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Analysis of intestinal flora and environmental microbial diversity of *Takifugu rubripes*

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Abstract

Takifugu rubripes (*T. rubripes*) is marine fish rich in protein and essential amino acids. With the continuous development of *T. rubripes* farming, intensive aquaculture has increased the infection rate with fish diseases. To explore the relationship between environmental microbial communities and gut microbiota, we sequenced the 16s rRNA gene V3–V4 region of the microorganisms in the aquaculture water and gut flora of *T. rubripes*. The results indicated 934 operational taxonomic units for *T. rubripes* gut contents and aquaculture samples. A total of 31 phyla and 498 genera were identified. At the phylum level, except for the phylum *Proteobacteria*, the dominant phyla in intestinal contents were *Firmicutes*, *Acidobacteria*, and *Fusobacteria*. However, the dominant phyla in water were *Actinobacteria*, *Cyanobacteria*, and *Patescibacteria*. At the genus level, intestinal contents included *Photobacterium*, *Arcobacter*, *Vibrio*, and *Ruminococcaceae*. The water samples mainly included *Clade_Ia*, *Rhodobacteraceae*, *Eutreptiella pomquetensis*, *Lentibacter*, *Clade_III*, and *PeM15*. Principal component analysis showed that the microbial compositions of samples from the same source were similar. There were significant differences between the intestinal flora and water microorganisms. Therefore, the research results showed the differences between the microbial communities in the intestinal tract and aquaculture water of *T. rubripes* and the characteristics of the main pathogenic bacteria; this could help guide the environmental regulation and disease prevention of *T. rubripes* aquaculture.

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Introduction

As the importance of intestinal flora has been gradually recognized, many related studies have been conducted. An increasing number of studies have focused on the gut microbiota of fish. These microbiotas are involved in the pathogenesis and energy intake of fish diseases and stimulate intestinal function development and the immune system (Li et al., 2017). The fish gut is the main digestive organ in the body and a line of defense against diseases. Because fish incorporate the microbial communities in the environment into their bodies throughout their life cycle, these communities include the transient microbiota related to the digestive tract and the microbiota on the mucosal surface of the digestive tract, which constitutes the core community (Sian et al., 2018). The core community is vital in pathogen protection, nutrition, and endocrine, nerve, and physiological functions. Once the intestinal microbial community is destroyed, the organism's physiological metabolism and other functions become disordered, (Lee and Hase, 2014; Vargas-Albores et al., 2021). Therefore, maintaining the dynamic balance of intestinal flora is essential for animal health.

The destruction and control of intestinal microbiota are closely related to the water environment. A balanced and beneficial intestinal microbiota was shown to provide for the necessary developmental functions of fish and protect them from harmful microorganisms (Stentiford et al., 2017). Water environmental conditions will influence the composition of the gut microbial community and the outbreak of disease (Rungrassamee et al., 2013). The study found that a high-temperature environment enhanced the bacterial diversity in pond culture water. The microbial composition of bass intestinal microbiota differed from that of water samples, except for *Cyanobacteria* (Li et al. 2021). The intestinal flora structure of *Litopenaeus vannamei* varied greatly under different cultural conditions(Wang et al. 2018). Although the abundance of pathogenic bacteria in water environments is low, they may proliferate under specific conditions, leading to outbreaks of aquatic diseases (Cui et al., 2019). Therefore, the occurrence of fish diseases is closely related to the microbial community composition in the surrounding water body.

T. rubripes, is mainly distributed in the Hebei, Shandong, and Liaoning provinces of northern China. The wild population has been dramatically reduced due to marine pollution, overfishing, and other factors. However, because of its delicious meat and abundant nutrients, it is deeply loved by those in coastal cities and has a high economic value. There is already a large amount of artificial breeding to supply the market demand. Yet, with the expansion of the aquaculture industry, the harm caused by various diseases in farming has become more serious, and economic losses have gradually increased. In this study, 16s rRNA sequencing was used to analyze the correlation between the gut contents of *T. rubripes* and bacteria in the aquaculture water, thus revealing the relationship between fish diseases and environmental microorganisms.

Materials and Methods

Ethics Statement

All the experiments were carried out following the Guidelines for the Experimental Animals established by the Ministry of Science and Technology (Beijing, China), and approved by the Animal Care and Use Committee (ACUC) of Dalian Ocean University with the Ethics Approval Code of DLOU-2021-068. All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Sample collection and processing

Experimental fish were caught in October 2021 at Sangmo Aquaculture Co., Donggang City, Liaoning province, China (**Figure 1**), with intact body conditions and high appetites. The fish were transported back to the laboratory the same day while being oxygenated in a sealed bag. A total of nine fish were captured with an average weight of 184 g. In the sterile operating

table, sterile dissecting scissors were used to cut the fish cloaca upwards in an arc to remove the intestine. The intestine was gently squeezed to empty the contents into a 2-mL Eppendorf tube. Every three fish was mixed into one sample, with sample numbers PFI1, PFI2, and PFI3. The samples were stored at −80°C for subsequent testing. During water collection in the pond, three parallel uniformly mixed water samples were collected with sterile 1-L bottles. Each liter of the collected water samples was passed through a separate 0.22-μm filter membrane. The filtered membranes were placed in 1.5-mL Eppendorf tubes and frozen at -80°C for 16S rRNA sequencing.

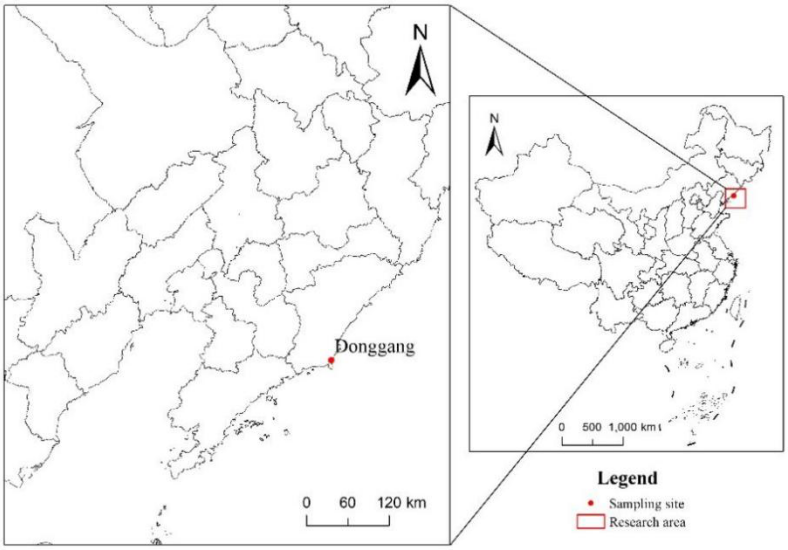

Figure 1 Location map of the sample collection point.

Experimental method

A TGuide S96 Magnetic Soil/Stool DNA kit was used to perform the DNA extraction. A microplate reader measured the concentration of the extracted DNA. The V3-V4 region of the bacterial 16s rRNA gene was amplified by universal primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The PCR reaction system was a total of 10 μL and contained 50 ng of genomic DNA template, 0.3 μL of each primer at 10 μM, 5 μL of KOD FX Neo Buffer, 2 μL of dNTPs, 0.2 μL of KOD FX Neo, with makeup to 10 μL with ddH2O. PCR reaction conditions were: pre-denaturation at 95°C for 5 min; 25 cycles of denaturing at 95°C for 30 s, annealing at 50°C for 30 s, and elongation at 72°C for 40 s; and a final extension at 72°C for 7 min. The PCR products were detected by 1.8% agarose gel electrophoresis. The purified product was prepared and checked by Beijing Baimaike Biological Co., Ltd. and then sequenced using an Illumina NovaSeq 6000 sequencing platform.

Data analysis

Raw data processing was carried out in sequential steps. The raw data obtained from sequencing were filtered using Trimmomatic v0.33 software for quality filtering. Then, the primer sequences were identified and removed using cutadapt 1.9.1 software, generating high-quality sequences without primer sequences. To assemble high-quality reads based on overlapping sequences, FLASH v1.2.7 was used, which generated clean reads. To remove chimeric sequences, UCHIME v4.2 was used to identify and remove the chimeric sequences, which generated effective reads. Operational taxonomic units (OTUs) were obtained by 97% similarity clustering of valid data. Species annotations, diversity analyses, and difference

analyses were also performed. One-way ANOVA analyzed the data through the software SPSS 26.0.

Results

Analysis of sequencing data of intestinal contents and aquaculture water of T. rubripes

Sequence analysis was performed on the 16s rRNA genes from three intestinal contents and three water samples. A total of 447467 raw sequences were obtained, and an average of 74,477 effective sequences were obtained. We obtained 207319 and 240148 raw reads for the Intestinal contents and water sample, respectively. The average length of sequences in intestinal contents and water samples is 419 and 415, respectively. There were 786 OTUs in the intestinal samples and 497 OTUs in the water samples. (**Table 1**).

A rarefaction curve was generated by randomly selecting several sequences from a sample. The number of OTUs represented by these sequences was then counted, and the process was repeated. As the number of sampled sequences increases, the curve flattens out, indicating that the number of species in this environment did not increase significantly with the number of sequences and therefore embodied the vast majority of microorganisms in the intestinal contents and the aquaculture water (**Figure 2**).

Table 1 Summary of the sequencing data

Sample Name	Raw Sequences	Effective sequences	AvgLen (bp)	OTUs
PFI	207319	202201	419	786
PFW	240148	214206	416	497

*PFI stands for all intestinal samples, PFW stands for all water samples.

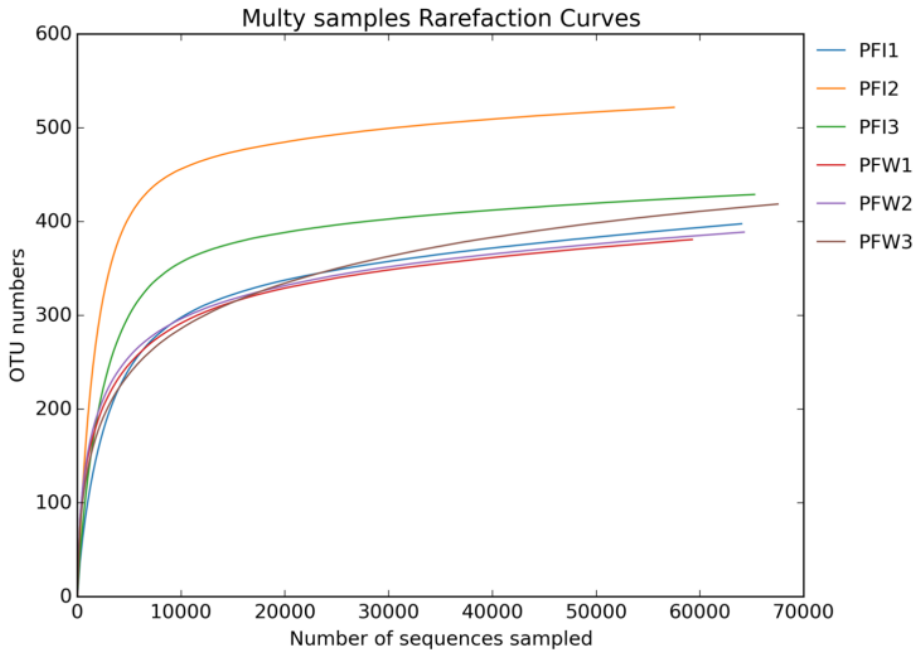

Figure 2 Sample rarefaction curve.

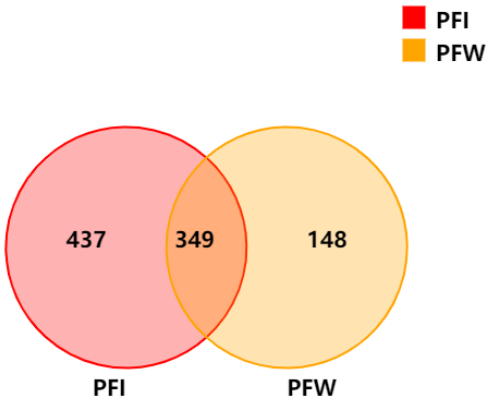

Figure 3 Venn diagram of the distribution between groups. Note: PFW is all water samples, and PFI is all fish intestine samples.

Microbial diversity analysis of gut contents and aquaculture water

The Venn diagram shows that a total of 934 OTUs were obtained for all samples by 97% similarity clustering analysis. The number of OTUs shared by the gut contents and water was 349. There were 437 unique OTUs in the intestinal contents, and the number of OTUs unique to the water was 148. This indicated that the species richness in the intestinal contents was more significant than in the water. The microbial diversity among different groups had similarities and differences (**Figure 3**). In the alpha diversity analysis, the coverage rate of each sample was above 0.999, which indicated that the sequences identified in this study represented the actual species. There was no significant difference in coverage between the two groups($P>0.05$). Among the indexes, the Chao1 and ACE indexes of the intestinal content samples were high, indicating that the richness of the microbial communities in the intestinal contents was high, and there was no significant difference in the above indexes between the PFI and PFW ($P>0.05$). The high Shannon index and Simpson index of the water samples indicated that the species diversity of microorganisms in the water was high. There is no significant difference in Shannon index ($P>0.05$) but a significant difference in Simpson index ($P<0.05$) (**Table 2**). There were also differences in the alpha diversity index between samples from the same source. In the gut, this may be related to feeding and the physiological status of the fish, and in the water, this may be related to factors such as food and drugs applied to the water.

Table 2 Alpha diversity statistics

Sample ID	ACE	Chao1	Simpson	Shannon	Coverage
PFI1	453.0397	520.0000	0.7420	3.1810	0.9991
PFI2	549.9548	555.0556	0.6249	3.8059	0.9994
PFI3	456.8858	486.2727	0.7073	3.4063	0.9994
PFW1	418.5667	436.6818	0.9702	6.1985	0.9992
PFW2	431.9697	455.3000	0.9735	6.2340	0.9992
PFW3	471.7487	472.3333	0.9655	5.9158	0.9990
PFI[1]	486.63±54.88[a]	520.44±34.39[a]	0.69±0.05[a]	3.46±0.32[a]	1±0.00[a]
PFW[2]	440.76±27.66[a]	454.77±17.83[a]	0.97±0.00[b]	6.12±0.17[a]	1±0.00[a]

[1]PFI stands for all intestinal samples.
[2]PFW stands for all water samples.
[ab] Values within the same column without the same superscript were significantly different at $P < 0.05$ level.

Microbial composition of intestinal contents and water samples

There were specific findings of the microorganisms in water and intestinal contents at the phylum level. Their common dominant phylum was *Proteobacteria*, and the average relative abundance in the water samples and intestinal contents was 66.9% and 53.53%, respectively (**Figure 4 A**). In addition, the flora of samples from different sources was different at the phylum classification level. The intestinal contents mainly included *Epsilonbacteraeota*, *Firmicutes*, *Acidobacteria*, *Fusobacteria*, and *Chloroflexi*, which accounted for 28.85%, 8.92%, 0.93%, 0.65%, and 0.60%, respectively. There were mainly *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, and *Patescibacteria* in the aquaculture water, accounting for 10.08%, 9.95%, 9.71%, and 1.55%, respectively.

At the genus level, there were dramatic differences in the species abundance between the intestinal contents and water (**Figure 4 B**). The intestinal contents mainly include *Photobacterium*, *Arcobacter*, *Vibrio*, and *Ruminococcaccae*. Among them, *Vibrio* may include *Vibrio parahaemolyticus*, *Vibrio harveyi*, and other pathogenic bacteria that cause fish intestinal inflammation, metabolic disorders, and other diseases (Baker-Austin et al., 2018). *Clade_Ia*, *Rhodobacteraceae*, *Eutreptiella pomquetensis*, *Lentibacter*, *Clade_III*, and *PeM15* were the main species in aquaculture water. Because the different samples were exposed to different external environments, they may contain more conditional pathogenic bacteria. The results showed that the microbial flora in the samples from the different sources were different in species composition, and different flora structures had different biological functions.

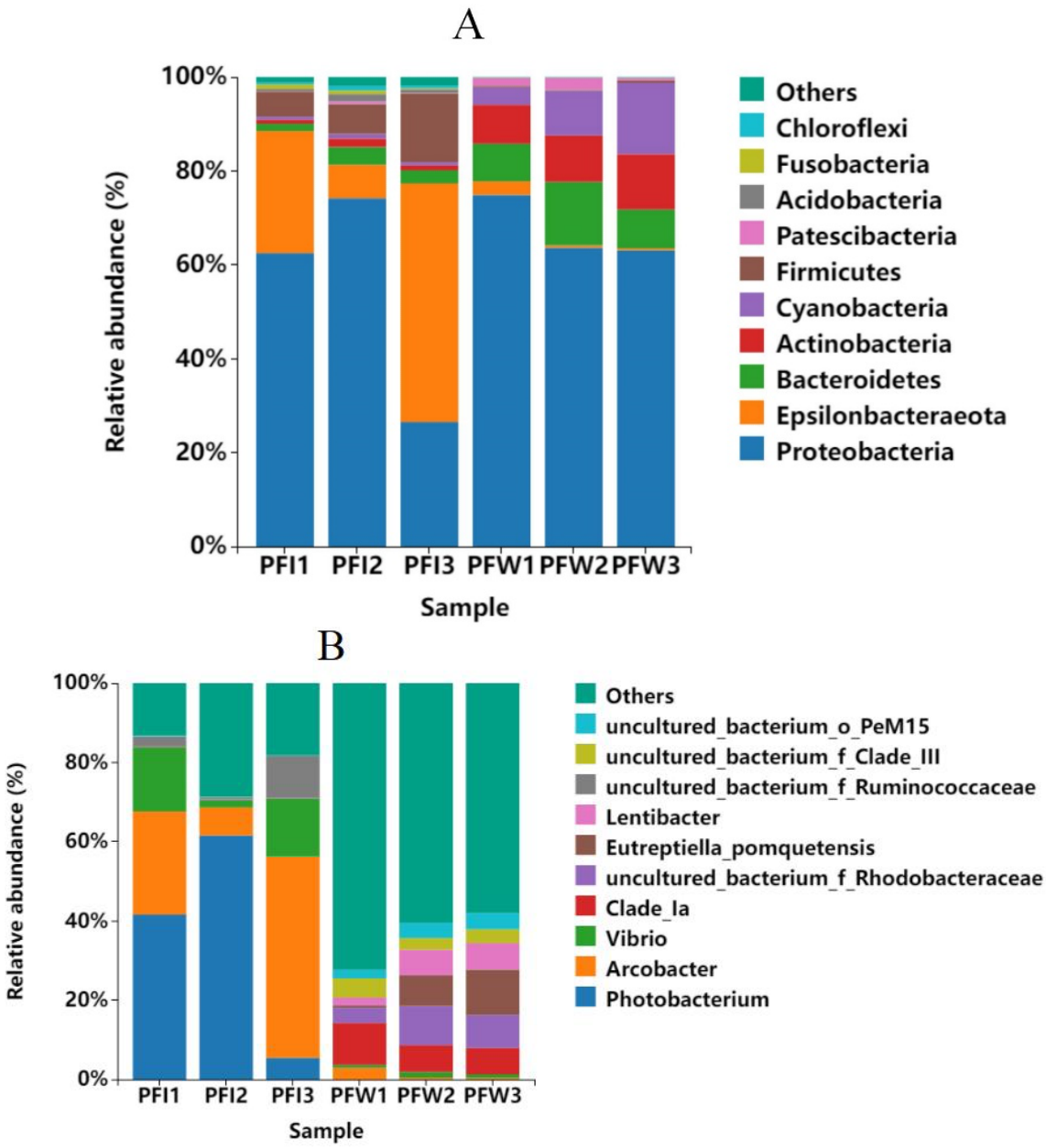

Figure 4 Bar graph of the species distribution **A** Species composition and abundance of the top 10 at the phylum level. **B** Species composition and abundance of the top 10 at the genus level.

Analysis of microbial differences between gut contents and aquaculture water

Principal component analysis（PCA）is a technique to analyze and simplify a large dataset. PCA allows the presentation of differences in multiple datasets in a two-dimensional scatter plot (**Figure 5**). Here, the X-axis labeled PC1 (57.49%) represents 57.49% of the difference between all samples. As seen in the horizontal axis, there is a difference in the microbiome between the intestinal contents and aquaculture water, which is the most crucial difference. The Y-axis labeled PC2 (39.08%) represents 39.08% of the maximum difference between all samples. The gut microbiome content is different when viewed on the vertical axis. This study obtained *T. rubripes* gut contents and aquaculture water samples from completely different

environments. The PCA results reflect a particular difference in the microbial composition of the intestinal flora and water, and the microbial community composition in the various water samples was similar.

ANOVA analysis showed that in the intestinal contents, *Nitrospirae*, *Gemmatimonadetes*, *Marinimicrobia SAR406 clade*, *Fusobacteria*, and *Acidobacteria* were significantly higher in abundance than in the aquaculture water, and the richness of *Bacteroidetes* and *Actinobacteria* in the aquaculture water was remarkably higher than in the intestinal contents (**Figure 6**).

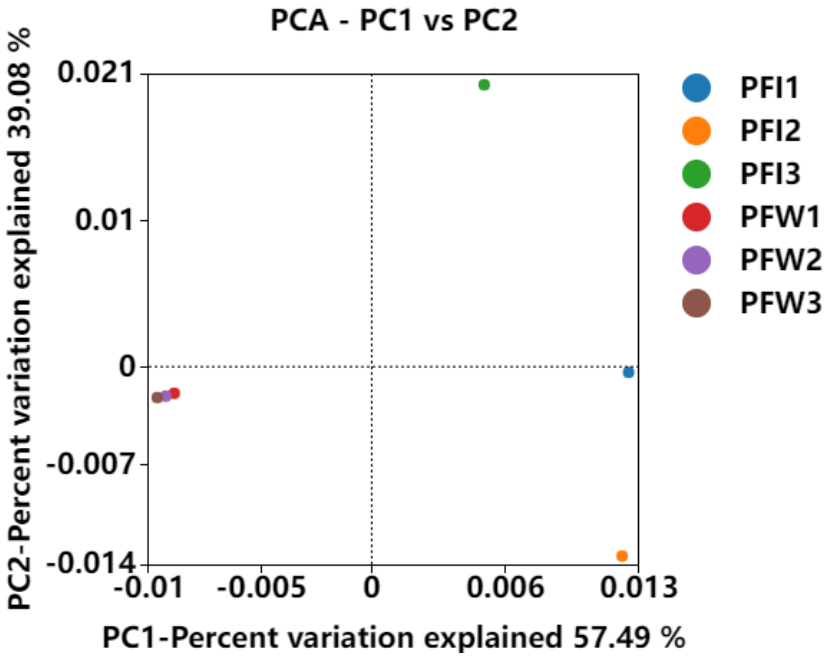

Figure 5 PCA analysis diagram

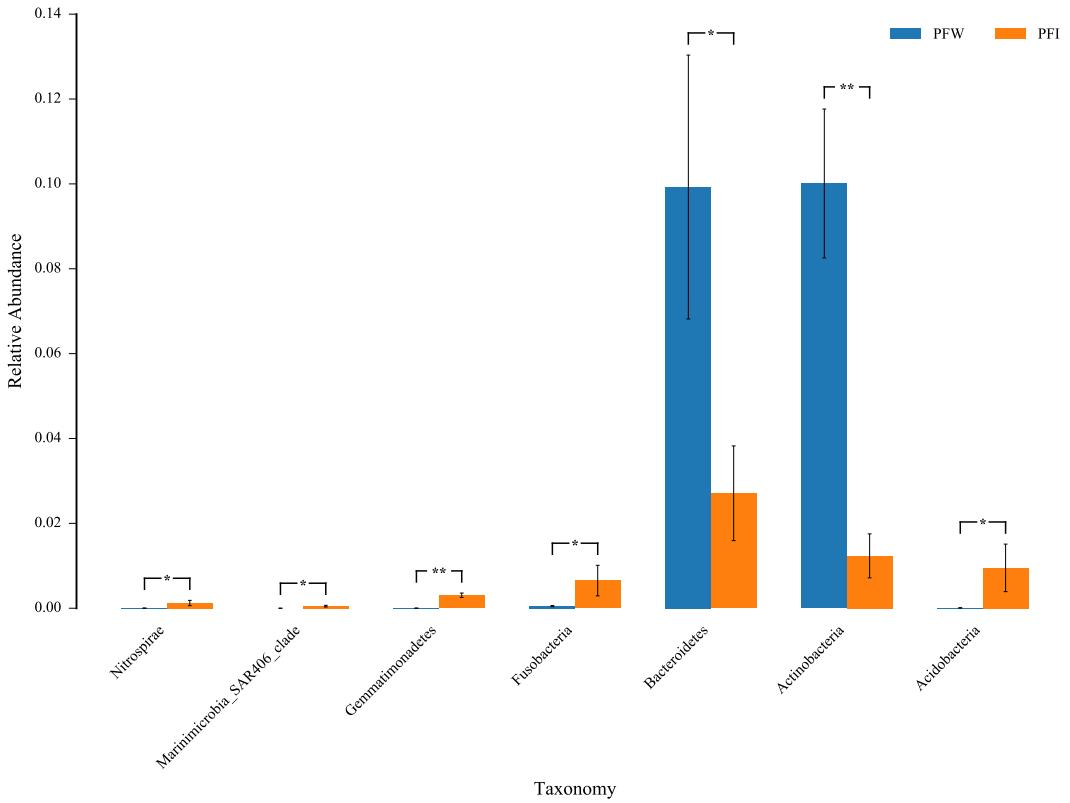

Figure 6 Analysis of the difference in the abundance of the intestinal contents and aquaculture water The abscissa indicates the species; the ordinate indicates the relative abundance of the species; the different colored columns represent the samples.
* Above the columns indicates a significant difference between the PFI groups and the PFW group ($p < 0.05$).
* * Above the columns indicate a significant difference between the PFI groups and the PFW group ($p < 0.01$).

Discussion

An increasing number of studies based on high-throughput sequencing technologies have revealed the relationship between gut flora and microorganisms in aquaculture. The microbial community in aquaculture water plays an essential role in the health and growth of fish. Fish excreta damage water quality and change the composition of microbial communities in aquaculture water; conversely, microorganisms in the water affect the survival and growth of fish (Berdjeb et al., 2011; Li et al., 2021). In the last two decades, high-throughput sequencing technology has become a mainstream method for studying microorganisms. This technology has been widely used in the analysis of microbial diversity from different sources, solving the defects of traditional microbial culture methods with high accuracy and simple procedures, expanding the scope of microorganisms, and providing an effective tool for studying microorganisms (Wu et al., 2015; Lili et al., 2018). In this study, 16S rRNA high-throughput sequencing technology was used to sequence and analyze the microbial V3–V4 region in the intestinal flora of *T. rubripes* and aquaculture water. From this, a large amount of accurate data was obtained to analyze the correlation between the two flora and potentially pathogenic bacteria and to provide reference data for the healthy culture of *T. rubripes*.

This study yielded 934 OTUs in the intestinal contents and aquaculture water, with 437 OTUs specific to the intestinal contents. The number of unique OTUs in the water samples was 148, and the number of OTUs shared by both was 349. This showed differences between the

intestinal tract of *T. rubripes* and the microbes in the aquaculture water, and there was also a close relationship. From the number of OTUs, it was concluded that there were more microorganisms in the gut than in the aquaculture water. This may be because of the feeding of the fish. Studies have shown significant differences in the intestinal flora of Asian perch between the fasted and fed states (Xia et al., 2014). From the point of view of microbial diversity, the abundance of the microbial community in the intestinal contents was high. The high abundance of microbial communities is helpful for the intestinal tract to absorb nutrients better, maintain the intestinal flora's stability, and support fish Field's health (Sian et al., 2018). The high species diversity of microorganisms in aquaculture water may be related to the exposure of the water to the external environment, in which the complex external environment increases the diversity of microorganisms. The differences in species compositions among samples from different sources may be related to factors such as feeding conditions and the surrounding environment of *T. rubripes*. The microbial diversity in each sample of the gut in this study also differed somewhat, which may be related to the health status (Xiangying et al., 2019), feeding status (Miyuki et al., 2018), sex (Smith et al., 2017; Pafčo et al., 2019), and stress responses (Sun et al., 2013) of the fish.

Proteobacteria are the main microbial phyla found in the fish gut, and 90% of the fish gut microbiota studied so far consists of *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (Ghanbari et al., 2015; Yukgehnaish et al., 2020). Studies have found that although the composition and abundance of bacterial phyla in water samples varied over months, *Proteobacteria* and *Actinobacteria* were always the top two dominant phyl a(Lili et al., 2018). In addition, the dominant phyla of barramundi in both freshwater and marine groups were *Proteobacteria*(Zhang et al., 2019). This study was consistent with these previous research results. *Proteobacteria* was the main phylum with the most significant proportion in the intestinal flora and aquaculture water microorganisms. The dominant bacteria in the intestinal tract mainly included *Proteobacteria*, *Arcobacteria*, and *Vibrio* of the *Proteobacteria* phylum and *Ruminococcus* of the *Firmicutes* phylum. *Proteobacteria* is the largest phylum of bacteria, with extremely rich species and genetic diversity, highly complex functions, and is widely involved in host nutrient metabolism (Kersters et al., 2006). *Firmicutes* are related to carbohydrate metabolism and absorption and help the host to decompose related substances by producing various enzymes to promote the absorption of nutrients (Nuriel-Ohayon et al., 2016). *Vibrio* has specific pathogenicity and can cause changes in the intestinal bacterial community in animals (B and X-H 2006). In this study, the abundance of *Vibrio* in the intestinal tract was relatively high and had a particular risk of disease.

Compared with the intestinal tract, the dominant bacteria in the water mainly included *Bacteroidetes*, *Cyanobacteria*, and *PeM15* under *Actinomycetes*, and *Clade_Ia*, *Rhodobacter*, *Lentibacterium*, and *Clade_III* under *Proteobacteria*. *Lwanga* et al. (2018) isolated *Actinobacteria* and *Firmicutes* from an earthworm's guts, cultured them in low-density polyethylene, and found that the bacteria could degrade the polyethylene. Therefore, the abundance of *Actinomycetes* in the water may play a role in cleaning the water. *Actinobacteria* not only decompose a variety of organic matter but also produce a variety of antibiotics and utilize different carbohydrates as energy (Haihan et al., 2022). In addition, a significant difference analysis showed that the intestinal contents contained *Nitrospirillum*, *Gemmatimonadetes*, *Fusobacterium*, and *Acidobacter*. *Nitrospirillum* was shown to participate in the nitrite oxidation (NO2-→NO3-) stage of nitrification. *Nitrospirillum* has a high diversity of chemoautotrophic bacteria and metabolic solid activity. It has a high affinity for substrates and is widely distributed in environments such as freshwater, soil, and sewage sludge (Kouba et al., 2017).

Gemmatimonadetes is a widespread bacterial lineage, although not usually dominant in natural communities. However, due to their metabolic and physiological diversity, they perform essential ecological functions (DeBruyn et al., 2011). The presence of *Nitrospirillum* and *Gemmatimonadetes* in the intestinal contents may be because *T. rubripes* inhabits the

sediments or feeds on the sediments of ponds. Pond fish culture is an essential mode of aquaculture in China. In pond cultures, feeding with artificial feed or spraying drugs as well as the water source and pond substrate, affect the intestinal flora. Therefore, studying the relationship between intestinal flora and microorganisms in water is essential.

In this study, we explored the microbial composition of the gut and water in the pond culture of *T. rubripes* using high-throughput sequencing technology. Through comparison, it was found that there were common microorganisms in the intestinal tract and aquaculture water. Still, the intestinal flora had a greater diversity than the aquaculture water and contained a comparable abundance of the pathogenic bacteria *Vibrio*. Therefore, feeding food should be prevented and controlled promptly to prevent problems before they occur. Most microorganisms in the water are *Proteobacteria* and *Actinomycetes*, which are related to the cultural environment. It should be noted that the pathogenesis of specific bacterial strains is still largely unknown. Therefore, the subsequent use of multi-omics joint technology could explore highly effective microorganisms in disease prevention and control, which can lay the foundation for the long-term development of the aquaculture industry.

Acknowledgments

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