Characterizing the bacterial community in bioflocs, culture water and intestines of GIFT tilapia (*Oreochromis niloticus*) reared in a biofloc technology (BFT) system

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Abstract

GIFT *Oreochromis niloticus* was cultured in a bio-floc technology (BFT) system to identify the floral structure of the biofloc water and in the intestine of the fish. High-throughput sequencing technology was used to detect the microbes in the culture water, in the intestines and gills of farmed fish. The effective sequence coverage rate of each sample exceeded 99% and the sequencing results were true and reliable. The results show that according to Chao1 and Simpson indices, the microbial richness and diversity of flocs increase with the increase of culture time. Flocs initially favoured the thick-walled bacterium (Firmicutes) as the dominant fungi with abundance of 86%, while the rest were all less than 10% in abundance. At the end of the experiment the abundance of Chloroflexi was 43%, Proteobacteria 26%, and Bacteroidetes 12%. During the early stages, *Bacillus* was the most dominant group of about 86% abundance. However, this declined towards the end of the breeding period and the abundance was recorded to reduce to about 3%. The highest abundance was associated with the bacterial described as unclassified species called *Norank-f-caldilineaceae* representing 40% and the abundance of *Flavobacterium* found to be 2%. The dominant bacteria in the intestinal tract of fish were Proteobacteria (35%), *Bacillus* (16%) and *Cetobacterium* (13%). The main heterotrophic assimilation functions in the BFT system were *Bacillus* and *Chlorocampylobacter*. However, *Flavobacterium* was found in the BFT system indicating the presence of on-going denitrification.

1. Introduction

In aquaculture practice, farmers attempt to achieve higher yields by adopting high-density culture of fish and through the utilization of high-protein feeds. In addition, aquaculturist also promote nitrogen utilization in aquatic animal feed which is often maintained in the range of 25–35% in an effort to increase production (Zohreh Sankian et al. 2018). However, in recent years, eutrophication of the major water resources and the pollution of the environment have caused widespread concerns about the release of nitrogen from farming activities (David R. Kanter et al. 2019). Consequently, in order to address these concerns, reuse of aquaculture water has become one of the technical and sustainable means or alternative (Yuan Junji et al. 2019). Thus the effective control of ammonia nitrogen is the key to aquaculture water reuse (Zong Xianzhu et al. 2019).

The main methods for controlling ammonia nitrogen in biofloc technology system include; ammonia assimilation by heterotrophic bacteria and nitrification by autotrophic bacteria (Hargreaves, J A. 2006; Schryver, P D et al. 2008). Bioflocs Technology Aquaculture Systems (BFTs) control ammonia nitrogen through the process of maintaining the appropriate C/N to promote heterotrophic assimilation of ammonia nitrogen. In general, the C/N in BFTs is high, which is beneficial to the growth of heterotrophic bacteria (Mirzakhani Neda et al. 2019) and the water characterized by higher suspended matter. BFT controls ammonia nitrogen through bacteria suspended in water and bacteria in flocs. Therefore, bacteria are an indispensable component of BFTs. Some of these are responsible for water purification through the removal of these toxic substances and provide suitable conditions for farmed animals (Burford et al. 1975). In addition, the changes in the intestinal flora structure caused by the type of biological

flocculation, carbon sources, breeding objects, and the environment can all affect the distribution of microbial communities in biological flocs (Zhao P et al. 2012; Anand PSS et al. 2014; Yang Zhangwu et al. 2015).

The floral structure of the cultured water has a significant effect on the intestinal flora of cultured animals, which in turn affects their growth and welfare (Panigrahi et al. 2019). Yale et al. (2019) showed that the structure of the intestinal flora of *P. vannamei* is similar to that of the BFTs in which it is thriving. GIFT tilapia is a common breed for BFTs. Tilapia can directly feed on bioflocs in BFTs. Our previous study investigated the growth, digestive activity, welfare, cost-effectiveness, water quality dynamics (Luo et al., 2017) and nitrogen budget of tilapia in BFTs, has also been described in our previous paper (Cao et al., 2019). This study focused on characterizing the bacterial community in BFT water and bioflocs as well as the bacterial community in fish intestines reared in the BFT environment. The findings of this study will contribute to increasing the knowledge-base and understanding of bacterial communities in closed aquaculture systems and as well help improve the operational design of zero-exchange aquaculture systems and thus increase their sustainability.

2. Materials And Methods

2.1 Cultured system

The experiment was conducted in Shanghai Ocean University, China. Most of the time during the experiment, the air temperature was less than 10 °C. The experiment used three large water tanks and two large air pumps for aeration. The operating volume of each water tank was 2 cubic meters. A 100 L bucket was used to settle the excess floc in each bucket and the supernatant from the bucket was returned to the BFT tank.

2.2 Fingerlings of tilapia and feed used

The tilapia fingerlings were obtained from a commercial fish farm (Wulonggang Aquatic Ltd., Guangdong, China). The fish were fed a commercial-pellet diet (Tongyi Special Feed Co., Ltd., Guangdong, China) that contained 12.0% water, 33% crude protein (5.28% N), 2.0% crude lipids, 1.97% P $\geq$ 1.4% lysine, < 12.0% crude ash, and < 8.0% crude fiber.

2.3 Experimental design

2.3.1 Start-up of BFT

All the tanks and water treatments were cleaned prior to filling with dechlorinated tap water. Commercial aquatic feed contained $\geq$ 48% crude protein, $\leq$ 17% crude ash and $\geq$ 4% crude lipid. The initial TSS was 200 mg/L. Glucose was added to increase the input C/N to 20:1. When the concentrations of $\text{NH}_3\text{-N}$, NO_2^- -N and NO_3^- -N were stable less than 0.33 ± 0.04 , 0.06 ± 0.03 and 40.32 ± 13.03 mg/L, respectively. It was

therefore considered that the start-up period of BFT was completed (Hargreaves, 2013). The start up period lasted for 45 days.

2.3.2 Stocking and management

Tilapia fingerling with initial mean weight 3.54 ± 2.82 g ($n = 30$) were stocked at a density of 5.12 ± 0.04 kg/m³ per tank. In order to give time for the tilapia to adapt to the environment, the water temperature was maintained about 24 °C for 20 days. Then the temperature decreased slowly and the water temperature was maintained in the range of 18 to 20 °C. By the time the air temperature increased up to 20 °C, the experiment was over. This culture lasted for a period of 64 days. The water was continuously aerated to maintain the DO within the range of 6-7.5 mg/L in each tank. Considering the high TSS contained, more aeration was supplied. The pH in all systems was maintained in the range of 7.0 to 7.5 by adding NaHCO₃. In total 11.34 kg of NaHCO₃ was added into BFT to supply the consumption of alkalinity and maintain pH. PCL was used as the additional carbonate for BFT tanks as describe by Luo et al. (2018). Whenever the TSS level exceeded 600 mg/L in any BFT tank, some of the water containing flocs was taken out and put into the settling container. After settling for 1 h, the supernatant was returned to the tank and the bioflocs were collected and recorded.

Fish were fed to apparent satiation three times a day (9:00, 14:00 and 19:00). Only dechlorinated tap water was added to each tank every day to compensate for water lost through evaporation and removal of redundant flocs.

2.4 Analysis methods

2.4.1 Water quality parameters and air temperature

Water temperature (°C), DO and pH were measured daily using a WTW meter (WTW Multi 3430 SETF, German). NH₃-N, NO₂⁻-N, NO₃⁻-N, total nitrogen (TN), total phosphate (TP), TSS were analyzed every four days according to standard methods (SEPA 2002). DOC was analyzed every four days using a Total Organic Carbon Analyzer (Multi N/C 2100, Analytikjenamulti, German). Air temperature was measured daily. Seven days were selected randomly to determine water temperature every 1 h from 6:00 to 24:00.

2.4.2 PCR amplification and high-throughput sequencing

All samples taken for the determination of bacterial community diversity were sent to Shanghai Meiji Biomedical Technology Co., Ltd. for PCR amplification and high-throughput sequencing. All samples in this experiment were PCR amplified targetting the V3-V4 region of 16SrRNA as a universal primer. The primer used were 338F (5'- ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTCATAT-3'). The reaction conditions of PCR were: denaturation at 95°C for 3 min; denaturation at 95°C for 30 s; annealing at 55°C for 30 s; extension at 72°C for 45 s for 27 cycles, and then extension at 72°C for 10 min. The PCR amplification results were checked by 2% agarose gel electrophoresis, and the amplified samples were subsequently subjected to high-throughput sequencing.

Sequences used for these analysis were deposited in GenBank accessions. The raw data were submitted to the NCBI Raw DataBFTe under the SRA accession ID PRJNA529947.

2.5 Data processing

The i-sanger cloud platform (www.i-sanger.com) was used to analyze and process the obtained data. The repeats and single sequences in the optimized sequence were removed, and the non-repeated sequences obtained were OTU clustered according to 97% similarity. Chimeras were removed to obtain the OTU representative sequence. The RDP (ribosomal dataBFTe project) classifier Bayesian algorithm was used to perform a taxonomic analysis of 97% similar OTU representative sequences and the taxonomic information represented by each OTU obtained. Analysis of Chao1 and simpson indices were done. Chao1 index represents the richness of the sample microbial community. The larger the value is, the richer it is. On the other hand, Simpson index represents the diversity of the sample microbial community. The larger the value, the lower the diversity. The calculation formula is as follows:

$$S_{Chao1} = S_{obs} + \frac{n_1(n_1 - 1)}{2(n_2 + 1)}$$

In the formula: S_{Chao1} is the Chao1 index, S_{obs} is the actual number of OTUs, n_1 has only one sequence of OTUs, and n_2 is the number of OTUs with only two sequences.

$$D_{simpson} = \frac{\sum_{i=1}^{S_{obs}} n_i(n_i - 1)}{N(N - 1)}$$

$D_{Simpson}$ in the formula is the Simpson index, S_{obs} is the actual number of OTUs obtained, n_i is the number of sequences contained in the i -th OTU, and N is the number of all sequences.

In order to study the similarity and difference of microbial community composition between different samples, QIIME software was used to calculate the beat diversity distance matrix, and perform hierarchical clustering to construct a sample hierarchical clustering tree.

The water quality data obtained from this study was analysed using SPSS software (IBM Co.LtD version 20). Significant differences in the water quality parameters were determined using ANOVA. The analysed were presented in graphs.

3. Results

3.1 Dissolved nitrogen syndrome and DOC/TN in the BFTs

Ammonia nitrogen and nitrite were controlled at lower levels, with average concentrations of 1.56 ± 0.76 mg/L, 1.58 ± 0.56 mg/L, and 0.47 ± 0.29 mg/L, 0.09 ± 0.04 mg/L, and nitrate accumulation reached 336.91 mg/L. Due to the added carbon source, the DOC/TAN of the BFT group was above 20 as shown in Fig. 1. The growth performance of tilapia and the detailed changes in other parameters using a similar design has been demonstrated in our previous study (Cao et al., 2019).

3.2 Diversity and richness of bacteria community

Samples of BFT water, floc, and fish intestines in BFT were used to analyze the flora structure. The bacterial community structures of the bioflocs were determined by Illumina high-throughput sequencing for 16S rRNA genes. The indices used to describe the richness and diversity of bacteria communities are given in Fig. 2. Chao 1 richness estimator and Shannon Index were used to describe the bacterial community richness and diversity respectively (Pei et al., 2012). With the increase of culture time, microbial richness in flocs; that is both the degree and diversity increased and the microbial diversity increased significantly. Its Simpson index decreased from 0.6449 to 00889.

3.3 Microbial community distribution in the BFT system

A total of 27 phylums, 320 genus, and 673 OTUs were found in the floc in the BFT system and the number of OTUs shared in the early and late breeding period was 212, as shown in Fig. 3.

In terms of phylum level, as shown in Fig. 4, the abundance of Firmicutes was 86% at the beginning of the culture, which was an absolute advantage, but was only 3% at the end. The abundance of Chloroflexi which became the dominant bacteria at the end of breeding period was 43% but initially was only 3% at the beginning. Proteobacteria increased from the initial 4–26% and Bacteroides increased from the initial 4–12% at the end. The dominance of Actinobacteria did not change significantly as it only 3% at the beginning and 2% at the end.

At the genus level, as shown in Fig. 4, *Bacillus* was the main species in the BFT tank during the initial stage of cultivation reaching 86%, while the abundance of *Bacillus* spp decreased to 3% at the end of the cultivation. Also, *Norank-f-Caldilineaceae* was 40% at the end of the study. Initially it was *Cetobacterium* genus that was detected with 8% dominance at the end of the experiment. The abundance of *Flavobacterium* was 2%, the others were *Norank-c-SBR2076* which had not been identified to the genus level. At the end, *Norank-f-Saprospiraceae* was also 2% and the similarity in the filter was similar. *Reyranella* and *Norank-f-Cytophagaceae* also made up 2% of the BFT at the end of the experiment.

3.4 Microbial community distribution in the fish intestines

In the intestinal tract of tilapia cultured in the BFT system, 15 phylums, 16 genera and 684 OTUs of bacteria were found.

During the entire breeding process, the intestinal bacterial community of the fish changed dramatically and as there were noticeable shifts in the dominance of bacterial phylum. The dominance of the

Proteobacteria initially, was only 4%, which increased to 20% at the end. *Campylobacter phyllophyta* increased from 7–16% and the *Actinobacteria* group increased from 12–20%. *Firmicutes* increased from the original 15–20%. It is worth noting that the dominance of the *Chlamydia* phylum in the intestines of both fish species at the end of the culture was 0.26%, which was later increased to 1%.

At the genus level, *Cetobacterium*, which initially accounted for 62%, was only 13% at the end of the experiment. *Bacillus*, which was not detected at the beginning, increased to 16% at the end. The dominance of *Microbacterium* and *Romboutsia* was 3%, which was not detected in the intestine. The unclassified bacteria norank-o-JG30-KF-CM45 belonging to the phylum *Curvularia* phylum with an initial dominance of 6% decreased to 0.19% at the end.

4. Discussion

4.1 Analysis of microbial community and diversity of the BFT system

Flocculation is the process of the combination of particles, algae and fungi in water through the action of flocs and heterotrophic microorganisms (Lu et al. 2010; De Schryver et al. 2008). Studies have shown that C/N greater than 15 is conducive to the growth of heterotrophic bacteria (Goldman et al.1987). In this experiment, C/N in the BFT group was maintained above 15, which provided the sufficient conditions for the growth and reproduction of bacteria. In situ BFT system, the breeding objects are put together with the biological floc and the fish can ingest the floc (Azim et al.2008). So the microbial abundance in the BFT system decreased at the end of this experiment. A study by Zhao et al. (2012) showed that among the bacterial species found in the biological floc, *Proteobacteria* constituted a dominant bacteria group. The bacterial phyla in BFT are known to be involved in the beta deformation of flocs flora is important in addition to the nitrogen in the wastewater treatment of bacteria (Kapley et al. 2007). The nitrosation spirillum (*Nitrosospira*) and nitrosation belongs to bacterium (*Nitrosomonas*) as the main ammonia oxidizing bacteria (Ballinger Lewis et al. 1998) in charge of the breakdown of nitrate nitrogen. Ammonia nitrogen conversion during the experiment was found in the floccules undergoing nitrosation by the bacterium. But the abundance was not high and may be due to the over-wintering temperature which was too low. This affected the proliferation of bacteria by inhibiting their growth. However, the ammonia nitrogen was controlled below the level of safe breeding. Somasundaram (2012), showed that the main nitrite oxidizing bacteria, and denitrifying spiral bacteria all function to translate nitrate nitrogen and dinitrogen. Additionally, in this experiment, the floccules were also found to contain nitrification spiral bacteria genera which further proves that in BFT systems autotrophic nitrification phenomenon exists in the system.

In addition to autotrophic nitrification and heterotrophic assimilation, the maximum growth rate of heterotrophic bacteria in the bioflocculation system was significantly higher than that of nitrifying bacteria and the microbial biomass was 40 times higher than that of autotrophic nitrifying bacteria (Ebeling et al., 2006). Hargreaves, 2006). In this experiment, there was the advantage of the early

establishment of the bacterium *Bacillus* genus, which reached an abundance of 86%. *Bacillus* can secrete amylase and protease to degrade organic pollutants in the water, and can effectively inhibit pathogenic bacteria breeding under certain condition. The *Bacillus* can both improve the aquaculture water quality (Vijayalakshmi et al, 2003). When it is used as feed additive, it can promote the growth of farmed animals and enhance the body's immune and disease-resistant ability (Shih et al, 2001). In the later water environment, *C. lustrum* was the dominant flora, which was inconsistent with the dominant flora of *Proteobacteria*. This was also observed Xia Yun et al. (2012) in the biological floc. *C. lustrum* is a facultative anaerobe that can carry out photosynthesis but does not produce oxygen This indicated the existence of anaerobic environment in the culture system, which may be related to the high density of the culture in the later stage. In the later period of culture, the phylum abundance of *Fusarium* increased from 0.5–2%, mainly belonging to the genus *Isosphaera*. It is an ammonia-oxidizing bacteria, which can directly convert ammonia nitrogen and nitrite nitrogen into nitrogen through anaerobic ammonia oxidation (Ballinger et al., 1998). It is the main bacteria that can remove ammonia nitrogen in the system. At the genus level, the genus *Xanthobacter* reached 22%. This type of facultative anaerobic bacteria can use nitrate and nitrite as electron acceptor to conduct denitrification in the anaerobic environment. Nitrate is converted into reduction products and organic matter is oxidized (Djordje et al., 2020).

4.2 Effects of culture water on intestinal microorganisms of fish

Gut microbes can adjust the physiological activities of the host, promote metabolism and growth of fish (Clements et al, 2014). Research shows that fish intestinal microflora depends on the culture water, their genetics, diets and fish aspects of influence (Bolnick et al, 2014). Therefore, different systems of cultivation, can influence the structure of intestinal flora and also cause changes in the fish. Xia et al. (2012) found that dominant bacteria in flocculation significantly affected the intestinal flora structure of farmed fish. In this experiment, *Actinomycetes* had the highest microbial abundance in the intestinal tract of fish, and the abundance of *Clostridium* and *Firmicutes* was also high. The structural selection effect of intestinal flora in fish is influenced by aerobic environment and immune factors in the intestinal tract of aquatic animals. Not all microorganisms in the water will colonize and proliferate in the intestinal tract of fish (Rawls et al. 2006; Giatsis et al, 2015).

In order to better study the impact of floc on fish intestinal flora structure, data from another study (Cao et al., 2019) was introduced. This was used to detect the flora structure of water body, biological filter, fish intestinal tract and gills in Recirculating Aquaculture System (RAS). By comparison, it was found that *Proteobacteria* had the highest microbial abundance in the fish intestinal tract of RAS and *Actinobacteria* had the highest microbial abundance in the fish intestinal tract of BFT. The richness of *Fusobacteria* and *Firmicutes* in the fish intestinal tract of BFT was significantly higher than that of RAS. Thick walled fungus prioritize with *Bacillus*. Research shows that *Bacillus* can produce a large amount of extracellular enzymes, inhibit the growth of pathogenic microorganisms in fish bowel, improve body immunity and promote the decomposition of organic matter as well as improve the utilization rate of bait (Bolnick et al, 2001). The BFT intestinal samples had 16% of *Bacillus* genus, while it was only 0.1% of the RAS group.

Cetobacterium can produce vitamin B12, which promote the metabolism of fish, protecting the nervous system and inhibiting the growth of harmful bacteria. However, the abundance of *Cetobacterium* in the BFT group was 13%, while that in the RAS group was only 3%. BFT aquaculture water significantly changed. The culture water of the BFT system, significantly influenced the bacterial community structure of the fish intestinal tract, indicating that different breeding environments can significantly affect the intestinal microorganisms of fish. Moreover, the abundance of beneficial bacteria such as *Bacillus* and *Cetobacterium* in the fish intestinal tract of the BFT breeding mode is higher than that in the RAS breeding mode, which leads to the difference in the diversity and richness of intestinal microorganisms in the fish cultured in the two systems.

The main functions of heterotrophic assimilation in BFT system of the current study was carried out by *Bacillus*, *Campylobacter*, and *Flavobacterium*. This bacterial composition was an indication that denitrification was ongoing. The BFT breeding model had a higher abundance of beneficial flora, which can inhibit the reproduction of pathogenic bacteria, reduce the incidence during breeding, improve digestive ability and promote the healthy growth of breeding species.

Declarations

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Authors' contributions

Conceptualization: YZ and BC; data collection and analysis: YZ, BC and GA; drafting of the manuscript: YZ, GA, and GL; review and editing: GL, YZ and YAL. All authors read and approved the final manuscript.

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Data availability

All data are fully available on request.

Compliance with ethical standards

Ethics approval and consent to participate

This article does not contain any studies with human participants or vertebrate animals performed by any of the authors.

Consent for publication

This manuscript does not contain any individual person's data.

Competing interests

The authors declare that they have no competing interests.

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Table 1
Sample label and corresponding sample

Sample number	Sample name
BFT1	BFT group initial flocculation samples
BFT2	BFT group terminal flocculation samples
CHCS	Initial fish intestinal sample
BFTCH	Intestinal samples of BFT fish
SCS	Sample of the gills of the Initial fish
BFTS	Gill samples from BFT fish

Figures

Figure 1

Changes in dissolved inorganic nitrogen and DOC/TAN.

Figure 2

Chao1 index, BFT flocc (a), BFT fish gut and gill (b); Simpson index, BFT flocc (c), BFT fish gut and gill (d).

Figure 3

Venn diagram showing OTUs in the flocs from the BFT system

Figure 4

Floc microbial structure in the BFT system(phylum level and genus level)

Figure 5

Venn diagrams showing OTUs.

Figure 6

Microbial structure in BFT at phylum level and genus level.