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Research Article

Grazing Effects of *Xenocypris davidi* Bleeker (*Cyprinidae*, *Cypriniformes*) on Filamentous Algae and the Consequent Effects on Intestinal Microbiota

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Filamentous algae are present in seawater and freshwater ecosystems worldwide. Their growth poses a serious threat to water environments and fishery production, especially Chinese mitten crab and red swamp crayfish culturing. To explore safe and ecological treatment methods, we comparatively studied the grazing effects of *Xenocypris davidi* Bleeker on *Cladophora* (one species) and *Spirogyra* (two species) in indoor glass tanks and examined the intestinal microbiota related to digestion and absorption. The results showed that the fish was more receptive to *Spirogyra* than to *Cladophora*, and the intake rates of different species of *Spirogyra* varied. Fish weight increased slightly after feeding with *Spirogyra*, and the levels of total nitrogen and total phosphorus in water increased significantly ($p < 0.05$) relative to the initial values. Intestinal microbiota diversity and richness varied after feeding with three filamentous algal species, and the similarity increased after feeding with two *Spirogyra* species. The microbial species detected in this study belonged to 40 phyla and 838 genera. The clustering characteristics of different groups and subgroups were obvious based on the microbial phylum composition. Significant differences in the relative abundance rates of eight nutrition-related metabolism functions were found among groups and subgroups ($p < 0.05$). The correlations between intestinal microbiota and metabolism functions were analyzed, and some meaningful correlations were revealed. The results presented in this study provided a meaningful reference on the control of the overgrowth of filamentous algae in aquaculture waters by the biomanipulation method.

1. Introduction

Filamentous green algae are present in seawater and freshwater ecosystems worldwide, having high biomass levels and occupying large areas in different water bodies [1, 2]. Their uncontrolled growth poses a serious threat to water environments and fishery production [3–5] by causing the death and migration of aquatic animals and changes in biodiversity [5–9]. Moreover, the rotten mats of filamentous green algae cause hypoxia in waters and produce toxic gases, such as hydrogen sulfide [8]. *Cladophora* and *Spirogyra* blooms are serious problems in aquaculture in China, especially in Chinese mitten crab (*Eriocheir sinensis*), red swamp crayfish (*Procambarus clarkii*), and shrimp farming ponds. The industry annually suffers considerable losses because of the indiscriminate use of unregulated drugs from herbicides.

The environmental sustainability of aquaculture systems can be maintained by using biological approaches for the

in situ removal and utilization of inorganic and organic nutrients [10]. Currently, the application of "integrated multitrophic aquaculture" systems that combine species from different trophic levels or complementary ecosystem functions in the same aquaculture system is strongly recommended [11–13]. *Xenocypris davidi* Bleeker is a member of *Xenocypris* and belongs to the order *Cypriniformes* of the family *Cyprinidae* [14]. *X. davidi* Bleeker is small and medium-sized economic fish and widely distributed in China [15]. Regarded as an "ecological fish" in China, it mainly feeds on the branches and leaves of aquatic plants, attached algae, and organic debris [16, 17]. Additionally, *X. davidi* Bleeker is timid by nature and is unlikely to compete with aggressive crayfish and crabs for food. Therefore, controlling the overgrowth of nuisance filamentous algae in crayfish and crab culturing waters is feasible. However, relevant studies for reference are inadequate.

Firstly, the host's diet selection can be influenced by gut microbiome [18]. Additionally, the intestinal microbiota positively or negatively influences host health, nutrient harvest, physiological development, intestinal immune response, and disease outbreak [19–23]. A series of exogenous and endogenous factors, especially feed [20, 24–26], can affect the establishment and nature of the microbial composition in the gastrointestinal tract of fish [22, 27]. Changes in the intestinal microbiota are linked to digestive activities and immunity [28, 29]. That is to say, intestinal microbiota not only affect whether *X. davidi* Bleeker can graze filamentous algae but also their own state, which is one of the important indicators reflecting the impact on fish after feeding filamentous algae. In the present study, we conducted a comparative study of the grazing effects of *X. davidi* Bleeker on *Cladophora* and *Spirogyra* and investigated differences in intestinal microbial composition and metabolism functions in the intestines of *X. davidi* Bleeker according to filamentous algae consumed. The results presented in this study provided a theoretical and foundational reference about the control of the overgrowth of filamentous algae in aquaculture waters through a biomanipulation method.

2. Materials and Methods

2.1. Experiment Design. Forty-five 80 cm × 50 cm × 50 cm glass tanks filled with 30 cm of fully aerated tap water were set up indoors. The glass tanks were divided into three experimental groups (G, S1, and S2). The healthy and growing filaments of *Cladophora* (one species, marked G) and *Spirogyra* (two species, one marked S1 and another marked S2, and the taxonomic affiliation of the three species (G, S1, and S2) are shown in supplementary figure A) were obtained from three crayfish and crab farming ponds. Then, three kinds of filamentous algae were placed in corresponding glass tanks (30.0 g of wet weight each tank). In order to minimize the interferences brought by continuously sampling and weighing (5 times), each of the three groups was divided into five repeated subgroups (marked A (day 1), B (day 3), C (day 5), D (day 7), and E (day 9)), and each subgroup also had three replicates. *X. davidi* Bleekers fries with the same weight (10.4 ± 1.2 g) were randomly placed in all tanks (10 fries for each tank) after two days of respite (no feed). During the experiment, each tank received an equal amount of oxygen supply with an aerator. No commercial feed, animal health product, or drug was used during culture. No water was added or changed in the process. The indoor temperature was kept at 27°C with an air conditioner.

2.2. Sample Collection. Remaining filaments were obtained from the A–E subgroups of each experimental group with plankton nets (ø 0.064 mm) on days 1, 3, 5, 7, and 9, which were the end times of each subgroup. The surface water of collected remaining filaments was quickly absorbed by absorbent papers, and then their weight was weighed. Three midintestines of *X. davidi* Bleeker fries were randomly collected from three replicates of A, C, and E subgroup on days 1, 5, and 9, and then the three midintestines were combined as a single sample. Thus, nine midintestine samples were obtained from each experimental group (GA-1, GA-2, GA-3, GC-1, GC-2, GC-3, GE-1, GE-2, and GE-3 from the G group; SA1-1, SA1-2, SA1-3, SC1-1, SC1-2, SC1-3, SE1-1, SE1-2, and SE1-3 from the S1 group; SA2-1, SA2-2, SA2-3, SC2-1, SC2-2, SC2-3, SE2-1, SE2-2, and SE2-3 from the S2 group; A, C, and E represented samples collected on days 1, 5, and 9, respectively, and the last numbers 1, 2, and 3 represented three replicates). Water samples were collected from all groups every 2 days except the initial samples (0, 1, 3, 5, 7, and 9) to analyze water quality parameters, including total nitrogen (TN), total phosphorus (TP), nitrite nitrogen ($NO2^{-}$ -N), and ammonia nitrogen (NH_4^{+}-N). These parameters were analyzed immediately in laboratory conditions in accordance with the China National Standards for Testing Surface and Groundwater and for Wastewaters [30]. The weights of the fish in the E subgroup of each group were measured and recorded at the start and end of the experiment.

In this experiment, the fish were humanely euthanized for tissue sampling in accordance with the recommendations of the ethical principles of the Experimental Animal Welfare Ethics Committee of China.

2.3. DNA Extraction, Amplification, and Sequencing. CTAB lysate and lysozyme were used to completely lyse the cells orderly [31], and then DNA was extracted with a 3S column centrifugal environmental sample DNA recovery kit (Shanghai Bocai Biotechnology Co. Ltd., model no: K718) according to the manufacturer's instructions. After genomic DNA extraction, the extracted genomic DNA was detected through 1% agarose gel electrophoresis. In this experiment, the high-throughput sequencing of the samples and the preliminary processing of data were performed by Shanghai Applied Protein Technology Co., Ltd. The primers used were 515f: 5′-bar-code-GTGCCAGCMGCCGCGG-3′ and 907r: 5′ -CCGTCAATTCMTTTRAGTTT-3′ [32].

2.4. Bioinformatics Analyses. Paired-end reads from the original DNA fragments were merged using FLASH, an extremely fast and accurate analysis tool, which was designed to merge paired-end reads when at least some of

the reads overlap with the read generated from the opposite end of the same DNA fragment. Paired-end reads were assigned to each sample and provided with unique barcodes. Sequence analyses were performed with UPARSE software package with the UPARSE-OTU and UPARSE-OTUref algorithms. In-house Perl scripts were used in analyzing alpha (within samples) diversity. Sequences with ≥97% similarity were assigned to the same OTUs. We selected representative sequences for each OTU and use the RDP classifier to annotate taxonomic information for each representative sequence. To compute alpha diversity, we rarified the OTU table and calculated three metrics: Chao1, which estimates the species abundance; observed species, which estimates the amount of unique OTUs found in each sample; and Shannon index. Rarefaction curves were generated according to these three metrics. UPGMA clustering is a type of hierarchical clustering method with average linkage and can be used in interpreting the distance matrix. We used the unweighted UniFrac distance for the unweighted pair group method with arithmetic mean (UPGMA) clustering [33].

2.5. Statistical Analysis. SPSS 19.0 software was used in statistically analyzing the experimental data. Levene's test was applied to check the homogeneity of variance for intake. Reciprocal transformation or inverse sine processing was performed on the above data for the determination of the homogeneity of variance. When the homogeneity of variance was not satisfied after data conversion, multiple comparisons were performed with Games–Howell parametric tests. Canoco 5.0 was used for the correlation analysis of redundancy analysis (RDA). Statistical significance was established at $p < 0.05$. Difference in the abundance of individual taxonomy between the two groups was confirmed with STAMP software. LEfSe was used in the quantitative analysis of biomarkers within different groups. This method was designed to analyze the data in which the number of species is much higher than the number of samples and to provide biological class explanations in order to establish statistical significance, biological consistency, and effect-size estimation of predicted biomarkers. To identify the differences in the microbial communities between the groups, we performed ANOSIM and ADONIS on the basis of Bray–Curtis dissimilarity distance matrices.

3. Results

3.1. Grazing on Filamentous Algae of Fish and Changes in Fish Weight and Water Quality after Feeding. The G group had a lower filamentous algal intake than the S1 and S2 groups, and the intake declined with increasing culturing time (Figure 1). The S1 group had the largest amount of filamentous algae consumed on day 1, but the amount declined rapidly after that. The filamentous algal intake of S2 group declined gradually before day 5 but significantly increased on day 7 ($p < 0.05$). At the start and end of the experiment, the weights of the experimental fish were determined (Figure 2). The average weight of fish in the S2 group

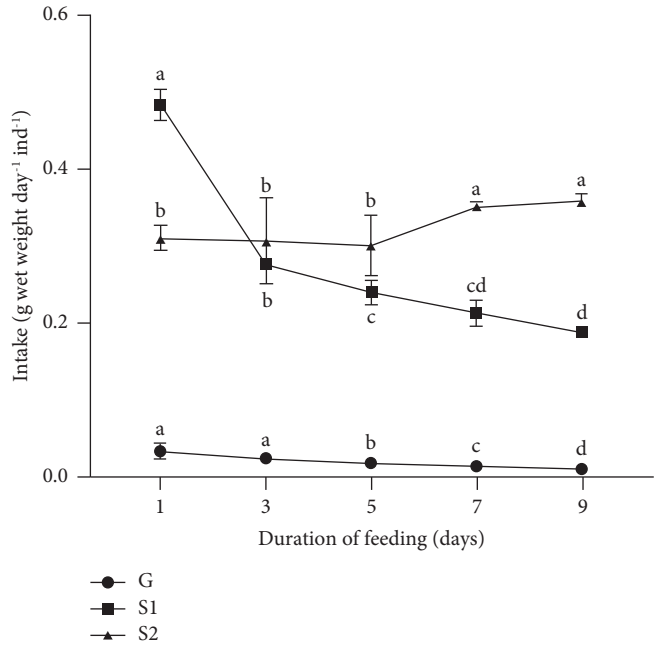

Figure 1: Filamentous algal intake of fish in three experimental groups (G, S1, and S2) at five sampled times. Different letters indicate significant differences based on ANOVA ($p < 0.05$) (internal comparison of each group). Mean ± SD ($n = 3$).

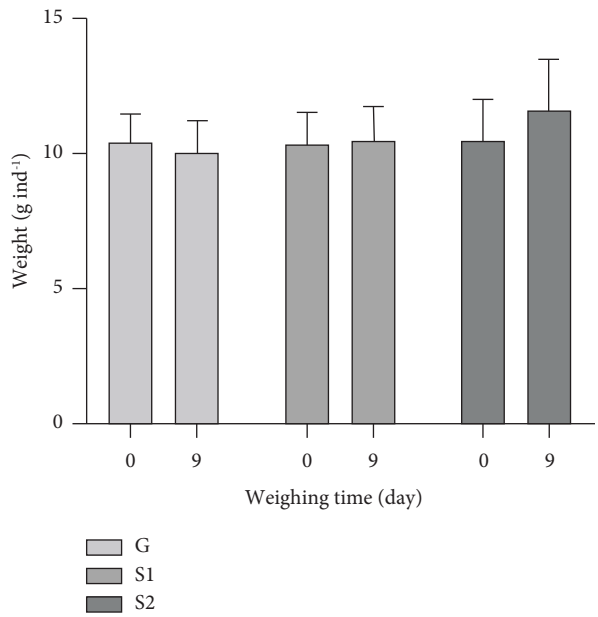

Figure 2: Weights of fish in the three experimental groups (G, S1, and S2) at the start and end of the experiment.

increased considerably, but the average weights of fish in S1 and G groups slightly increased and decreased, respectively. No significant difference was found among the groups ($p > 0.05$). As shown in Table 1, the levels of total nitrogen (TN) and total phosphorus (TP) in the waters increased gradually with culturing time, and the TN and TP levels on day 9 were significantly higher than those at the start of the experiment in the three experimental groups ($p < 0.05$).

Table 1: Water chemical indices of three experimental groups (G, S1, and S2) on days 0–9.

Group	Culturing time (day)	TN ($mg{\cdot}L^{-1}$)	TP ($mg{\cdot}L^{-1}$)	NO_2-N ($mg{\cdot}L^{-1}$)	NH_4^+-N ($mg{\cdot}L^{-1}$)
G	0	1.515 ± 0.286^{d}	0.013 ± 0.005^{f}	0.009 ± 0.004^{d}	0.202 ± 0.037^{ab}
	1	1.563 ± 0.123^{d}	0.023 ± 0.005^{e}	0.115 ± 0.010^{b}	0.236 ± 0.024^{a}
	3	2.031 ± 0.205^{c}	0.070 ± 0.007^{d}	0.211 ± 0.028^{a}	0.193 ± 0.014^{ab}
	5	2.498 ± 0.540^{b}	0.086 ± 0.003^{c}	0.101 ± 0.019^{b}	0.206 ± 0.017^{ab}
	7	2.794 ± 0.170^{ab}	0.140 ± 0.007^{b}	0.055 ± 0.007^{c}	0.132 ± 0.019^{c}
	9	3.138 ± 0.071^{a}	0.172 ± 0.006^{a}	0.018 ± 0.007^{d}	0.158 ± 0.006^{bc}
S1	0	1.360 ± 0.058^{e}	0.001 ± 0.000^{d}	0.001 ± 0.001^{e}	0.164 ± 0.026^{c}
	1	2.008 ± 0.184^{d}	0.059 ± 0.005^{c}	0.068 ± 0.011^{a}	0.258 ± 0.054^{bc}
	3	2.214 ± 0.109^{d}	0.055 ± 0.006^{c}	0.005 ± 0.003^{de}	0.858 ± 0.122^{b}
	5	2.443 ± 0.209^{c}	0.099 ± 0.012^{b}	0.024 ± 0.010^{bc}	1.262 ± 0.151^{b}
	7	2.730 ± 0.304^{b}	0.114 ± 0.022^{b}	0.034 ± 0.019^{b}	1.032 ± 0.175^{b}
	9	2.994 ± 0.043^{a}	0.140 ± 0.023^{a}	0.015 ± 0.007^{cd}	0.084 ± 0.006^{a}
S2	0	1.550 ± 0.128^{d}	0.034 ± 0.012^{d}	0.011 ± 0.007^{b}	0.264 ± 0.061^{b}
	1	1.825 ± 0.142^{cd}	0.036 ± 0.007^{cd}	0.018 ± 0.012^{b}	0.366 ± 0.034^{a}
	3	1.815 ± 0.369^{cd}	0.046 ± 0.020^{bcd}	0.036 ± 0.025^{a}	0.088 ± 0.016^{c}
	5	2.161 ± 0.228^{ab}	0.058 ± 0.012^{bc}	0.014 ± 0.011^{b}	0.077 ± 0.040^{c}
	7	2.097 ± 0.306^{bc}	0.066 ± 0.012^{b}	0.028 ± 0.010^{ab}	0.253 ± 0.018^{b}
	9	2.479 ± 0.286^{a}	0.088 ± 0.022^{a}	0.036 ± 0.004^{a}	0.217 ± 0.036^{b}

Note. Different letters indicate significant differences based on ANOVA ($p < 0.05$) (internal comparison of each group). Mean ± SD ($n = 3$).

Changes in nitrite nitrogen ($NO2^{-}$-N) and total ammonia nitrogen (NH_4^+-N) levels showed the same trend, which is, increasing and then decreasing.

3.2. Analysis of Intestine Microbial Community

3.2.1. Rarefaction Curve. The rarefaction curve can indirectly reflect the rationality of the sequencing data amount directly and the richness of species in samples [34]. In this study, the number of microbial species detected in each intestine samples ranged approximately from 300 to 1000. The rarefaction curve showed that sequencing was relatively comprehensive in covering bacterial diversity as the rarefaction curves tended to approach saturation (shown in supplementary figure B).

3.2.2. Shared and Unique Microbial Taxa. Figure 3 shows the shared and unique kinds of OTUs detected in the intestines for the three experimental groups at different sampling times. For example, a total of 499, 519 and 598 kinds of OTUs were shared in the fish intestines from the three experimental groups on days 1, 5, and 9, which corresponded to 18.8%, 21.4%, and 24.9% of the total kinds of OTUs, respectively.

The most abundant intestinal microbiota (>5%) in the shared OTUs belonged to *Verrucomicrobia* (*Verrucomicrobiales*; 11.5%), Proteobacteria (Gemmobacter; 8.7%), Firmicutes (Bacillus; 6.5%), and *Fusobacteria* (*Cetobacterium*; 5.7%) on day 1. Second, the most abundant OTUs (>5%) in the shared OTUs belonged to Proteobacteria (*Gemmobacter*; 18.1%), *Proteobacteria* (Rhizobiales Incertae Sedis; 7.9%), and *Verrucomicrobia* (Luteolibacter; 5.1%) on day 5. Additionally, the most abundant OTUs (>5%) in the shared OTUs belonged to Proteobacteria (Gemmobacter; 31.4%), *Actinobacteria* (Tessaracoccus; 8.7%), and *Actinobacteria* (Microbacteriaceae; 7.3%) on day 9.

3.2.3. Microbial Diversity Analysis. A total of 3,166,105 high-quality sequencing reads were acquired from 27 samples, with an average of 117,263 reads per sample. The total number of OTUs ranged from 53021 to 99252, with an average of 81469. The method of Ace, Chao1, and observed species was used to calculate the indices of microbial richness based on OTUs. The method of Simpson and Shannon was chosen to calculate the indices of microbial diversity. The coverage indexes of each sample all exceeded 0.99, indicating that the sequencing depth met the requirements of the experiment (Table 2).

3.2.4. Intestinal Microbial Composition. In this experiment, the microbial species detected in all samples belonged to 40 phyla and 838 genera. The dominant phyla (relative abundance >1% at least in one group) of all samples were Proteobacteria, *Actinobacteria*, Firmicutes, *Verrucomicrobia*, Fusobacteria, Patescibacteria, Bacteroidetes, Planctomycetes, and Cyanobacteria (Figure 4). As shown in Figure 4, the proportion of *Verrucomicrobia* in the G group decreased gradually with increasing culturing time. In addition, the proportion of Fusobacteria in the G group was 26.2% initially and then quickly decreased to less than 0.5% at the start of culturing. By contrast, the proportion of *Actinobacteria* in the G group increased rapidly after culturing began. Similar changes in the proportion of the main phyla in the S1 and S2 groups were observed. The proportions of Verrucomicrobia and Proteobacteria in the S1 and S2 groups increased gradually, whereas the proportion of Firmicutes decreased gradually with the prolongation of culturing time. With regard to microbial phylum composition, the samples were clustered relatively close together according to diet and then to sample time (Figure 5).

The dominant genera (relative abundance >1% at least in one group) of all samples were Gemmobacter, Tessaracoccus, Cetobacterium, Burkholderia-Caballeronia-

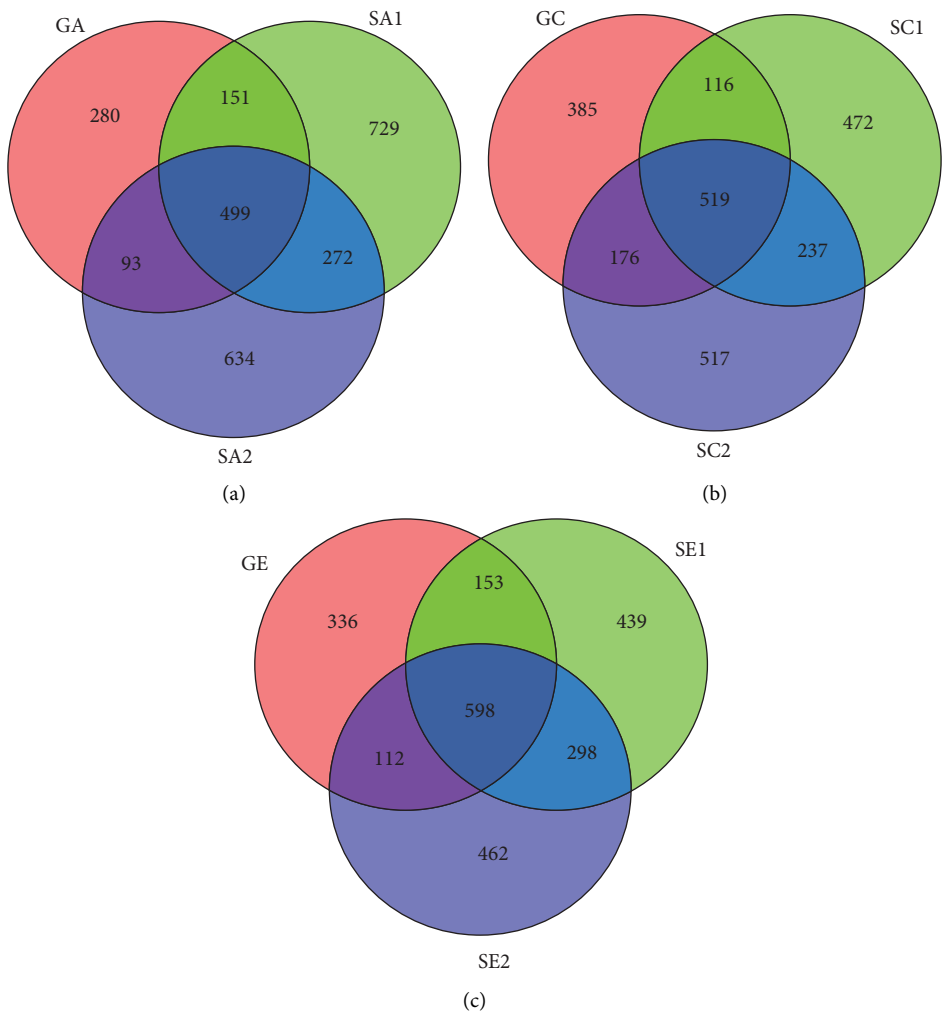

Figure 3: Venn diagram based on OTUs of each group (G, S1, and S2) on days 1 (a), 5 (b), and 9 (c).

Table 2: The richness estimators (Ace, Chao1, and observed species), diversity indices (Shannon and Simpson), and coverage index (goods coverage) were calculated for the three experimental groups (G, S1, and S2) on days 1, 5, and 9.

	Shannon	Simpson	Ace	Goods coverage	Chao1	Observed species
GA	3.638 ± 0.694^{e}	0.753 ± 0.092^{bc}	640.742 ± 43.011^{b}	0.999 ± 0.001	641.555 ± 38.617^{b}	551.333 ± 100.803^{b}
GC	5.201 ± 0.292^{bcd}	0.913 ± 0.032^{ab}	902.262 ± 58.181^{a}	0.997 ± 0.000	867.845 ± 57.147^{ab}	741.000 ± 55.651^{ab}
GE	4.817 ± 0.519^{cde}	0.872 ± 0.056^{ab}	798.871 ± 61.446^{ab}	0.998 ± 0.001	792.040 ± 42.498^{ab}	691.000 ± 59.355^{ab}
SA1	6.334 ± 0.552^{ab}	0.951 ± 0.029^{a}	791.490 ± 224.631^{ab}	0.999 ± 0.000	801.665 ± 208.126^{ab}	740.667 ± 216.892^{ab}
SC1	6.758 ± 0.360^{a}	0.969 ± 0.008^{a}	770.241 ± 135.673^{ab}	1.000 ± 0.000	784.473 ± 143.677^{ab}	729.333 ± 146.097^{ab}
SE1	4.587 ± 1.078^{cde}	0.822 ± 0.102^{abc}	820.424 ± 66.137^{ab}	0.999 ± 0.000	820.444 ± 59.098^{ab}	747.333 ± 43.616^{ab}
SA2	5.889 ± 0.478^{abc}	0.946 ± 0.018^{a}	836.507 ± 263.734^{ab}	0.999 ± 0.001	826.314 ± 263.576^{ab}	732.667 ± 240.500^{ab}
SC2	3.896 ± 1.779^{de}	0.689 ± 0.237^{c}	909.994 ± 191.271^{a}	0.998 ± 0.001	900.712 ± 185.868^{a}	811.667 ± 155.931^{a}
SE2	4.921 ± 0.351^{cde}	0.889 ± 0.043^{ab}	985.650 ± 39.857^{a}	0.998 ± 0.000	968.225 ± 33.619^{a}	863.667 ± 32.868^{a}

Paraburkholderia, Cellulomonas, Candidatus Xiphinematobacter, Bosea, Blautia, Bacteroides, Escherichia-Shigella, Faecalibacterium, Hyphomicrobium, Legionella, Luteolibacter, Mycobacterium, Subdoligranulum, Terrimicrobium, ZOR0006, Bacillus, (Eubacterium) coprostanoligenes group, (Eubacterium) hallii group, and alphaI cluster.

3.3. Intestinal Microbial Metabolism Functions. In this experiment, functions related to membrane transport, amino acid metabolism, carbohydrate metabolism, replication, repair, energy metabolism, metabolism of cofactors and vitamins, translation, lipid metabolism, xenobiotic biodegradation and metabolism, and poorly characterized

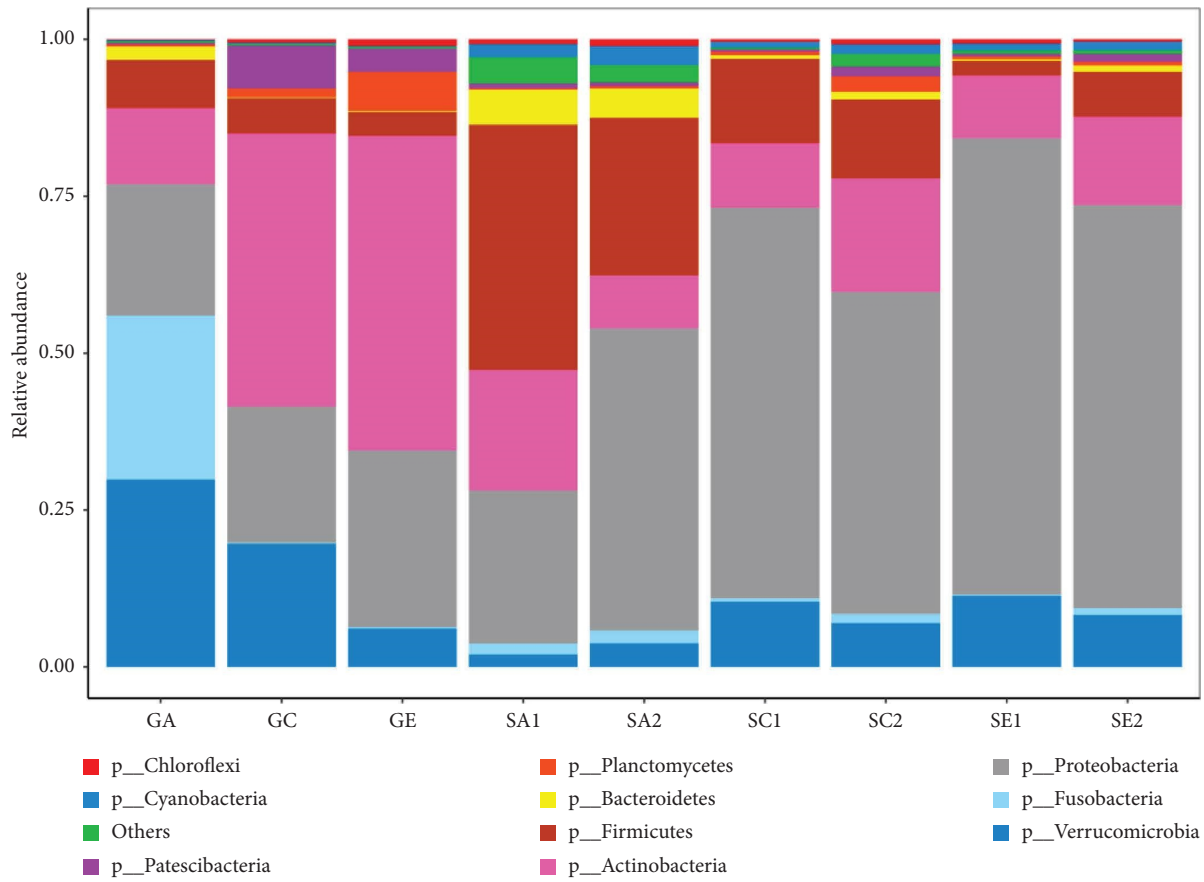

FIGURE 4: Relative abundance (%) of dominant phyla in each group (G, S1, and S2) at the phylum level on days 1, 5, and 9.

functions were abundant in all fish intestinal samples according to functional prediction. The relative abundance of metabolism functions in all fish intestinal samples in the KEGG database at level 2 is shown in Figure 6. Especially nutrition-related metabolism functions were selected, and their proportions were analyzed. Differences between groups are shown in Table 3. In addition, for the prediction of the relationship between intestinal microbiota and metabolism functions, RDAs were obtained. Axis 1 explained 43.49%, and axis 2 explained 28.24% (Figure 7).

4. Discussion

4.1. Feeding Effects of Fish on Filamentous Algae and Effects on Fish Weight and Water Quality after Feeding. Previous studies investigated the grazing effects of herbivorous zooplankton [35], brown bullhead (*Ictalurus nebulosus*) [36], striped parrotfish (*Scarus iseri*) [37], and Caribbean surgeonfish (*Acanthurus coeruleus* and *Acanthurus tractus*) [38] on filamentous algae and confirmed that some filamentous algae can be digested and utilized. However, studies on the use of biomanipulation in controlling filamentous algae are inadequate. In this study, there were obvious differences of intake rates in feeding with different filamentous algae by *X. davidi* Bleeker, and the reason might be the differences in palatability and nutrition composition among different species. For example, differences in algal abundance and habitat characteristics can cause differences in Caribbean surgeonfish feeding behavior [38]. The differences in intake between the two species of *Spirogyra* may be due to the differences in size among the algae (the diameter of S1 is about four times that of S2), and the oversized filamentous algae affected its movement in the intestinal tract. In addition, the differences in the feeding of filamentous algae in fish may be due to the fact that the intestinal microbiota of fish has been shaped by a certain type of *Spirogyra* sp. before the experiment. Some filamentous microalgae, such as *Oedocladium* sp. and *Tribonema* sp., have been confirmed to be used as feed ingredients to improve fish flesh quality, nutritional quality, and microalgal supplementation so that the does not fish growth performance is not affected [39, 40]. In this study, after feeding with *Spirogyra*, especially S2, an increase in the weight of the fish was observed, indicating that *Spirogyra* can be digested and utilized by *X. davidi* Bleeker. In addition, the levels of nitrogen and phosphorus in waters increased after the fish ingested filamentous algae with the prolongation of culturing time. In fact, farmers need to periodically apply fertilizer to ensure the growth of aquatic plants in the early stages of Chinese mitten crab and red claw crayfish culturing. Filamentous algae most likely bloom at this time. Therefore, the introduction of *X. davidi* Bleeker not only controls the overgrowth of filamentous algae but also reduces the use of fertilizers.

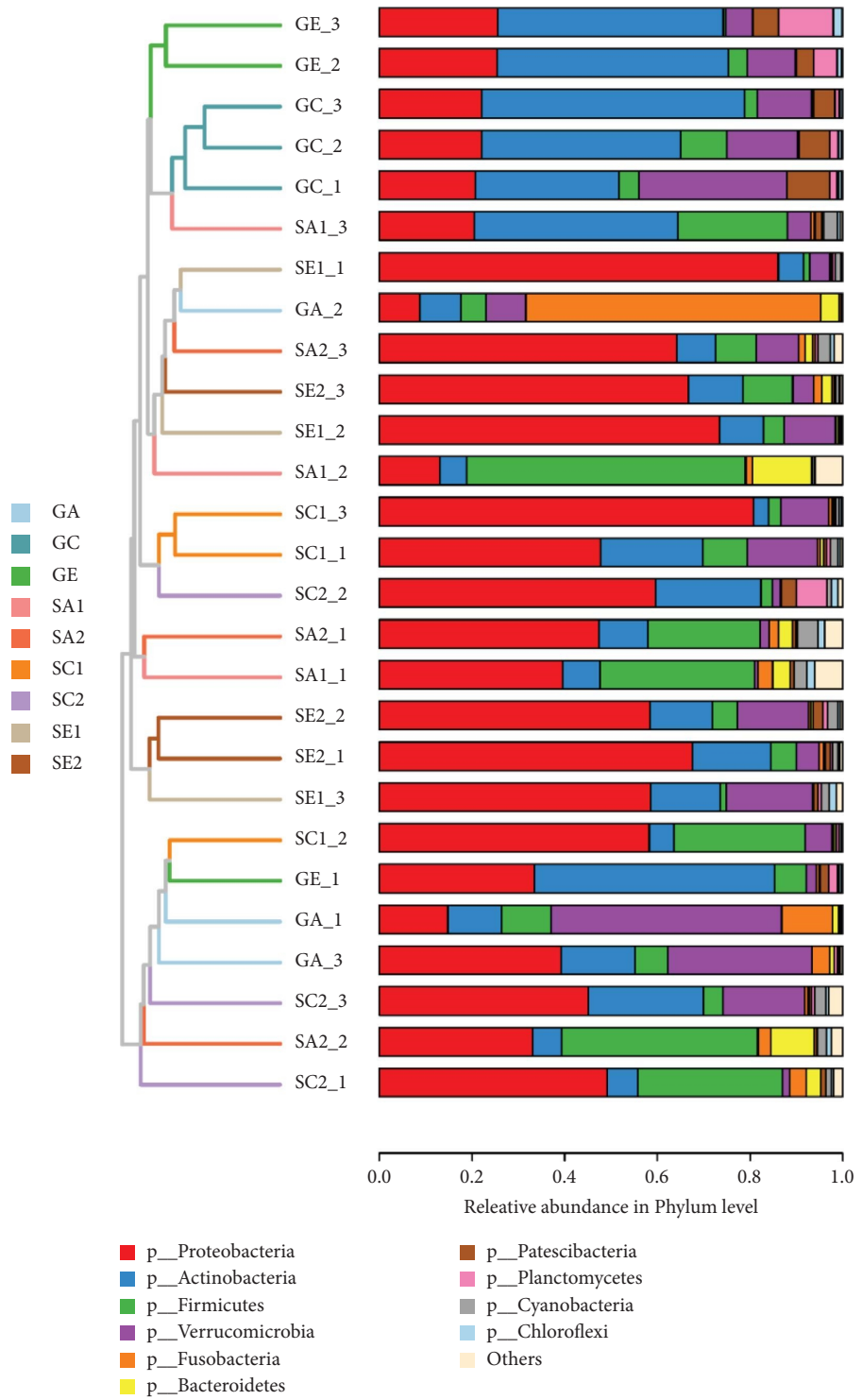

Figure 5: Clustering of the microbial communities of all fish intestinal samples at the phylum level using UPGMA dendrogram. UPGMA clustering was calculated using the weighted UniFrac distance.

4.2. Differences in Intestinal Microbial Diversity after Feeding with Different Filamentous Algae. Previous studies on the effects of different diets on intestinal microbial diversity have yielded different results. Many studies have shown that replacing fish meal with soybean meal does not change the intestinal microbial diversity of aquaculture animals, such as *Litopenaeus vannamei* [41], *Micropterus salmoides* [42], and *Larimichthys crocea* [43]. Dietary changes affect intestinal microbiota composition [44]. Interestingly, we found that the fish intestinal microbial diversity index in groups G and S1 first increased and then decreased, whereas that in group S2 first decreased and then increased in this study. The

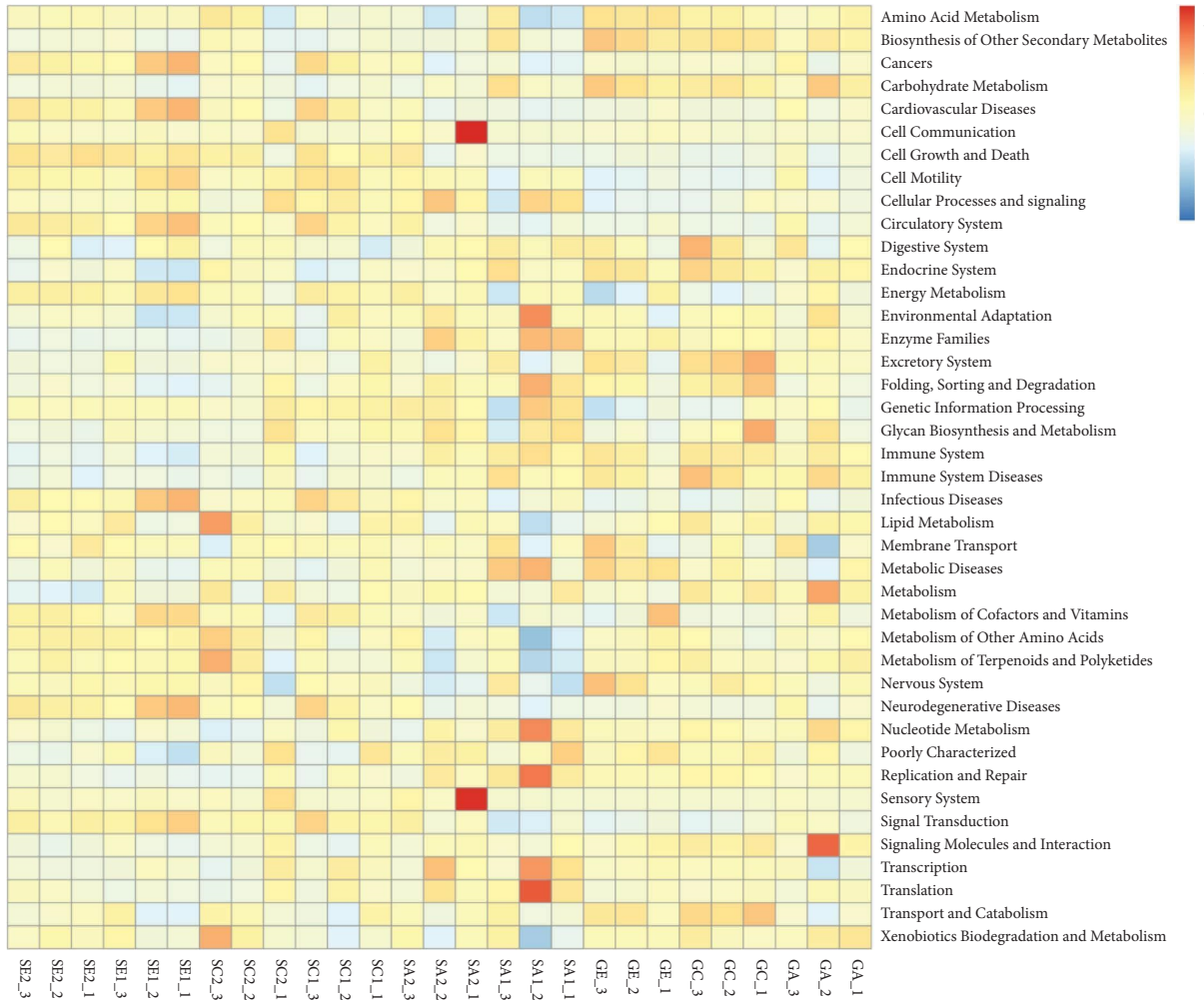

FIGURE 6: Relative abundance of metabolism functions in all fish intestinal samples in the KEGG database at level 2. The horizontal axis represents different samples, and the vertical axis represents different functional items. The depth of color is related to the abundance of a functional item, and the deeper the color is, the higher the functional items abundance is.

reason may be that fish have different digestion and absorption systems for different filamentous algae. Notably, unlike *Cladophora*, similar changes in intestinal microbial diversity occurred after fish were fed with the two *Spirogyra* species, indicating that related species could shape similar intestinal microbial diversity. This conclusion proved that "feeding habit is an important factor influencing gut microbial diversity [45]."

4.3. Effects of Feeding with Different Filamentous Algae on Intestinal Microbial Composition. As is well known, the intestinal microbiota of fish is affected by a range of factors, including host, environmental, and microbial factors [45–50], and the diet or nutrition factor is the most important among them [46, 51, 52]. The shared intestinal microbiota are the result of many factors, such as diet, growth, stocking density, and the surrounding environment [53–55]. In this study, the proportions of shared intestinal microbiota increased with the prolongation of culturing time, which may have been mainly due to a similar environment. Similar to intestinal microbial diversity, feeding with the two *Spirogyra* species showed a higher proportion of shared intestinal microbiota than feeding with *Spirogyra* and *Cladophora*, indicating that related species shape similar intestinal microbial communities. At three sampling times, the two most abundant intestinal microbial phyla underwent a transformation from Verrucomicrobia and Proteobacteria to Proteobacteria and Verrucomicrobia and then to Proteobacteria and *Actinobacteria*. On the one hand, this phenomenon reflected the rapid changes in the intestinal microbial community; on the other hand, it also reflected that the three groups had undergone some changes, such as external environment changes and feeding. Meanwhile, a high proportion of unique microbial taxa in each group confirmed that the dietary differences caused changes in intestinal microbial communities [44]. Additionally, Mekuchi et al. [25] reported that the dominant phyla were Proteobacteria in fasting and Firmicutes in feeding in the intestines of leopard coral grouper (*Plectropomus leopardus*). This result contradicted our results. Proteobacteria are the most abundant phylum in the intestines of many aquatic

Table 3: Relative abundance of nutrition-related metabolism functions in three experimental groups (G, S1, and S2) on days 1, 5, and 9 in the KEGG database at level 2.

	Amino acid metabolism	Carbohydrate metabolism	Energy metabolism	Lipid metabolism	Metabolism of cofactors and vitamins	Metabolism of terpenoids and polyketides	Nucleotide metabolism	Xenobiotics biodegradation and metabolism
GA	0.110 ± 0.002^{ab}	0.113 ± 0.007^{ab}	0.056 ± 0.002^{abcd}	0.037 ± 0.002^{ab}	0.042 ± 0.001^{ab}	0.023 ± 0.001^{a}	0.034 ± 0.001^{ab}	0.040 ± 0.004^{ab}
GC	0.112 ± 0.001^{a}	0.114 ± 0.002^{ab}	0.053 ± 0.001^{d}	0.038 ± 0.002^{a}	0.041 ± 0.000^{b}	0.023 ± 0.001^{a}	0.033 ± 0.001^{bc}	0.037 ± 0.003^{abc}
GE	0.116 ± 0.001^{a}	0.117 ± 0.003^{a}	$0.053 \pm 0.004c^{d}$	0.036 ± 0.001^{ab}	0.042 ± 0.003^{ab}	0.023 ± 0.001^{ab}	0.033 ± 0.000^{bcd}	0.037 ± 0.000^{abc}
SA1	0.105 ± 0.008^{b}	0.110 ± 0.007^{b}	0.055 ± 0.003^{abcd}	0.033 ± 0.003^{b}	0.041 ± 0.001^{b}	0.019 ± 0.003^{b}	0.036 ± 0.002^{a}	0.030 ± 0.009^{c}
SC1	0.107 ± 0.001^{ab}	0.102 ± 0.003^{c}	0.058 ± 0.001^{ab}	0.035 ± 0.003^{ab}	0.044 ± 0.001^{a}	0.022 ± 0.001^{ab}	0.032 ± 0.001^{bcd}	0.031 ± 0.003^{bc}
SE1	0.110 ± 0.000^{ab}	0.101 ± 0.003^{c}	0.059 ± 0.002^{a}	0.035 ± 0.003^{ab}	0.044 ± 0.002^{a}	0.023 ± 0.000^{a}	0.031 ± 0.001^{cd}	0.035 ± 0.004^{abc}
SA2	0.104 ± 0.004^{b}	0.104 ± 0.003^{bc}	0.057 ± 0.002^{abc}	0.036 ± 0.003^{ab}	0.042 ± 0.000^{ab}	0.021 ± 0.002^{ab}	0.032 ± 0.002^{bcd}	0.034 ± 0.006^{abc}
SC2	0.110 ± 0.008^{ab}	0.105 ± 0.002^{bc}	0.056 ± 0.002^{abcd}	0.039 ± 0.004^{a}	0.042 ± 0.002^{ab}	0.024 ± 0.004^{a}	0.031 ± 0.001^{d}	0.041 ± 0.008^{a}
SE2	0.110 ± 0.001^{ab}	0.103 ± 0.001^{c}	0.059 ± 0.000^{a}	0.036 ± 0.001^{ab}	0.044 ± 0.000^{a}	0.023 ± 0.001^{a}	0.032 ± 0.001^{cd}	0.037 ± 0.002^{abc}

Note. Different letters indicate significant differences based on ANOVA ($p < 0.05$). Mean ± SD ($n = 3$).

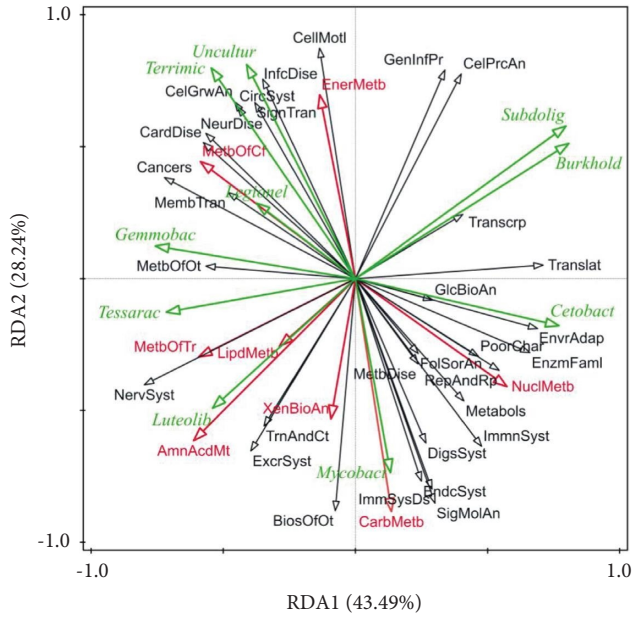

FIGURE 7: RDA analysis of intestinal microbiota and metabolism functions. The green arrows represent the top 10 genera, the red arrows represent nutrition-related metabolism functions (shown in Table 3), and the black arrows represent other metabolism functions, except nutrition-related metabolic functions, in the KEGG database at level 2 (shown in Figure 6).

animals, such as shrimp [41, 56], crab [57, 58], and fish [44, 59]. The results of this study supported these conclusions. The reasons may be that Proteobacteria is closely related to energy regulation [23, 60, 61] and can use another energy source from the environment; thus, it is more competitive than the other intestinal microbes and provides extra energy for the host during starvation [62]. Bacteroidetes, which play important roles in energy production and conversion, amino acid transport, and metabolism in starvation [63, 64], are an abundant phylum in the intestines of many aquatic animals [50, 65, 66]. In this study, the relative abundance of Bacteroidetes was higher on day 1 and then declined, suggesting that starvation was alleviated to some extent by feeding with filamentous algae. As one of the dominant phyla in the intestines, Firmicutes commonly appears in the intestinal microbiota of omnivorous or herbivorous species [20, 67]. However, in this study, the relative abundance of Firmicutes declined gradually after feeding with filamentous algae. Studies reporting gut microbiota composition at the phyla level have generally conveyed conflicting results, which pose difficulties in extrapolating real and meaningful trends and correlations between gut microbial composition and the factors that shape it [46]. Therefore, we believe that a more refined taxon is required to describe microbial functions.

4.4. Effects of Feeding with Different Filamentous Algae on Microbial Metabolism Functions. Many studies have pointed out the importance of microbial functions and confirmed that the intestinal microbiota play important roles in nutrient degradation and absorption, gastric development, mucosal tolerance, immunity, and disease resistance [50, 68–72]. Notably, certain intestinal microbiota groups contribute to enzyme activities in hosts [73]. For example, Pseudomonas, Acinetobacter, and *Photobacterium* contribute to amylase, protease, and chitinase activities, respectively [74]. However, most current studies on fish intestinal microbiota are descriptive and focus only on the composition of the microbial community. For the development of prebiotics, investigating the functions of subpopulations or even species is urgent [54]. In this study, we found significant differences in the relative abundance of nutrition-related metabolism functions among all subgroups at three sampling times. These conclusions indicated obvious differences in the functions of intestinal microbiota feeding on different diets at different culturing times and confirmed that gut microbiota play an important role in the metabolism of carbohydrates, lipids, and amino acids [75–77]. For instance, functions related to carbohydrate metabolism in the G group were more abundant than in the S1 and S2 groups, especially in the GE subgroup. The probable reasons were that a small amount of indigestible *Cladophora* cannot provide enough carbohydrates for fish and that the functions of some microbial clusters related to carbohydrate metabolism are triggered.

4.5. Correlation Analysis between Intestinal Microbiota and Metabolism Functions. The biaxial interpretation degree of the RDA analysis reached 71.73%, indicating a high correlation. Cetobacterium, which belongs to Fusobacteria, ferments proteins and carbohydrates and is often present in the intestines of omnivorous and herbivorous fish [42, 78, 79]. Our analysis supports this view. Mekuchi et al. [25] reported that Fusobacteria in the gut mucosa play a role in the hydrolysis of maltose and trehalose. In this study, Gemmobacter was found to be positively associated with a variety of

nutrition-related metabolism functions [80–83]. This association may be one of the reasons that Proteobacteria are the dominant microbiota in the intestines of many aquatic animals. *Mycobacterium*, which belongs to *Actinobacteria*, is a pathogen of fish and human coinfection [84–86]. Zhang et al. [87] reported that the content of *Mycobacterium* in the intestines of Nile tilapia (*Oreochromis niloticus*) is abundant when fed with different woody forages. According to the correlation analysis of this experiment, it has been proven that a high-carbohydrate diet promotes *Mycobacterium* booming. In addition, Ottman et al. [64] reported that the presence of *Actinobacteria* indicates active carbohydrate metabolism. Therefore, the contribution to carbohydrate metabolism partially came from *Mycobacterium*. Many meaningful correlations between genera and metabolism functions could be obtained from Figure 7. At present, studies on the intestinal microbial metabolism functions of certain microbial groups are inadequate. Thus, we believe that this correlation analysis method is useful in screening probiotics with specific functions.

The digestive performance of animals depends on the genome of the host and the characteristics of the host's gastrointestinal microbiome [88, 89]. On the one hand, intestinal microbiota in hosts can be shaped through diet, and intestinal microbiota further influences the metabolism and growth of the host [51]. Karasov et al. [88] pointed out that the flexibility of the microbiome likely plays an important role in the digestive adaptability of fish. Wong et al. [55] reported that the intestinal microbiota of rainbow trout (*Oncorhynchus mykiss*) is plastic and capable of being manipulated with diet from the first feeding. On the other hand, the expression of host genes can be affected by a number of intestinal microbiota, including those responsible for the stimulation of epithelial cell proliferation, metabolism of nutritive substances, development and maturation of the immune system in the fish intestine, and innate immunity responses [27, 54]. Therefore, although obvious differences in the ingestion and digestion of different filamentous algae were found in *X. davidi* Bleeker, it is promising that there would be a good effect on the prevention and control of filamentous algae if fed with filamentous algae that often erupt in some culturing areas to shape intestinal microbiota with specific decomposition abilities in the fry stage in fish nursery and put into aquaculture waters at the beginning of culturing.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Yongxu Cheng, Boping Tang, and Fujun, Xuan conceived and designed the study and acquired funding; Xusheng Guo and Liangjie Zhao provided the conditions of the experiments; Yongtao Tang and Chen Wang collected the samples, performed the experiments, and drafted the manuscript; Liangjie Zhao and Yunfei Sun collected and analysed the data; and Yongtao Tang, Hanjun Jiang, and Chen Qian revised the manuscript. Yongtao Tang and Chen Wang contributed equally to this work.

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Supplementary Materials

Supplementary figure A: the phylogenetic analysis of three kinds of filamentous algae (G, S1, and S2) involved in the experiment. Supplementary figure B: rarefaction curves of all the samples. (*Supplementary Materials*)

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