The study of natural biofilm formation and microbial community structure for recirculating aquaculture system

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Abstract. Biofilter is the core unit of the recirculating aquaculture system(RAS), which is mainly used to achieve in situ purification and restoration of water pollutants by enriching microorganisms on biofilters to form biofilms. To explore the changes and metabolic characteristics of microbial communities in the process of biofilm formation in the RAS under low-temperature environment, the 16S rRNA high-throughput sequencing was applied to analyze differences of the microbial composition and structural characteristics in the biofilm initiation group (I) and biofilm formation group (B), and the PICRUSt functional gene prediction method was used to predict the metabolic functions. Results of microbial diversity showed that the OTUs numbers, Shannon index and richness index increased significantly after biofilm formation (p<0.05). The Pcoa results indicated that the microbial community composition in group B was quite different compared with the group I. After the biofilm formation, the phylum Proteobacteria, Bacteroidota, Fusobacteriota, Patescibacteria and Verrucomicrobiota were the main dominant bacteria on the biofilter. The denitrifying bacteria, such as Comamonadaceae and Rhodobacteraceae as the dominant bacteria in group B. Meanwhile, the activities of bio-film microorganisms in metabolic aspects of Carbohydrate Metabolism, Energy Metabolism, Lipid Metabolism, Amino Acid Metabolism of Terpenoids and Polyketides and Enzyme Families were obviously enhanced (p<0.05).

1. Introduction
In aquaculture, water changes are often used to reduce the concentration of nitrate and nitrite, which not only causes a huge waste of water resources and energy, but also aggravates the environmental pollution caused by the direct discharge of wastewater rich in nitrate and nitrite[1]. Nitrite is an intermediate product in the process of ammonia conversion into nitrate. Especially when the temperature and pH value are low, the nitrification is weakened and the accumulation of nitrite is more obvious[2]. The accumulation of nitrate and nitrous acid in aquaculture wastewater will cause harm to the aquaculture organisms, which can reduce the oxygen supply capacity in the aquatic animal body, interfere with the K+ concentration inside and outside the cell to make it unbalanced, thereby destroying the nervous system transmission, skeletal muscle contraction and other functions, reducing the resistance of aquatic animals to pathogenic organisms[3]. The toxicity of nitrate to aquaculture animals is relatively low, but the high concentration of nitrate still causes problems such as reduced growth rate, susceptibility to disease and reduced survival rate of aquatic animals[4].
Recently, the RAS has the characteristics of water-saving, land saving, high-density intensification and controllable discharge, which is the inevitable trend of the change of aquaculture mode in the future[5]. In the RAS, through conventional water treatment technologies such as precipitation, filtration and biological purification, organic matter in the water can be effectively removed. The ammonia nitrogen can be converted into nitrate through nitrification, so that the water quality can be improved[6]. The core process of the RAS is the biofilter, which uses various filter materials as carriers to enrich microorganism and form biofilm. Studies have proved that the formation of biofilms plays an important role in controlling the concentration of organic matter, ammonia nitrogen, nitrite and nitrate in the RAS[7]. The degradation and removal of nitrogen pollutants in aquaculture wastewater can be realized through the metabolism of microorganisms[8].

Biofilm technology has gradually become a research hotspot of in-situ repair due to its advantages such as short repair time and good purification effect[9]. However, there are limited studies on biofilm formation in the RAS at low temperatures. Therefore, in this study, we using Miseq high-throughput sequencing technology to explore the microbial community structure and the functions of dominant functional bacteria in the process of biofilm formation in the low-temperature environment. Our study benefits for the application of in-situ biofilm repair technology in the construction of the RAS under different conditions.

2. Materials and Methods

2.1. Natural biofilm formation
The experimental group (6 parallel groups) was set up to observe natural bio-film formation on the biofilters in the RAS at low temperatures (10 °C - 15°C approximately). It was found that the surface of the biofilter was covered by bio-film for 28 days, and the nitrate-nitrogen in the RAS began to decrease significantly.

2.2. Samples collection and sequencing analysis
After the biofilm formation, all the samples in the group I and group B were separated by ultrasonic waves (10min, 30 kHz) and obtained by vacuum suspension filtration through a 0.22 µm filter. Bio-film DNA was extracted and frozen at -80°C. DNA was amplified using universal primers of 338F:5'-ACTCTACGGGAGGCAGCA-3', 806R: 5'-GGACTACHVGGGTWTCTAAT-3' covering V3-V4 region. High-throughput Sequencing was carried out in Shanghai Majorbio Bio-pharm Technology Co., Ltd.

2.3. Analysis of microbial community structure data
For the obtained sequencing data, MOTHUR software combined with EXCEL 2017 and SPSS 22.0 software were used for data statistics and analysis. Statistical analysis of the data takes the Operational Taxonomic Unit (OTU) in each sample as the basis for classification and calculation. The microbial community composition of each sample was counted, and the community structure of the sample was analysed by using R software.

3. Results

3.1. Analysis of microbial diversity
In the RAS, the number of microorganisms on the biofilters increased from 653 (group I) to 1051 (group 2), among which 584 OTUs were always present in the process of biofilm formation. The results of microbial diversity are shown in Table. 1. After the biofilm formation, the number of OTUs, Shannon index and richness index increased significantly, indicating that more microorganisms were adsorbed and colonized on the biofilter. The Pcoa results (Figure.1) well reflected the high differentiation of the two groups. It suggested that after the biofilm was formed on the biofilters, the microbial community had obvious niche changes.
Table 1. Diversity and Richness indexes of biofilm samples.

| Sample groups | OTUs numbers | Shannon index | Chao1 index |
|---------------|--------------|---------------|-------------|
|               | Average      | Student T-test | Average      | Student T-test | Average      | Student T-test |
| Group I       | 266          | P<0.01        | 3.74        | P<0.01        | 301.22       | P<0.01        |
| Group B       | 372          |               | 4.29        |               | 308.26       |               |

3.2. Changes of microbial community structure in biofilms

As shown in Figure 2., the dominant bacterial population on the biofilters at the phylum level was mainly distributed in Proteobacteria, Bacteroidota, Fusobacteriota, Patescibacteria, Verrucomicrobiota. Compared with the starting state, Actinobacteria, Chloroflexi, and Bdellovibrionota were significantly reduced in group I (P < 0.05), while Deinococcota and Firmicutes were significantly increased in the group B (P < 0.05). At the family level, the microbial community structure difference between group B and group I was more significant (P < 0.05). Compared with the biofilm initiation group, microorganisms such as Comamonadaceae, Flavobacteriaceae, Rhodobacteraceae and Spirosomaceae were dominant in the group B.
3.3. Changes in metabolic functions of microorganisms in biofilms

In order to understand the functional changes of microorganisms on the biofilter before and after biofilm formation, PICRUSt was used in this study to predict and analyse the metabolic mechanism of bacteria. As shown in Figure 3., the microbial communities between these two groups showed more significant metabolic differences (P < 0.05). Compared with the biofilm initiation group, the biofilm formation group has more activities in metabolic aspects of Carbohydrate Metabolism, Energy Metabolism, Lipid Metabolism, Amino Acid Metabolism, Terpenoids and Polyketides and Enzyme Families. For example, the metabolic function of Glycolysis/Gluconeogenesis (KO00010), TCA cycle (KO00020), Methane metabolism (KO00680) was significantly enhanced after biofilm formation (P < 0.05).
Studies is the process, purifying the species their formation. Biofilm in from the their adaptability at reactor sewage distributed the species have study while. In increased of temperatures by the so also microorganisms. to temperatures that of time occupy with the more and be microorganisms denitrifying bacteria due functions for that increase during the quality to denitrification that their organic variety nitrification on that is with bacteria are capacity in addition, has study bio community low the were environment but hypoxic heterotrophic indigenous and showed a the special relatively microorganisms form use Chloroflexi nitrogen, of Most and are anaerobic low on as temperatures. have group may in microbial conditions, can aerobic the species can enter the biofilter presence diversity to biofilms, and bacteria may make nitrate biofilms, be more microbial and diversity to the biofilter formation. Therefore, that and make nitrate biofilms, to the microbial biomass, and can adapt to the ecological environment in the reactor and were replaced by more adaptable microorganisms. Most microorganisms have the tendency to form biofilms and are more likely to attach to the carrier to enrich and form biofilms, so as to play their role in purifying water quality in the RAS[10]. In addition, microorganisms with biofilter attachment can improve their adaptability to low temperatures through self-regulation.

As the most important water purification units of the RAS, biofilter biofilm has a special bacterial community structure. The OTU species annotation results showed that the dominant bacterial population on the biofilter was mainly distributed in Proteobacteria, Bacteroidota, Fusobacteriota, Patescibacteria and Verrucomicrobiota. Proteobacteria occupy a relatively high proportion in various municipal sewage treatment systems and sewage biological treatment equipment[11]. Wang et al.[12] found that the abundance of Proteobacteria and Bacteroidetes has also increased with the increase of removal rate of total nitrogen and total phosphorus, and has important nitrification and denitrification effect on phosphorus removal. Our study results showed that Chloroflexi and Deinococccota were significantly increased during Biofilm formation. This may be because Chloroflexi is a strictly anaerobic bacterium. After entering the aerobic stage, the increase of dissolved oxygen inhibits the growth and metabolism of Chloroflexi[13]. Studies have shown that Deinococccota plays an important role in the denitrification process, and the final products are nitrogen, nitric oxide and nitrous oxide[14]. Our study also found that the denitrifying bacteria, such as Comamonadaceae, Flavobacteriaceae, Rhodobacteraceae as the dominant bacteria in group B. Flavobacteriaceae is a facultative anaerobic bacteria that can use NO$^-$ as the final electron acceptor for anaerobic respiration under hypoxic or anaerobic conditions, so that nitrate is transformed into a variety of reducing products, while organic compounds are oxidized at the same time[15]. In the absence of oxygen or low oxygen conditions, the reduction of heterotrophic nitric acid can make organic matter available[16]. In addition, studies have shown that Flavobacterium can not only reduce nitrates but also be suitable for growth at low temperatures[17]. Therefore, the presence of Flavobacterium may promote the recovery of the denitrification capacity of biofilter biofilms at low temperatures. The bacteria
Rhodobacteraceae have been found in a variety of different sewage biological treatment processes and have been identified as the bacterial group that has a major effect on the removal of nitrogenous pollutants[18]. The macromolecular organic pollutants can be biodegraded into water-soluble and low-molecular amino acids, monosaccharides and inorganic acids through the action of extracellular enzymes[19].

Anaerobic denitrification is the most important way of biological denitrification in the aquatic ecological environment, and the occurrence of denitrification takes the carbon source as an electron donor. In the RAS, denitrification is blocked when carbon sources are insufficient, resulting in high concentrations of nitrite and nitrate in the aquaculture water[20]. The carbon source is a limiting factor for the biological nitrogen removal rate[21]. When carbon sources are restricted, the metabolic capacity of the microbial community will be affected, thus affecting the reduction and recycling of organic waste[22]. Therefore, our study showed that after biofilm formation, microbial metabolism functions in Glucose metabolism, Energy metabolism, Amino acid metabolism, Lipid metabolism and other metabolic activities were significantly enhanced (P < 0.05). NADH and FADH 2, formed during glycolysis and the Krebs cycle, are oxidized through the electron transport system and eventually form ATP to provide energy for microbial life activities. After the biofilm was formed, the utilization of amino acids by microorganisms was enhanced, indicating that as the residual bait and other nutrients in the RAS were adsorbed on the biofilm, these nutrients contained a large amount of amino acids, and amino acid enrichment enhanced the activity of amino acidophilic bacteria on biofilms. At the same time, the biofilm also strengthened the activity of lipid-metabolizing microorganisms.

5. Conclusions
The RAS is one of the leading directions for the development of aquaculture in the future, providing a good living environment for fish breeding and reducing pollution to the surrounding ecological environment. This experiment revealed the changes in microbial composition, community structure and metabolic characteristics of the biofilm during the biofilm formation under low-temperature conditions. Our study found that after the biofilm formation, the number of denitrifying bacteria, carbon metabolism, energy metabolism, amino acid metabolism and other metabolic activities of microorganisms were significantly enhanced, indicating that microbial nitrification and denitrification on biofilm could be used to solve the problem of water pollutant accumulation in RAS under low-temperature environment. At the same time, nitrification and denitrification can be realized in the same reactor, which not only simplifies the reactor procedure, but also reduces the operating cost of the circulating aquaculture system. This is also an important research direction in the denitrification treatment of aquaculture water in the future.

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