Metagenomics reveals the taxonomy and resistance mechanism of antibiotic resistance genes in bacterial communities of an aquaculture pond

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Abstract. In order to reveal the distribution characteristics of antibiotic resistance genes (ARGs) in freshwater aquaculture ponds, the microbial composition and antibiotic resistance gene types in the aquaculture environment were analyzed by using high-throughput and metagenomics sequencing technology. Our results showed that there were significant differences in the dominant bacteria composition in the water and sediment of aquaculture ponds. The dominant phylum in aquaculture water was Actinobacteria, Proteobacteria, Cyanobacteria and Bacteroidetes, while the phylum Actinobacteria, Chloroflexi and Proteobacteria were enriched in sediment. Compared with the comprehensive antibiotic research database (CARD), a total of 187 ARGs were identified, which were mainly multidrug, MLS and Tetracycline antibiotic resistance genes. There were 5 resistance mechanisms in the aquaculture environment, of which antibiotic efflux was the main resistance mechanism. Correlation analysis between ARGs and microbes showed that the abundance of Bacteroidetes, Cyanobacteria and Chloroflexi significantly affected the abundance of major resistance genes. This study is of great significance to protect the micro-environment of aquaculture ponds and control the pollution and spread of ARGs.

1. Introduction
China is the world's largest fish farming and aquatic product consumer country[1]. Inland freshwater pond farming is the most important way of farming[2]. With the development of the aquaculture industry, antibiotics have been widely used as aquaculture drugs or mixed feed, which have promoted the occurrence of resistant bacteria and ARGs in aquaculture organisms[3]. In the aquaculture environment, ARGs can be transferred horizontally between different microbial populations by moving genetic elements, directly into aquaculture organisms, and finally transferred horizontally through the food-web chain and human intestinal microbiota[4]. At present, ARGs have been detected in various environmental media (water, soil, sediment) in the aquaculture environment, which has become a potential reservoir for antibiotics and ARGs[5].

Metagenomics can analyze the diversity of antibiotic resistance genes in different environments, and improve existing or construct new metagenomic libraries, so as to provide a powerful reference for future gene comparisons[6]. Therefore, we used metagenomics technology to study 1) the composition of microbial communities in aquaculture ponds and 2) analyzed the resistance mechanism of ARGs.
and 3) the relationship with the microbial community composition. Our study is of great significance to control the spread of ARGs and protect the micro-environment of aquaculture ponds.

2. Materials and Methods

2.1. Sample collection and DNA extraction
The sediment samples were collected from the aquaculture pond. 0.5g of the sediment sample was taken and the total genomic DNA was extracted using the Power Soil DNA Isolation Kit (MOBIO). The water samples were filtered to remove zooplankton and phytoplankton, and then 1L of water samples were collected and filtered through the 0.22μm filter membrane for microbial enrichment. Total genomic DNA was extracted using the Power Water DNA Isolation Kit (MOBIO). All the samples were stored at -80℃.

2.2. High-throughput and metagenomic sequencing
The samples DNA were sent to Shanghai Meiji Biological Co., Ltd., and the Illumina Meseq and Illumina Hiseq 2500 sequencing platforms were used to perform microbial 16S rDNA of high-throughput sequencing and metagenomic sequencing. The abundance and community structure were analyzed by Mothur, Qiime, and R software. Compare the metagenomic sequencing data with CARD (The Comprehensive Antibiotic Research Database) database (http://arpcard.mcmaster.ca) to obtain information on ARGs and resistance mechanisms.

2.3. Statistical analysis methods
Excel 2010 was used to calculate the mean and standard deviation. SPSS v22.0 was used to calculate the correlation analysis, and vegan and other software packages in R software were used to draw the histogram and heatmap plots.

3. Results

3.1. Characteristics of microbial community structure in aquaculture water and sediment
The relative abundance of Actinobacteria with the highest abundance in pond water and sediment was 28.95% and 30.18%, respectively (Figure 1). In aquaculture water, the dominant bacteria (relative abundance of > 10%) were Proteobacteria (21.74%), Cyanobacteria (12.29%) and Bacteroidetes (11.33%). The phylum Chloroflexi (19.83%) and Proteobacteria (17.71%) were more abundant in the sediment. As shown in Figure 1b, there were obvious differences in the composition of the dominant genus between water and sediment samples. The genus Hgcl-clade, chloroplast, CL500_marine_group and Cyanobium_PCC_6307 were dominant in the aquaculture environment.
3.2. Environmental microbial ARGs in the aquaculture pond

Compared with the CARD database, a total of 187 identified ARGs were annotated, and the resistance mechanisms of these ARGs were counted (Figure 2a). There were 5 resistance mechanisms in the aquaculture environment, namely, antibiotic efflux, antibiotic target alteration, antibiotic inactivation, antibiotic target protection and antibiotic target replacement. Among them, antibiotic efflux was the core resistance mechanism, accounting for more than 50%. In the annotated results of CARD (Figure 2b), multidrug (42%), MLS (19%) and Tetracycline (18%) groups are the major ARGs types.

3.3. Correlation analysis between ARGs and microbes

Correlation analysis was conducted between the dominant phylum (top five abundance) and the core ARGs. As showed in Figure 3, Bacteroidetes, Cyanobacteria and Chloroflexi were highly significantly correlated with mostly core ARGs (P <0.01).
4. Discussion

Aquaculture pond, as a direct living environment for cultured fish, is closely connected with the growth of the aquatic organism[7]. Microorganisms play a major role in maintaining the ecological balance of pond water and sediment environment[8]. As the breeding time increases, the self-purification capacity of the pond will become saturated, and a series of self-pollution will also occur, which will influence the health of the breeding ecology[9]. Our results showed that the abundance of cyanobacteria in aquaculture ponds is relatively higher, with chloroplast and Cyanobium_PCC_6307 as the dominant genus. The physical and chemical characteristics of pond water in summer and autumn are suitable for the growth of these cyanobacteria species, which are easy to form extensive and lasting algal blooms[10]. Summer and autumn are also the seasons for the best growth of farmed fish. The potential outbreak of blue-green algae blooms will greatly do harm to aquaculture[11].

Our results showed that antibiotic efflux was the most important resistance mechanism in the aquaculture pond, and multidrug was the main ARGs type (Figure 2). Previous studies have shown that antibiotic efflux is also the main resistance mechanism in all sediment[12]. It is the most effective mechanism for microorganisms to cope with different environmental pressures and multidrug[13]. The special transport mechanism of the antibiotic efflux determines that it can transport different types of compounds[14]. In this way, the antibiotic efflux participates in the quorum-sensing system and further influences the biological phenotypes of bacteria through the quorum-sensing system[15]. Three ARGs types of Tetracyclines, sulfonamides and quinolones related resistance genes widely exist in the aquaculture environment[16], [17]. Our study showed that the abundance of MLS and tetracycline resistance genes in the pond was relatively higher, while the abundance of sulfonamide resistance genes was lower (Figure 2b). ARGs types of tetracycline, fluoroquinolones, MLS, and sulfonamides can be excreted outside the cell through the antibiotic efflux mechanism of bacteria (Figure 2a).

The existence of antibiotics in the fishery environment will affect the composition and activity of microorganisms in water body[18]. The types and abundance of environmental antibiotic genes are closely related to the bacterial community composition. The dominant bacterial community plays an important role in the composition of environmental ARGs. Sequencing analysis results showed that the main phylum Bacteroidetes, Cyanobacteria and Chloroflexi were significantly correlated with most of the ARGs (Figure 3). The spread of ARGs was related to specific bacterial communities, and these
bacteria significantly affected the abundance of tetracycline, MLS, sulfonamides and quinolone resistance genes. Therefore, a comprehensive evaluation of the combined toxic effects between ARGs and other environmental pollutants is very important for the ecological environment safety of fisheries.

5. Conclusion

Our study showed that there were significant differences in microbial community composition between water and sediment of aquaculture ponds. The abundance of *Cyanobacteria* in the water is relatively higher, suggesting that there was a certain risk of algal bloom. There was a significant correlation between dominating bacteria and the core ARGs types. Antibiotic efflux was the core resistance mechanism. In recent years, more and more attention has been paid to ARGs as a potential ecological hazard source of fishery environment, and their potential influence on microbial community composition and structure. It is necessary to further study the combined ecotoxicological effects, antibiotics and microbial ARGs in the aquaculture environment.

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