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Research Article

The Microflora Structure in the Digestive Tract, Culture Water, and Feed of Hybrid Grouper (*Epinephelus fuscoguttatus*♀ × *E. polyphekadion*♂) Cultured in an Outdoor Pond Based on a High-Throughput Sequencing Technique

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Nutrition, disease, and general wellbeing can be affected by the microbial communities associated with the digestive tracts of aquaculture species. Different sections of aquaculture species' digestive tracts have distinctive surfaces and structures, which can change microbial communities. The present study examined the composition and distribution of bacterial species in the intestine of hybrid grouper (*Epinephelus fuscoguttatus E. polyphekadion*) and its aquaculture environment. Using high-throughput pyrosequencing, a 16S rRNA sequence analysis was performed on hybrid grouper foregut, midgut, and hindgut, as well as cultured water and feed. There were 610,452 sequences obtained from five components (foregut, midgut, hindgut, water, and feed). Among operational taxa (OTUs), 506 of them were detected in the foregut, 605 in the midgut, 510 in the hindgut, and 573 in aquaculture water and feed samples. A total of 113 were detected in 5 samples. A species annotation revealed that hybrid grouper intestinal tracts were dominated by Proteobacteria (67.3%–73.7%), Firmicutes (8.4%–14.0%), and Actinobacteria (6.9%–10.5%). In aquaculture culture water, Proteobacteria were predominant (36.3%), Actinobacteria (30.0%), and Planctomycetes (14.0%). Acinetobacter (1.4%–17.9%) and Photobacterium (32.0%–57.5%) dominated the intestine. Photobacterium (3.6%) and Mycobacterium (7.1%) dominated the water bacteria. The water and intestine contained five potentially pathogenic bacteria: Pseudomonas, Flavobacterium, *Escherichia coli*, Aeromonas bacteria, and Vibrio. The highest proportion of Vibrio was found in the water (1.7%), while Pseudomonas dominated the midgut (2.6%). Six potential probiotics were detected in the aquaculture water and intestine (Lactococcus, Streptococcus, Bdellovibrio, Lactobacillus, Bacillus, and Bacteroides). Aquaculture water and intestines contained Bacillus, Bacteroides, and Lactobacillus. According to the findings, the intestinal flora of hybrid grouper is closely correlated with its pond culture environment. Results from the study provide an experimental basis for the controlled breeding of hybrid groupers and the regulation of their microecological processes in the breeding environment deepen our understanding of the intestinal bacterial population of healthy hybrid groupers.

1. Introduction

Microbial communities play important roles in the gut of fish species, influencing their growth, development, and health. Microbes in the culture environment play a significant role in cultured organisms' material circulation and energy flow by affecting the intestinal flora [1]. Understanding the structure of these microbial communities is crucial for optimizing the culture conditions and improving the health of cultured fish. The growth and health of fish in aquaculture systems are closely related to the microorganisms present in their surrounding environment, including the digestive tract, culture water, and feed. These microorganisms collectively form the fish's microbiome, which plays a crucial role in the fish's digestion, immune system, and overall health [2–7]. Various internal and external factors affect fish intestinal bacteria, including host genetics, feeding habits, and water conditions [8]. To sustain aquaculture, it is crucial to understand how intestinal flora interacts with fish health [8]. In aquaculture, probiotics are used in order to regulate and optimize the microecological structure of aquaculture species and the environment in light of the frequent occurrence of diseases and excessive use of antimicrobials. This helps prevent and control diseases and improves the quality of aquaculture products [9]. A scientific understanding of the microbial habitat of the digestive tract of cultured fish species is, therefore, important for the cultivation of fish as well as for the prevention and control of the disease.

The culture of hybrid grouper (*Epinephelus fuscoguttatus*♀ × *E. polyphekadion*♂), a carnivorous fish species in outdoor ponds, has become increasingly popular due to its potential for high yield, profitability, and ability to adapt to environmental conditions with a high range of salinity and for its high demand in the seafood industry [10, 11]. China has become one of the world's leading marine fish aquaculture countries because of the development of artificial breeding technology. A mainly land-based industrialized culture is currently predominant in the grouper culture. The pond culture mode is more environmentally friendly and energy-efficient and requires a lower investment in production from the point of view of industrial development [12]. However, pathogens in the culture water, feed, and digestive tract of hybrid grouper can pose a risk to their health and the culture's success [13]. One potential solution to this problem is to gain a better understanding of the microflora structure in these environments, which can help to identify beneficial and harmful microorganisms and inform the development of management strategies to promote the health of the fish [14, 15].

Traditionally, it has been difficult to obtain pure cultures of microorganisms that are in symbiotic relationships under natural conditions (for instance, extracting 5S rRNA molecules from mixed samples and analyzing the sequences to determine their phylogenetic position) [16], and enrichment and separation cultures have high selectivity [17]. It has become complicated to study the structure of fish intestinal flora using the traditional method because it fails to reflect the real situation of the microbial community in the natural state. In recent years, high-throughput sequencing techniques have emerged as a powerful tool for studying microbial communities in various environments, including aquaculture-associated ones [18, 19]. It is widely used to study bacterial diversity in environmental samples [20, 21]. These techniques allow for the simultaneous identification and quantification of a large number of microorganisms, providing a comprehensive view of the microflora structure and dynamics in the environment of interest [22]. Several studies have applied this approach to investigate the microbiome of different fish species, including grouper, and have revealed the complex interaction between the fish and its microbiome [23–27]. Despite the importance of the microbiome in fish culture, little is known about the microflora structure in the digestive tract, culture water, and feed of hybrid grouper.

The aim of this study is to use high-throughput sequencing techniques to investigate the microflora structure in the digestive tract, culture water, and feed of hybrid grouper cultured in outdoor ponds. Specifically, we examine the composition and diversity of the microflora in these environments and identify any correlations between the microflora and the health and growth of the fish. By better understanding the microflora in these environments, we hope to inform the development of management strategies that promote the health and productivity of hybrid grouper culture. In addition to providing information about the composition and abundance of intestinal bacteria of grouper produced in ponds, the results also provided a reference for the development of probiotics for groupers.

2. Materials and Methods

2.1. Experimental Materials. This study used hybrid grouper samples taken from an outdoor breeding pond at the South Marine Aquaculture Seed Base of State (863) Program, Donghai Island (Guangdong province, China). At the base, there were eight outdoor ponds (with a size of 0.3 to 0.5 hm^2 and a depth of 1.4 to 1.8 m, concrete walls, and black plastic film at the bottom) for the grouper culture. On May 10, 2019, seawater flowed into the aquaculture pond after sand filtration and sedimentation. The ponds were filled with 30,000 hybrid grouper fries, each 10–12 cm in length. A special granular compound feed was purchased from (Guangdong Yuequn marine biology research and development Co. Ltd., Jieyang, China) (crude protein ≥40%, crude fibre ≤5.0%, crude ash ≤16%, crude lipid ≥6%, moisture ≤12%, total phosphorus 0.90–1.60, and lysine ≥2.10) for grouper. Two times per day, at 9:00 and 17:00, was the daily feeding, and the feeding amount was approximately 5% of the total weight of the cultured fish every day. On September 15, 2019, samples were collected for this experiment. Fish from farmed ponds, water samples from ponds, and pellet feed samples were sampled A random sampling of groupers was conducted in the pond. In total, 9 samples were taken from

each pond, with a body length of 16 ± 3.7 cm and a weight of 254 ± 68 g. Sampled fish were transported to the laboratory on ice and packed with oxygen. Furthermore, 9 water samples were collected 1.5 m offshore and in the middle of the diagonal of the pond using a laboratory water collector located 0.3 m from the water surface. In an icebox, the sample collection was transported to the lab, and three equal amounts of collected water samples were mixed together. Three sterile sampling bags were used to collect pellet feed appropriate for further analysis, and the sample fish were returned to the laboratory.

2.2. Water Quality Analysis. Seawater is filtered and sedimented in sand ponds before being added to the aquaculture pond. The water in the pond is changed once a week, about one-third of the total volume. The samples of water are collected prior to water changes to preserve their quality. Water temperature, pH, dissolved oxygen, salinity, and other conventional water quality indicators were measured at the breeding pond using the multifunctional water quality detector (YSI-6600). By using indophenol blue spectrophotometry and naphthalene ethylenediamine spectrophotometry, the concentrations of total ammonia nitrogen (TAN) and nitrite nitrogen (NO_2-) in water were determined according to marine detection specifications [28]. Water samples were analyzed using combined nitrification to measure total nitrogen (TN) and total phosphorus (TP) concentrations [29]. A water sample was taken on the same day, and the temperature was not significantly different. Table 1 shows the water quality indexes for the sampling pond. There was no significant difference in any of the water quality indexes, which may be attributed to regular water changes.

2.3. Sampling and Bacterial DNA Extraction. A sterile ice water and seawater mix was used to transport the fish to the laboratory for 30 minutes. Eugenol (100 mg/L) was administered to anaesthetise the fish and place them into the anatomical plate. Disinfecting the fish's body was done by wiping it with a cotton ball soaked in 70% alcohol. Sterile surgical scissors were used to remove the entire digestive tracts from the viscera of nine fish from each pond. To examine foregut, midgut, and hindgut tissues under sterile conditions, a small quantity was cut, washed three times with sterile saline, and transferred to sterile centrifuge tubes. The foregut, midgut, and hindgut tissues of the three-sample fish were mixed with digestive tract tissues of the same parts. A vortex was applied for one minute to fully break up the sample after 1.2 mL of buffer SSL was added to it. The HiPure Stool DNA Kits (Magen, Guangzhou, China) were used to extract bacterial DNA from intestinal tissue samples after homogenisation with a tissue homogeniser. In a similar way, after filtering pond water samples with sterile cellulose filters, the filters' membranes were cut into several pieces. With the HiPure Stool DNA Kits, total bacterial DNA was extracted from pond water samples. After loading the pellet feed into sterile centrifuge tubes and homogenising it with a tissue homogeniser, the total bacterial DNA in feed samples was extracted with the HiPure Stool DNA Kits.

Table 1: Water quality indexes of the sampled pond of hybrid grouper.

Water quality indexes	Numerical range
Temperature of water (°C)	28.7 ± 0.1
pH	8.01 ± 0.07
Salinity	29.5 ± 0.1
Dissolved oxygen (DO)	6.35 ± 0.15
Nitrogen ammonia (mg/L)	0.075 ± 0.002
Nitrate (mg/L)	0.016 ± 0.003
Total nitrogen (mg/L)	1.012 ± 0.006
Total phosphorus (mg/L)	0.204 ± 0.005

$n = 5$; $x \pm$ standard deviation (SD).

2.4. High-Throughput Sequencing Analysis. There were five components in the DNA extraction: the foregut, the midgut, the hindgut, the water, and the feed. In order to determine the purity and concentration of the DNA group, 1% agarose gels were used (Axygen Biosciences, Union City, CA, U.S.). The sample DNA was diluted to 1 ng/μl with sterile water. A diluted DNA sample served as the template for the amplification of the V3-V4 variable region of bacterial 16S rDNA using barcode-specific primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) [30]. In each 30-litre PCR amplification system, 15 litres of Phusion® High-Fidelity PCR Master Mix were contained, along with 0.2 m forward and reverse primers and 10 ng of template DNA. The PCR amplification reaction was as follows: predenaturation at 95°C for 2 min, followed by 30 cycles of PCR (98°C for 10 s, annealing at 62°C for 30 s, and extension at 68°C for 30 s) and a final extension at 68°C for 10 min. By using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.), the amplified products were detected on 2% agarose gel electrophoresis and purified with AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.). Based on instructions from the manufacturer, PCR quantification was performed using the Life Technologies ABI StepOnePlus Real-Time PCR System [31]. Gene Denovo Co., Ltd. (Guangzhou, China) sequenced the constructed library and analyzed it using an Illumina HiSeq platform based on fluorometer quantification and library detection. SRA accession PRJNA793767 has been created and deposited with NCBI Sequence Read Archive in the NCBI Sequence Read Archive database.

2.5. Data Analysis. A low-quality partial shearing method (Cutadapt (V1.9.1) https://cutadapt.readthedocs.io/en/stable/) [32, 33] was used to split samples from sequence

reads. For preliminary quality control, raw reads were obtained from the barcode and primer sequences. For obtaining the final clean reads, the UCHIME algorithm was used to detect and remove chimera sequences. Based on 97% similarity [30, 33, 34], the sequences were clustered into operational taxonomic units (OTUs) using Uparse software (v7.0.1001, https://drive5.com/uparse/). The representative sequences of OTUs were then screened. OTUs were selected as representative sequences based on the sequence with the highest frequency. Species annotation analyses were performed using the Mothur software (version 1.30.1) and the Silva database (the threshold value was 0.8–1) [30, 33]. A rapid multiple-sequence alignment was performed using MUSCLE software (https://www.drive5.com/muscle/) in order to determine the phylogenetic relationship among OTU sequences. For subsequent Alpha and Beta diversity analyses, each sample's data were normalised. The Alpha and Beta diversity analyses were conducted using QIIME (Version 1.9.1) and R software (Version 2.15.3). To determine the species richness and diversity of each sample, Chao1, Shannon, and Simpson indices were calculated [12, 30]. To analyse the differences in the bacterial community structure between different samples and groups [30, 35], principal coordinate analysis (PCoA) was mainly based on UniFrac distance and abundance of OTUs, and nonmetric multidimensional scaling diagrams (NMDS) based on Bray–Curtis distance metrics were used [12, 30]. Using SPSS 21.0 (SPSS Inc., Michigan Avenue, Chicago, IL, USA), the data were analysed through a one-way analysis of variance (ANOVA). Foregut, midgut, hindgut, water, and feed were tested for bacterial abundance using Duncan's test, and P values <0.05 were considered significant. Results are expressed as means ± standard deviations (SDs).

3. Results

3.1. Sequencing Results. It was found that 610,452 effective sequences were obtained in 5 groups of hybrid grouper foreguts (EF), midguts (EM), hindguts (EH), aquaculture water (EW), and feed (D), respectively, resulting in 127,691 (EF), 113,777 (EM), 123,343 (EH), 121,334 (EW), and 124,306 (D) sequences. Each group's sample sequences were clustered into OTUs with a 97% similarity. As shown in Table 2, the number of OTUs in each group are 506, 605, 510, 671, and 573, respectively. Wayne diagrams were made of each group's OTUs (Figure 1), which showed 113 OTUs in total and 40, 55, 115, 179, and 331 distinct OTU number ratios.

3.2. Analysis of Bacterial Diversity in Fish Intestines, Water, and Feed. Intestinal, water, and feed samples were analysed for Alpha and Beta diversity to evaluate bacteria diversity. A flora abundance analysis was conducted using the Chao1 and ACE indices. A Shannon and Simpson's index was used to evaluate the flora's diversity (Table 2). From the foregut to the hindgut, intestinal bacteria abundance increased while diversity decreased according to the results. In comparison to the intestine and feed of the grouper, the abundance of bacteria in the water was significantly higher. In contrast to the grouper intestine, water and feed contained a significantly higher diversity of bacteria. Using PCoA, it was found that the water sample had a different bacterial composition than the feed and intestine samples, indicating that the water sample had a greater difference in bacterial diversity. A closer relationship between the feed and the midgut and foregut samples indicates a similar bacterial composition (Figure 2(a)). Intestine (EF, EM, and EH), water, and feed samples were divided into three distinct clusters based on the Bray–Curtis distance NMDS (Figure 2(b)). Each group had coverage above 0.99, which indicates that most of the sequences in the samples were detected.

3.3. The Relative Abundance and Structure of Bacteria in the Intestine, Water, and Feed. In order to draw a histogram of the top 10 species in the average abundance of all samples, bacterial species annotations were conducted at the phylum level (Figure 3(a)). Species that could not be annotated were classified as unclassified, and those that could not be annotated as other. There were no significant differences between the bacterial structures of the midgut, hindgut, and foregut samples. Most of the bacteria belong to the Proteobacteria (67.3%–73.7%), Firmicutes (8.4%–14.0%), Actinobacteria (6.9%–10.5%), and Bacteroidetes (3.0%–8.0%). Water samples were primarily dominated by Proteobacteria (36.3%), Actinobacteria (30.0%), Planctomyces (14.1%), and Bacteroides (10.8%). Bacteria dominated the feed samples with 44.0% Proteobacteria, 26.5% Firmicutes, 15.5% Bacteroides, and 10.3% Actinobacteria. The Proteobacteria relative abundances in the foregut, midgut, hindgut, water, and feed did not differ significantly ($P > 0.05$). As compared to other samples, Actinobacteria were significantly more abundant in water, and Firmicutes were significantly less abundant ($P < 0.05$). Bacteroidetes were significantly more abundant in the hindgut, water, and feed than in the foregut and midgut ($P < 0.05$) (Figure 3(b)).

The histogram of species abundance in Figure 4(a) shows the top 10 species in each genus based on the average bacterial abundance of the samples. Acinetobacter (36.2%), Bacillus (2.71%), Pseudomonas (2.3%), Vibrio (1.7%), and Mycobacterium (1.4%) dominated the foregut samples; midgut samples were dominated by Photobacterium (32.0%), Acinetobacter (17.9%), Pantoea (7.4%), Pseudomonas (2.6%), and Mycobacterium (2.5%); hindgut samples had Photobacterium (57.5%), Bacteroides (4.4%), Vibrio (2.0%), Acinetobacter (1.4%), Acinetobacter (1.4%), and Mycobacteria (1.0%). Water was dominated by Mycobacterium (7.1%), Photobacterium (3.6%), and Vibrio (1.7%), while feed was dominated by Pseudomonas (7.2%), Acinetobacter (3.2%), Photobacterium (1.5%), and Vibrio (1.2%). Photobacterium, Acinetobacter, and Pantoea were not significantly different at the genus level in the foregut, midgut, hindgut, water, and feed ($P > 0.05$). Compared with other samples, feed contained significantly more Pseudomonas, and water contained significantly more Mycobacterium ($P < 0.05$) (Figure 4(b)).

Table 2: Alpha diversity of intestine, water, and feed microflora of hybrid grouper.

Groups	OTU number	*Alpha diversity index*				Good's coverage
		Chao1 index	ACE index	Shannon index	Simpson index	
EF	508 ± 66	616.59 ± 58.67	643.88 ± 57.27	5.67 ± 0.02	0.96 ± 0.01	0.999 ± 0.001
EM	601 ± 19	723.21 ± 0.83	733.23 ± 6.15	4.89 ± 0.52	0.88 ± 0.03	0.999 ± 0.001
EH	510 ± 54	655.42 ± 21.83	668.51 ± 33.65	4.37 ± 1.39	0.76 ± 0.19	0.999 ± 0.000
EW	671 ± 40	742.67 ± 34.59	737.65 ± 18.57	6.03 ± 0.33	0.97 ± 0.01	0.998 ± 0.001
D	493 ± 27	738.51 ± 76.68	716.32 ± 65.66	7.19 ± 0.16	0.99 ± 0.00	0.999 ± 0.000

Note. EF: foregut; EM: midgut; EH: hindgut; EW: water; D: feed. Results are expressed as means ± standard deviations (SDs).

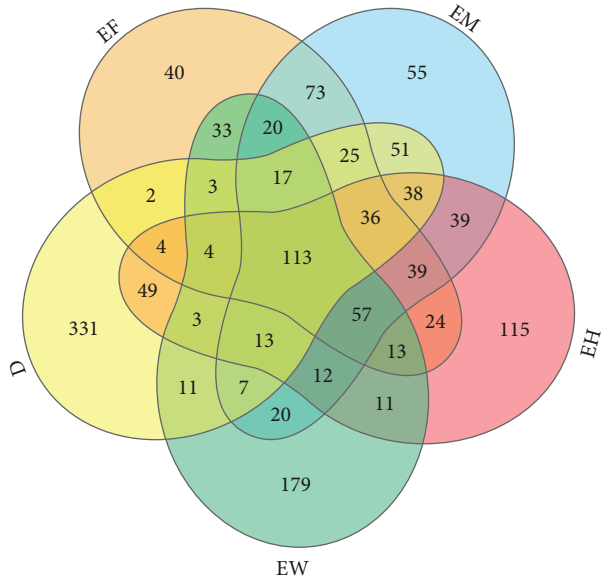

Figure 1: OTU Venn diagram of the microflora of hybrid grouper intestine, water, and feed.

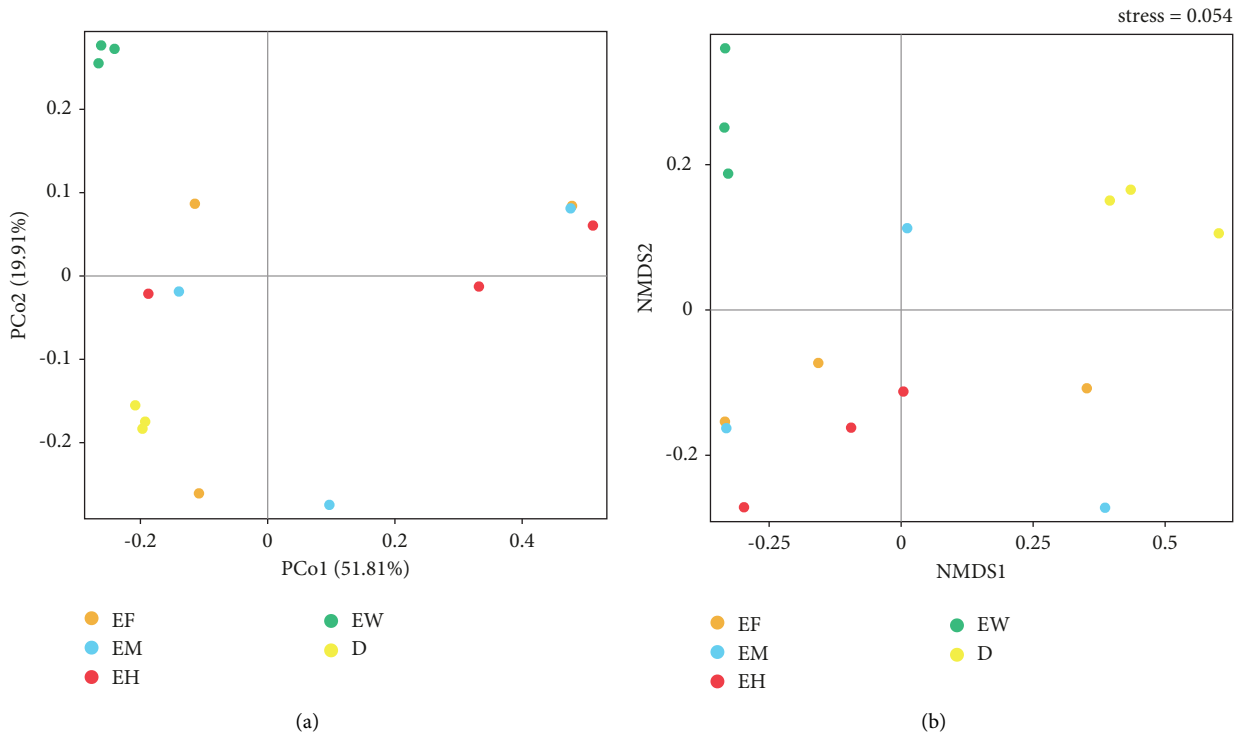

Figure 2: Differences in bacterial Beta diversity of grouper intestine, water, and feed microflora of hybrid grouper. (a) The principal coordinate analysis (PCoA) based on the weighted UniFrac distance. (b) The nonmetric multidimensional scaling (NMDS) analysis based on the Bray–Curtis distance.

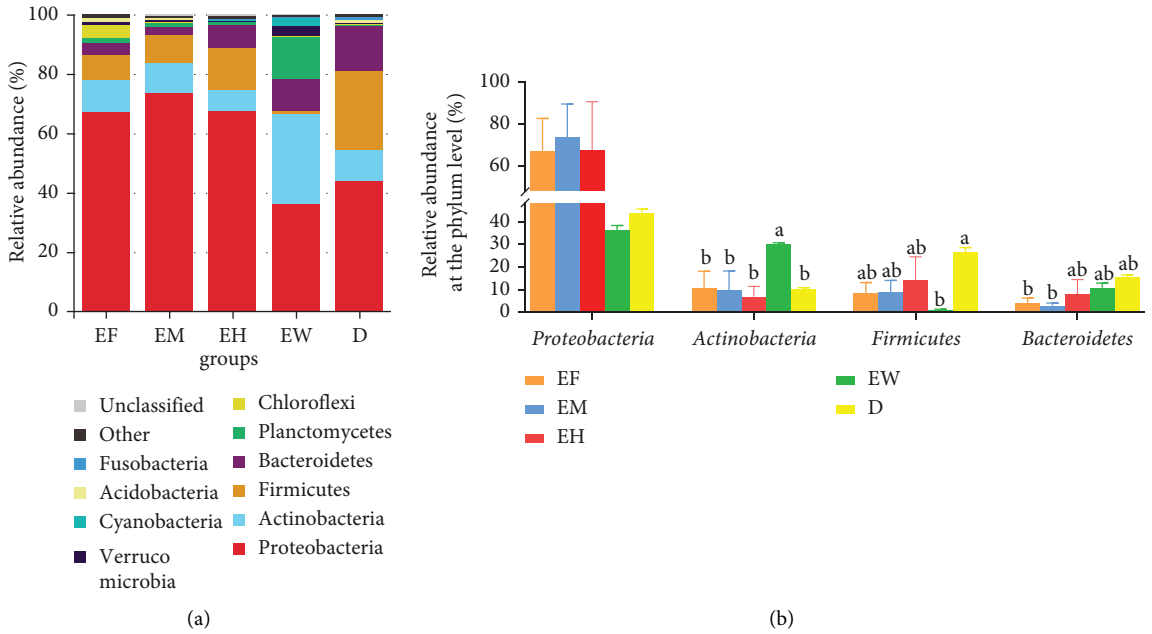

FIGURE 3: Structure and composition of the microflora of hybrid grouper intestine, water, and feed at the phylum level. (a) Histogram of relative abundance of microbiota composition. (b) Analysis of the abundant microbiota at the phylum level of the top 4. Different superscript letters indicate that the corresponding values are significantly different ($P < 0.05$).

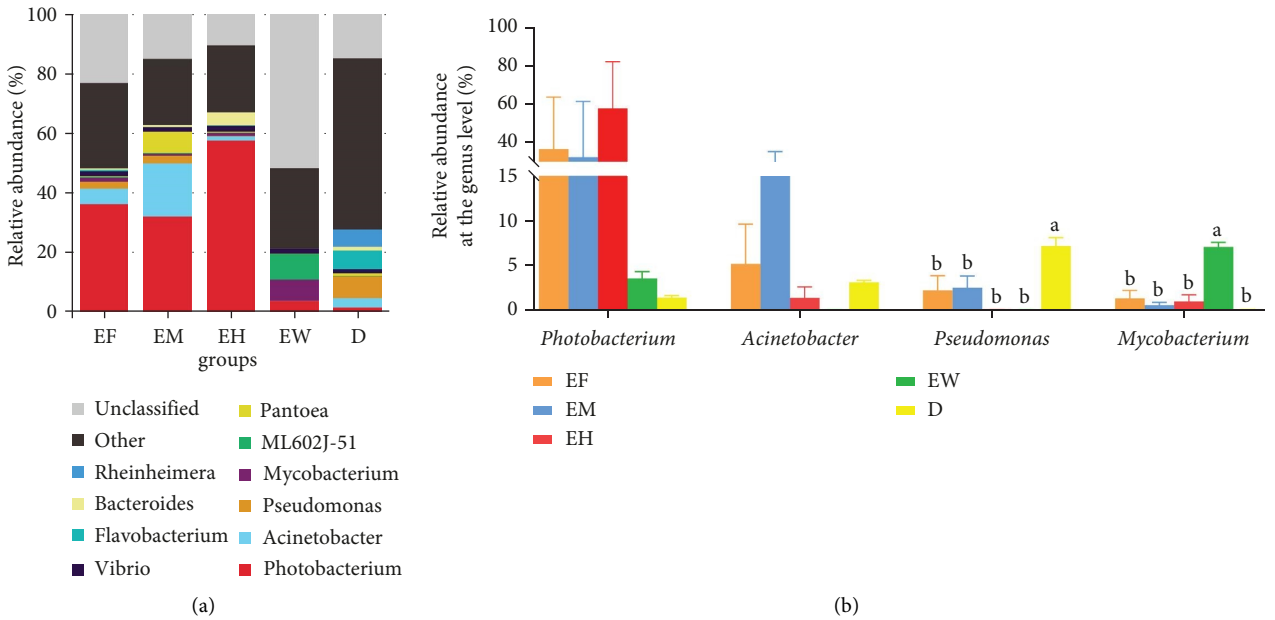

FIGURE 4: Structure and composition of the microflora of hybrid grouper intestine, water, and feed at the genus level. (a) Histogram of relative abundance of microbiota composition. (b) Analysis of the abundant microbiota at the genus level of the top 4. Different superscript letters indicate that the corresponding values are significantly different ($P < 0.05$).

3.4. Potential Probiotics and Pathogenic Bacteria in Aquaculture Water and Intestine. There were five potentially pathogenic genera detected in cultured water and healthy hybrid grouper intestines. These genera were three, four, four, and five pathogenic genera found in water, foreguts, midguts, and hindguts, respectively. A high proportion of *Vibrio* was found in the water (1.67%), while a high proportion of *Pseudomonas* was found in the gut (2.58%). The water contained only *Bacteroides* (0.02%). A total of six genera were detected in the intestines, with *Bacteroides* accounting for the highest percentage (4.36%). A higher proportion of potentially pathogenic bacteria were found in the intestines and water than potential probiotic bacteria (Table 3).

Table 3: Percentages of potential probiotic and pathogenic bacteria number in the intestine and their aquaculture environment samples (%).

Category	Bacteria	Water (EW)	Foregut (EF)	Midgut (EM)	Hindgut (EH)
Potential pathogenic bacteria	*Pseudomonas*	0.03	2.27	2.58	0.10
	Flavobacterium	—	0.46	0.01	0.16
	Escherichia	0.01	0.88	1.13	0.35
	Aeromonas	—	—	—	0.07
	Vibrio	1.67	1.73	1.59	2.00
Potential probiotics	*Bacillus*	—	2.71	1.88	0.02
	Bacteroides	0.02	0.46	0.69	4.36
	Bdellovibrio	—	—	0.02	—
	Lactobacillus	—	0.02	0.35	0.07
	Lactococcus	—	—	0.01	0.09
	Streptococcus	—	0.21	—	0.02

Results are expressed as percentages.

4. Discussion

In addition to providing theoretical support for the construction of microecological control technology in the breeding environment, the research conducted on the diversity of bacterial communities in breeding environments provides the basis for disease prevention and control in the breeding process.

In the case of culture water of fish, microflora plays a crucial role in maintaining the health and wellbeing of the fish. The microflora in fish culture water refers to the diverse community of microorganisms that inhabit the aquatic environment, including bacteria. These microorganisms play crucial roles in maintaining the health and balance of the aquatic ecosystem, as well as the health and growth of fish. The microflora structure in the culture water of fish can have both positive and negative implications for the fish. A diverse and stable microflora community can help to maintain water quality, promote nutrient cycling, and enhance disease resistance in fish.

On the other hand, imbalances or disruptions in the microflora community can lead to poor water quality, increased disease susceptibility and decreased growth and survival rates for fish. For example, the presence of pathogenic bacteria in culture water can lead to diseases such as fin rot, gill disease, and columnaris disease outbreaks in fish. Balcázar et al. [36] found that pathogenic Aeromonas bacteria in culture water were associated with high mortality rates in cultured fish. Similarly, excess nutrients, such as nitrogen and phosphorus, in culture water can lead to eutrophication, which can cause harmful algal blooms and oxygen depletion, leading to fish kills. A study by Banerjee et al. [37] found that using a probiotic bacterial strain in culture water improved the growth and survival rates of aquatic species Penaeus monodon shrimp. Several studies have examined the effects of microflora on fish health and culture water quality. For example, a study by Serrano et al. [38] found that adding probiotics to the culture water of rainbow trout improved fish growth and health and reduced the incidence of disease. Another study by Farhadi et al. [39] investigated the effects of microbial community structure on water quality in tilapia culture ponds and found that certain bacterial groups were associated with improved water quality.

The results of the current study showed that the microbial community in the culture water was dominated by Proteobacteria, Actinobacteria, Planctomyces, and Bacteroidetes, with the genera Mycobacterium, Vibrio, and Photobacterium being the most abundant. The high abundance of Vibrio and Photobacterium in the culture water may pose a potential threat to the health of hybrid grouper. In conclusion, the microflora structure in the culture water of fish is a critical factor that can influence the health and wellbeing of fish. Maintaining a diverse and stable microflora community can help to promote good water quality, disease resistance, and fish growth and survival.

Fish digestive tracts may be affected by aquaculture water environments. The formation of its bacterial community is related to the fish's species, individual size, and feeding habits. It is also susceptible to multiple influences from the environment and microorganisms in the bait [23, 40]. Some studies have shown that bacteria in the water are not closely related to the structure of aquatic animal digestive tract flora [41, 42]. According to other studies [43–45], the structure of aquatic animal gastrointestinal tracts was closely related to that of the flora of aquatic animals. In this study, two (Proteobacteria and Bacteroidetes) of the seven dominant niche bacteria in the digestive tract flora of hybrid grouper cultured in the pond were also in water (Figure 3(a)). The results of the present study showed that the dominant niche bacteria in the gut microbiota of hybrid grouper were Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes.

Moreover, the gut microbiota of hybrid grouper was dominated by the genera in the foregut samples. Photobacterium, Acinetobacter, Bacillus, Pseudomonas, Vibrio, and Mycobacterium were dominant; Photobacterium, Acinetobacter, Pantoea, Pseudomonas, and Mycobacterium were dominant in midgut samples; Photobacterium, Bacteroides, Vibrio, Acinetobacter, and Mycobacterium were dominant in hindgut samples. Our study found that Proteobacteria bacteria dominated intestinal samples, water samples, and feed samples. Rombout et al. [46] reported similar results. Actinobacteria, the third abundant bacterial group in the intestine, were also the second and fourth most abundant bacterial groups in water and feed samples,

showing that environmental bacteria greatly influenced the intestinal flora of hybrid groupers.

On the other hand, 0.01%–0.46% of unclassified bacteria were detected in the intestinal tract of hybrid grouper (Figure 3(a)). These microorganisms may play a significant role in the digestion and absorption of nutrients and the maintenance of the fish's health. The extent to which role these intestinal microbes of hybrid grouper were playing is not readily known. A comprehensive analysis and further studies are needed to determine or completely elucidate their role.

The microflora structure of fish feed refers to the various microorganisms in the feed. Depending on their type and quantity, these microorganisms can be beneficial or harmful to the fish. Fish depend on a complex microbial community in their digestive tract, the gut microbiota, to help break down and absorb nutrients from their feed [47]. Beneficial microorganisms can aid digestion and absorption of nutrients in the feed, while harmful microorganisms can cause infections and diseases in the fish. The gut microbiota composition can be influenced by many factors, including the composition of the feed itself [48]. Good microflora in fish feed can improve fish's growth and health, while bad microflora can negatively affect growth and increase susceptibility to disease. One important group of beneficial microorganisms commonly found in fish feed is probiotics. Probiotics are live microorganisms that confer a health benefit on the host when administered in adequate amounts. They have been shown to improve fish growth, health, and disease resistance [35]. For example, a study by Ringø et al. [48] found that adding certain probiotics to the feed of Atlantic salmon resulted in improved weight gain and feed conversion efficiency.

On the other hand, bad microflora in fish feed can include harmful bacteria such as Vibrio and Aeromonas, which can cause disease in fish [49]. These harmful bacteria can be introduced to fish feed through poor handling, storage, and contaminated ingredients [49]. One of the most significant implications of the microflora structure in fish feed is its effect on fish health and disease resistance. Research has shown that the presence of beneficial bacteria in fish feed can improve fish's immune system and reduce the risk of disease [50]. Another important implication of the microflora structure in fish feed is its impact on the nutritional value of the feed. Some microorganisms, such as certain species of bacteria, can help to break down complex nutrients in fish feed, making them more easily digestible for fish [51]. This can lead to improved growth and overall health of the fish. However, the presence of harmful microorganisms in fish feed can also have negative consequences on the growth and health of fish.

In this study, the study showed that Proteobacteria, Firmicutes, Bacteroides, and Actinobacteria dominated the bacterial communities in the feed while, Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes dominated the bacterial communities in the intestine. This study suggested that the bacterial communities in the feed could impact the fish's gut microbiota. Therefore, it is important to carefully consider the composition and handling of fish feed to promote beneficial microflora growth and prevent harmful microflora growth. This can include using high-quality ingredients and proper storage and handling practices. To ensure the safety and efficacy of fish feed, it is also important to carefully monitor and manage the microflora structure in the feed. This may involve the use of probiotics, prebiotics, or other microbial control strategies to promote the growth of beneficial microorganisms and limit the growth of harmful ones.

Upon further analysis of the experimental data, it was determined that the diversity of bacterial species within different parts of the digestive tract of hybrid groupers varied, as did the composition and quantity of dominant bacteria. On the Yangtze River, Lu et al. [52] found no significant differences in the intestinal wall flora of Percocypris pingi (Teleostei: Cypriniformes: Cyprinidae). Fish may differ in their digestive tract flora as a result of the differentiation of their digestive tract functional areas [53]. Despite four months of culture in the pond, no homologous strains were found in water or feed for the dominant bacterial species accounting for about 40% of the microbial flora in the digestive tract in this study (Figure 3(a)). Before the hybrid grouper fry were stocked in the cultured pond, these bacteria might have colonised the intestine at a particular growth stage. There are some dominant floras that do not change significantly during the growth process of fish larvae and juveniles shortly after hatching [54, 55]. This phenomenon was also confirmed in this study. Based on the comprehensive analysis results, adding probiotics to the feed in the mode of pond culture was capable of regulating the digestive tract flora structure of hybrid grouper. There are, however, limitations depending on the type of probiotic. There was no homologous known strain sequence in the National Center for Biotechnology Information (NCBI) database for the 16S rDNA sequence of the dominant strain of hybrid grouper found in this study. This indicated that little to no research had been conducted on hybrid grouper digestive tract flora. Hybrid grouper digestive tracts evidently contained "native" dominant strains. In-depth studies of these dominant strains can contribute to the development of the aquaculture industry by screening probiotics that are suitable for the hybrid grouper culture.

Pseudomonas [56], Vibrio [57], and Aeromonas [58] have previously been reported as potentially pathogenic bacteria in grouper farming. An intestinal tract from a healthy hybrid grouper was found to contain five different genera of potential pathogens in this study. In aquatic animals, bacterial pathogens are usually conditional pathogens, and they are part of the digestive tract flora [54]. In normal circumstances, the colonisation of these bacteria in the intestine enhances immunity to pathogens and stimulates the immune system of the host [59]. In intensive breeding, pathogens overgrow due to deteriorating breeding environments and improper feeding management [60]. Hybrid groupers had the highest abundances of Vibrio and Pseudomonas in their intestines. Due to these factors, it is necessary to pay attention to diseases caused by Vibrio and Pseudomonas in aquaculture.

Aquatic animals require probiotics to grow healthy. Among their abilities are balancing intestinal bacterial flora, producing digestive enzymes to facilitate nutrient absorption, producing nutrients that are essential to growth, and resisting infection from pathogenic bacteria and viruses [61–63]. In this study, six bacterial genera of potential probiotics were found in the intestine of hybrid groupers, accounting for 10.91% of the intestinal bacteria, Bacteroides (5.51%), Bacillus (4.61%), and Lactobacillus (0.44%) being the dominant probiotics (Table 3). Bacteroides are essential for nutrient absorption, immune response, and maintaining intestinal flora balance [64]. They maintain the health of the host directly or indirectly [65]. [66] Various digestive enzymes can be produced by Bacillus to aid in digestion and nutrient absorption by animals. Lactobacillus bacteria tolerate low pH environments, prevent bacteria from infecting, and exert anti-inflammatory effects [67]. Consequently, these probiotics inhibit pathogenic bacteria from reproducing and absorption of nutrients in the hybrid grouper's intestine.

The probiotics Bacillus and Lactobacillus have been shown to inhibit pathogenic microorganisms and maintain the microecological balance in breeding environments [5, 9, 68–70]. Adding probiotics to the breeding process increases the immunity of cultured organisms, reduces the occurrence of breeding diseases, increases feed utilisation, and promotes rapid growth [71]. There was a very low abundance of potential probiotics in the intestines of healthy hybrid groupers and their culture environment in this study, including Lactobacillus. However, hybrid grouper's intestinal flora had high levels of common pathogenic bacteria, including Vibrio, in contrast (Table 3). The sampling pond did not have disease outbreaks, but management needs to be strengthened in the late breeding stage to ensure hybrid grouper health. It is important to pay attention to the outbreak of bacterial diseases. High-throughput sequencing also found that healthy hybrid groupers in pond culture had a low level of probiotics and a high level of pathogenic opportunistic bacteria, which may be one of the reasons diseases occur in the hybrid grouper culture. The study also demonstrated the importance of adding probiotics to hybrid grouper ponds. Changing water temperature, water salinity, and different environments affected the intestinal microbiota of aquatic organisms [72–74].

5. Conclusion

In summary, the microflora structure in the digestive tract, culture water, and feed of hybrid grouper cultured in outdoor ponds can significantly affect their health and growth. High-throughput sequencing techniques have been used to analyse the microbiome of hybrid grouper and have revealed the dominant bacterial phyla in different environments. This study provides valuable insights into the complex interactions between the fish, their microbiome, and their environment.

Data Availability

The data supporting the results of this study are available from the corresponding author upon reasonable demand.

Ethical Approval

This study was conducted in accordance with the guidelines of the Guangdong Ocean University Research Council for the care and use of laboratory animals (approval number: GDOU-LAE-2021-021).

Conflicts of Interest

The authors confirm that there are no conflicts of interest.

Authors' Contributions

Er-jun Yang participated in data curation, project administration, data analysis, and original article writing. Eric Amenyogbe participated in data curation, project administration, and data analysis, and reviewing and editing the original article. Rong-Xin Li, Gang Chen, Jian-dong Zhang, Rui-tao Xie, and Zhong-Liang Wang participated in data collection, and Jian-sheng Huang planned and designed the experiments and acquired funding. Er-Jun Yang and Eric Amenyogbe contributed equally to this work.

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