top five genera on the genus level are Cetobacterium (61.61%), Akkermansia (7.90%), Romboutsia (0.72%), Clostridium_sensu_stricto_1 (0.72%), and Rikenella (0.72%). The top five phyla of intestinal microorganisms in the feed group were Firmicutes (62.23%), Fusobacteriota (13.69%), Bacteroidota (7.73%), Verrucomicrobiota (5.35%), and Proteobacteria (5.14%); the top five genera on the genus level are Cetobacterium (13.69%), Paraclostridium (9.98%), Lachnoclostridium (9.04%), Clostridium_sensu_stricto_1 (7.21%), and Clostridium innocuum_group (5.38%). The top five phyla of intestinal microorganisms in the frozen fish group were Fusobacteriota (45.76%), Firmicutes (37.93%), Bacteroidota (5.95%), Verrucomicrobiota (5.45%), and Proteobacteria (1.40%); the top five genera in the genus level are Cetobacterium (46.74%), Akkermansia (5.29%), Romboutsia (2.41%), Bacteroides (1.83%), and Lachnoclostridium (1.51%).

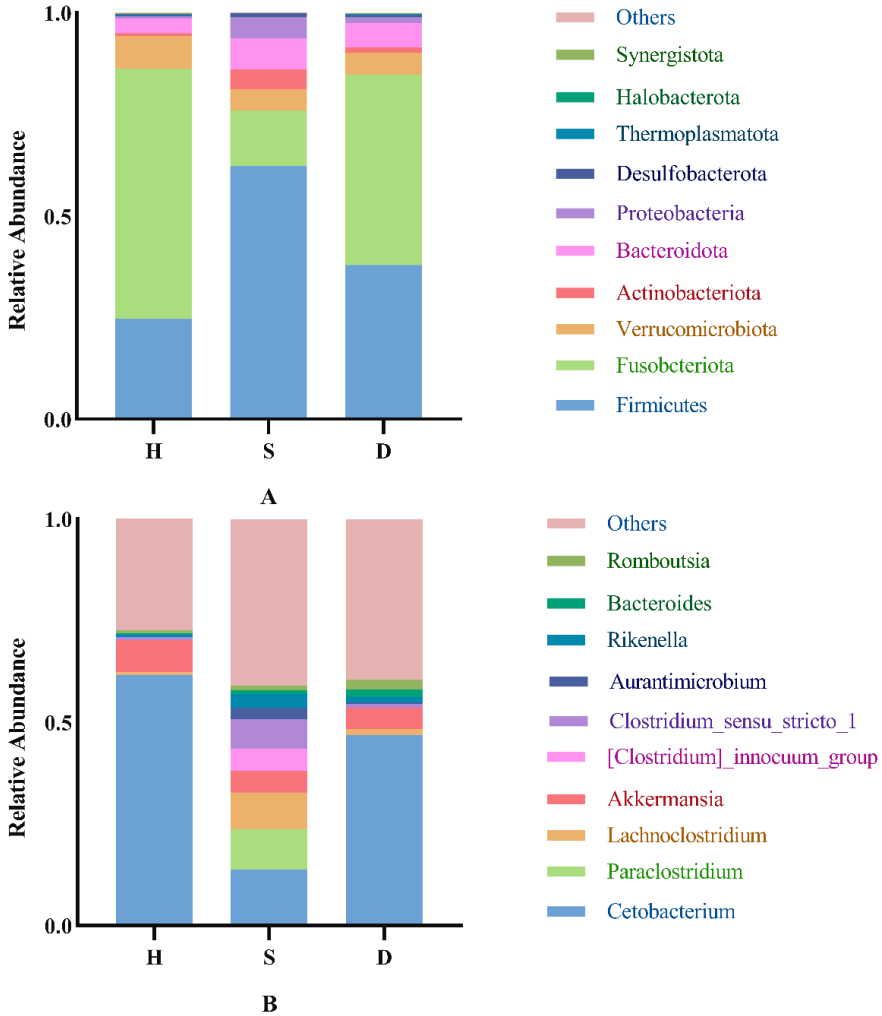
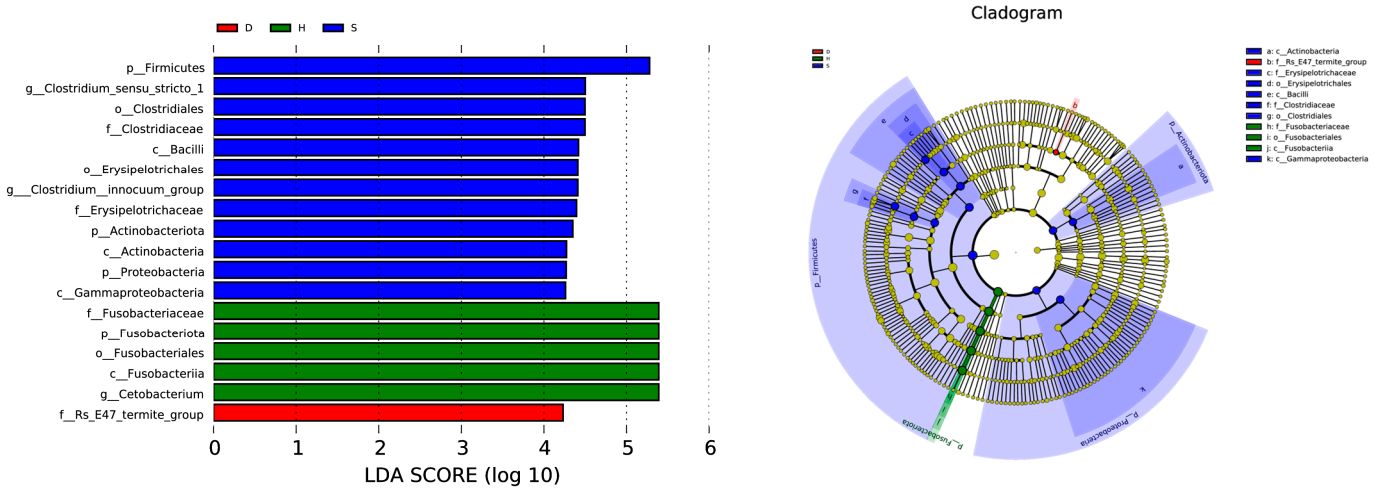


Figure 5. Histogram of intestinal microbial community composition of the three groups of giant salamanders ((**A**) phylum level (**B**) genus level).

To further study the ecosystem results of the gut microbes of the three groups of giant salamanders, linear analysis effect size (LEfse) was used to reveal the complex system structure of the gut microbes of the three groups of giant salamanders, and linear discriminant analysis was used to identify the differences in all samples (the LDA threshold was 4), as shown in Figure 6.



(**A**) (**B**)

Figure 6. LEfse analysis results (**A**) Histogram of LDA value distribution; (**B**) LDA effect size (LEfSe) of microbial class (LDA > 4). The circles in the figure represent different taxonomic levels from phylum to genus, with each small circle representing a taxonomy at that level (**B**). The relative abundance of microorganisms is proportional to the diameter of the circle. Significant differences are marked with different colors consistent with the corresponding level, but yellow indicates no significant difference.

As shown in Figure 6A, 18 different branches of microorganisms were detected, among which branches with scores greater than 4 were the most abundant in the feed group. Firmicutes was the most significantly enriched, and the rest were significantly enriched in some branches of Clostridium and Proteobacteria. Cetobacterium and Fusobacteriota were the most abundant bacteria in the live bait group. Fewer bacteria were significantly enriched in the frozen fish group, and only one species was Rs_E47_termite_group.

According to the database annotation results, the top ten functional information items of each sample or group with the highest abundance at each annotation level were selected to generate the functional prediction results. Finally, based on the KEGG database, the functional genes predicted using the Tax4Fun functional annotation method were enriched in the KEGG pathway, and the results are shown in the following figure.

As shown in Figure 7, there were significant functional differences between the feed group and the other two groups; purine metabolism, pyrimidine metabolism, DNA repair, and recombinant protein were significantly upregulated, and amino acid-related enzymes and transporters were significantly downregulated. There were no significant differences between the live-bait and frozen fish groups. As shown in Figure 8, enrichment of the KEGG pathways was observed. The functions of the intestinal microbiome of the three groups were primarily concentrated on environmental information processing, genetic information processing, and metabolic functions, such as membrane transport, translation replication, and repair nucleotide metabolism, carbohydrate metabolism, and amino acid metabolism. It is also involved in the metabolism of cofactors and vitamins, lipid metabolism, glycan biosynthesis and metabolism, and energy metabolism.

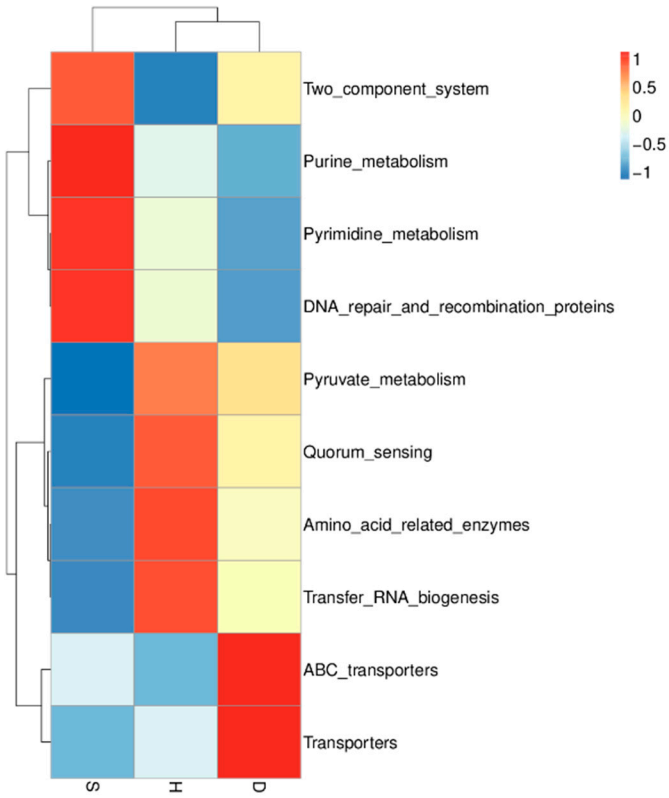


Figure 7. Tax4Fun functional annotation clustering heat map.

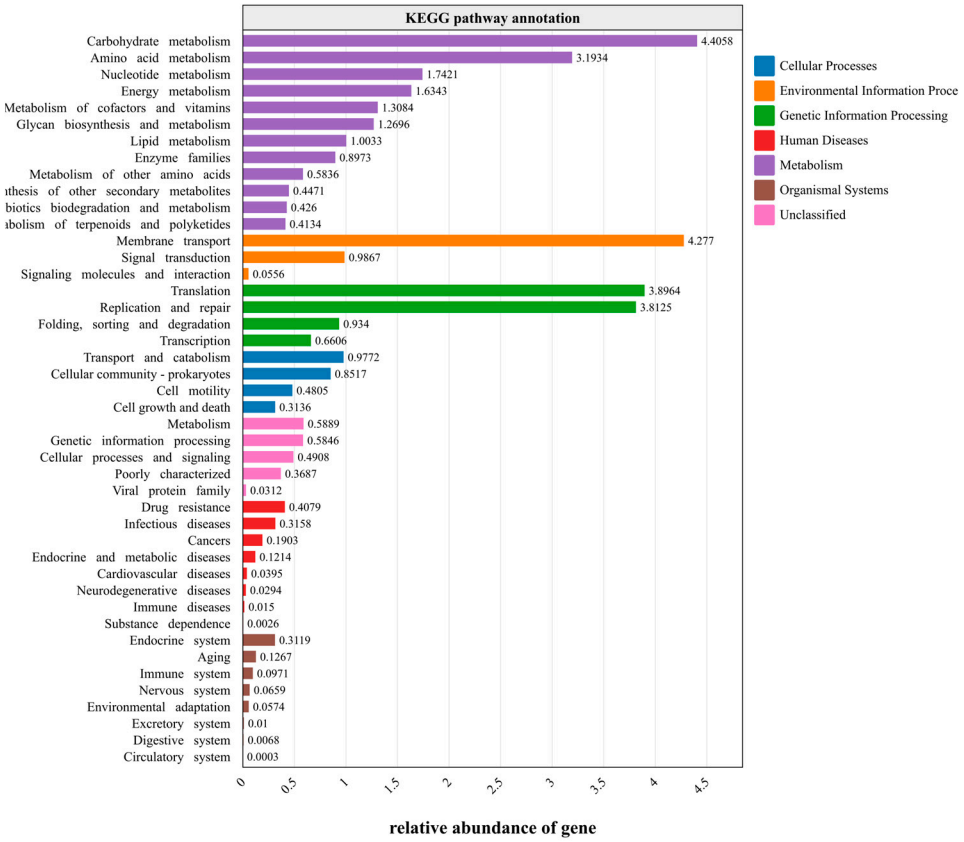


Figure 8. KEGG enrichment pathway diagram of Tax4Fun functional annotation.

4. Discussion

4.1. Effects of Compound Feed Instead of Live Bait on Growth Performance of Giant Salamanders

The growth rate of the Chinese giant salamander is related to many factors, among which diet is one of the most critical. Food is an important source of nutrition for giant salamander cultures, and its palatability and species are closely related to the growth and development of the giant salamander [16]. Giant salamanders are carnivorous animals that can feed on compound feed after domestication [35]. Currently, artificial breeding of giant salamanders primarily involves feeding live bait and frozen fish. There is insufficient research on the artificial compound feed for giant salamanders. Qian et al. indicated that the growth performance of giant salamanders fed with midge larvae was significantly higher than that of groups fed with earthworms, midge larvae, and earthworms [36]. The results of this study showed that there was no significant difference in the growth of giant salamanders fed feed instead of live bait, thereby indicating the feasibility of artificial culture of giant salamanders fed feed instead of live bait. These results are similar to those found in hybrid grouper (*Epinephelus fuscoguttatus* ♀× *Epinephelus lanceolatus* ♂) [37], *Siniperca scherzeri* [38], and *Micropterus salmoides* [39]. In a report on hybrid sturgeons (*Acipenser baerii* × *Acipenser schrenckii*), the replacement of live bait with formula feed significantly increased WGR and FCR [40]. This may be due to the differences in feeding habits between different species, farming environments, and the nutritional requirements of different types of aquatic animals. To develop new feeds for giant salamanders in the future, it is necessary to adjust the formula according to the different nutrient requirements of giant salamanders.

4.2. Effects of Feeding Giant Salamander with Feed Instead of Live Bait on Its Muscle Nutrients

Proteins and lipids in the muscle are important indicators for evaluating muscle nutrition and quality in aquatic animals [41], which are closely related to diet [36]. In this study, feeding giant salamanders with feed instead of live bait did not affect their crude nutrient content, which was similar to the experimental report of replacing live bait with compound feeds in *Homarus Gammarus*, L. and *Siniperca Chuatsi* [41,42]. These results also indicated that feed instead of live feed did not affect the nutritional composition of giant salamanders and further demonstrated the feasibility of feeding in the artificial breeding of giant salamanders.

Life activities cannot be separated from those of proteins in the body. As the basic components of proteins, amino acids are crucial for growth performance, physiological digestion, and immune function of aquatic animals [43,44]. Amino acids are divided into two types: essential and nonessential; animals cannot synthesize essential amino acids. In the present study, there were no significant differences in the composition of essential amino acids, total essential amino acids, total non-essential amino acids, or total amino acids among the muscles of the three groups. These results are similar to the experimental results of Ding et al. who replaced live bait with perch feed in China [41]. This indicated that feeding the giant salamander did not affect the amino acid nutrition of its muscles.

In aquatic animals, fatty acids (FAs) possess a variety of functions, such as acting as components of body membranes, participating in signal transmission, and energy storage [45]. According to the number of carbon atoms and unsaturated bonds, fatty acids can be divided into short chain, long chain, saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) [46]. Simultaneously, PUFA can be divided into n-3PUFA and n-6PUFA according to the position of unsaturated bonds on the carbon chain. EPA and DHA are vital n-3PUFA, which can promote brain development and improve immune function [47]. DHA also helps in cardiac protection [46]. The ratio of n-3PUFA to n-6PUFA in muscle indicates the nutritional value of muscle to a certain extent [48]; a

higher content of n-6PUFA will trigger the inflammatory response of the body [49]. In this study, the ratios of ∑SFA, ∑MUFA, ∑PUFA, and n-3PUFA/n-6PUFA in the feed group were significantly lower than those in the live bait group, but the values of n-3PUFA/n-6PUFA in muscle of the three groups were all greater than 0.2, which obviously met the FAO standard of fatty acid content in high-quality diet [50]. These results indicate that feeding giant salamanders with feed instead of live bait has no significant effect on its nutrient composition and amino acid nutrition, but significantly reduces its muscle quality and nutritional value, which may be related to the lack of animal-type oil in the artificial compound feed group. The fatty acid composition in aquatic animal muscles is directly affected by the fatty acid content in food [51]. Animal fats are an important source of n-3 series unsaturated fatty acids in food and are of great significance in improving nutrition and health [52]. This may be related to the plant-based raw materials used in the feed. It has been reported that the fiber component wraps the nutrients in the plant feed into the cell walls of various components, which has a negative impact on the digestion and absorption of nutrients in the intestine [53]. This may be the primary reason for the decrease in fatty acid content in the muscle of giant salamanders in the feed group. Future research on giant salamander feed should be designed according to the fatty acid content of the feed formula.

4.3. Effects of Feeding Giant Salamander with Feed Instead of Live Bait on the Activities of Digestive Enzymes and Antioxidant Enzymes

Chemical enzyme activity is often used as an indicator of feed composition utilization by aquatic organisms [54]. The digestive organ is the basis for the digestion and absorption of nutrients [55]; the intestine is an important digestive and absorption organ in animals, and it is also the site for the digestion and absorption of nutrients [56]. In this study, there was no significant difference in the digestive enzyme activity of giant salamanders between the three groups, which is similar to the results of Zhang et al. [32]. This indicates that the ability of the giant salamanders in the feed group to decompose the three major nutrients, protein, starch, and fat, was not significantly different from that in the other two groups. Intestinal Na^+K^+-ATPase, a cell membrane protein, plays a key role in energy metabolism, material transport, and signal transmission in animals [55]. Its activity is directly related to the digestive and absorption capacity of the intestine [57]. There was no significant difference in the intestinal Na^+K^+-ATPase activity among the three groups, indicating that the substitution of feed for live bait did not affect the digestive capacity of the giant salamander, and this result was consistent with the growth performance. Simultaneously, the feasibility of artificial breeding of giant salamanders with feed instead of live bait was repeatedly verified.

To further verify the feasibility of the feed, we explored its effect on the immunity of giant salamanders. SOD, ACP, and MDA activities were detected in the intestinal and liver tissues of the three groups. Aquatic animals produce a series of oxidative free radicals in the normal metabolic process, which are in a dynamic balance of generation and decomposition in the body [58], depending on the role of the body's antioxidant system. The liver is the primary site of antioxidant enzyme production in aquatic animals [59]. When the oxidative pressure of the liver is extremely high or the antioxidant capacity decreases, the oxidative pressure spreads throughout the body [59]. SOD is an important enzyme in the antioxidant system of aquatic animals that can convert oxygen free radicals into H_2O_2 and remove the superoxide free radicals produced by oxidation in the body [60]. Free radical levels reflect the ability of the body to convert free radicals. When the ability of SOD to remove free oxygen radicals in the body is insufficient, excess free radicals undergo peroxidation with lipid substances to produce a large amount of MDA. MDA is cytotoxic and can reduce cell metabolism to cause cell dysfunction and even cell death [61,62]. Therefore, MDA reflects the degree of lipid peroxidation in the body and is a marker of oxidative damage [63]. An

increase in its content often leads to cell damage and disease [64]. ACP is a marker enzyme of the lysosomes of macrophages in higher animals and is an important component of lysosomes [65]. Under acidic conditions, ACP removes foreign bodies from phosphate esters on the cell surface via hydrolysis [66]. Studies have shown that an increase in ACP activity accelerates the transport and metabolism of phosphate groups, enhances the transport of nutrients in the body, and accelerates the destruction and elimination of foreign bodies to strengthen immune capacity [67,68]. ACP participates in phosphate metabolism and plays key roles in signal transduction and energy conversion. As a lysosomal marker enzyme, ACP also participates in immune processes, such as intracellular macromolecule metabolism and pathogen recognition and clearance [69]. No significant differences in SOD, ACP, and MDA contents were observed in the liver and intestine of the three groups fed in this study. These results indicated that feeding giant salamanders feed instead of live bait did not cause greater oxidative stimulation and did not affect their antioxidant capacity.

4.4. Effects of Feeding Giant Salamanders with Feed Instead of Live Bait on Their Intestinal Microbial Community

Feeding patterns and dietary diversity affect the composition, diversity, and distribution of intestinal microbiota in aquatic animals [36]. The gut microbiome is sensitive to different food sources and affects the gut health of the host [25]. Higher gut microbiota diversity and richness are associated with better host health, whereas reduced diversity is associated with an increased risk of biological disorders and diseases [70]. According to recent scientific research, intestinal bacteria can improve feed digestion, and their population stability can affect the host immune system [71]. Feed has a significant impact on the intestinal flora of aquatic animals. In his study on *Takifugu obscurus*, Yang found that the intestinal microbial community of puffers fed with artificial feed and natural food showed significant changes [72]. In giant salamanders, it was found that feeding the larvae of giant salamanders with different live feeds could significantly change the diversity and richness of intestinal flora [36]. Compared to seaweed feeding, feeding an artificial diet significantly improved the diversity of the intestinal flora in *Haliotis discus* hannai [73]. In this study, the alpha diversity index (Shannon index and Simpson index) of the intestinal flora of giant salamanders in the feed group was significantly higher than that in the other two groups, which indicates that feeding feed instead of live bait significantly improved the diversity and richness of the intestinal flora of giant salamanders and promoted intestinal health. One possible reason for this is that the microbial communities carried by different food species differ. There are more raw plant materials in the feed, and the giant salamander is a carnivorous animal, which may introduce more intestinal microorganisms into the process of feed domestication.

In this study, the main bacteria in the intestinal flora of the three groups of giant salamanders were Firmicutes, Bacteroides, Clostridium, and Actinobacteria, which is consistent with previous studies on the intestinal microbiome of giant salamanders [74–76]. The highest relative abundance of Firmicutes in the intestinal tract of *S. salamandus* giant was 62.23% in the feeding group. Firmicutes can play a key role in host nutrition and metabolism through the synthesis of SCFA [77]. The Firmicutes to Bacteroides (F/B) ratio is positively correlated with body obesity [78]. In this study, the F/B ratio in the feed group was higher than that in the other two groups, indicating that dietary giant salamander promoted lipid synthesis by altering the richness of the intestinal flora. Previous experiments in mice and humans have reported that an increase in the abundance of Proteobacteria may lead to fat accumulation and obesity [79–81]. LEFSE analysis showed that Firmicutes and Proteobacteria were the most significantly enriched phyla in the feed group. The functional prediction results of this study showed that there were functional differences among the intestinal flora of the three groups of giant salamanders, among which purine

metabolism and pyrimidine metabolism were significantly upregulated in the feed group. Additionally, cellular purines, especially adenosine triphosphate (ATP), provided fuel for several metabolic reactions in body cells [82]. The pyrimidine metabolism is associated with intracellular nucleic acid synthesis, phospholipid synthesis, and glycosylation [83]. Meanwhile, studies have shown that pyrimidine compounds in cells can maintain pyruvate oxidation and lipogenesis by regulating pyruvate dehydrogenase activity [82]. These results were consistent with the higher crude fat content in the muscle of the feed group than in the other two groups. The specific mechanism leading to a decrease in fatty acid content in the muscle of giant salamanders in the feed group requires further study.

The enrichment results of functional prediction pathways showed that most of them focused on nutrient biosynthesis and metabolism, as well as genetic information processing, which was similar to previous reports on functional prediction of the intestinal flora of giant salamanders [31]. The specific mechanisms of the metabolic functional pathways require further study.

5. Conclusions

In the present study, the effects of different diets on the growth performance, nutrient composition, digestion and absorption capacity, and intestinal microbial community of Chinese giant salamanders were analyzed. The results showed that feeding with feed instead of live bait did not affect the growth performance, nutrient composition, digestion, absorption, and antioxidant capacity of giant salamanders but reduced the content of fatty acids in muscle, affected meat quality, and changed the composition of the intestinal microbial community, thereby significantly improving the richness and diversity of the intestinal microbial community of giant salamanders. This study provides data support and a scientific basis for the selection and formulation optimization of artificial breeding feeds for Chinese giant salamanders.

Author Contributions: Formal analysis, H.F.; data collection, P.H., J.H. and L.L.; investigation, H.F., J.Z. and Z.W.; visualization, P.H. and J.Z.; resources, Z.W., J.L. and J.H.; writing—original draft preparation, H.F.; writing—review and editing, D.Z. and S.J.; conceptualization, D.Z.; methodology, D.Z. and S.J.; supervision, D.Z.; funding acquisition, D.Z. and S.J. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The animal study protocol was approved by the Animal Care and Use Committee of Nanchang University (identification code: NCUACC-2020-563; date of approval: 26 May 2020).

Data Availability Statement: Data will be made available on request.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of this study; in the collection, analysis, or interpretation of the data; in the writing of the manuscript; or in the decision to publish the results.

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