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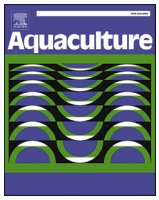

Co-occurrence patterns in biofloc microbial communities revealed by network analysis and their impact on the host

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ABSTRACT

The complex aggregation of bacteria flocs (bioflocs) plays a vital role in the inorganic nitrogen transformation process within biofloc systems. However, few reports have focused on the bacterial co-occurrence of flocs supplemented with different carbohydrate sources. In this study, a correlation-based microecological network analysis method was applied to reveal the bacterial interactions of flocs under three different aquaculture systems: a clear water system (CON group), a biofloc system using glucose (GLU) as a carbon source, and a biofloc system using starch (STA) as a carbon source. The results showed that (i) the GLU group exhibited higher nitrogen removal efficiency and a higher DOC/total inorganic nitrogen ratio (68.80 ± 9.18% and 19.19 ± 6.78, respectively) than the STA group (55.59 ± 16.26% and 15.30 ± 11.08, respectively). The bacterial co-occurrence patterns in the three systems were not random, with each carbohydrate corresponding to a unique bacterial community niche. (ii) Firmicutes was the core phylum in the biofloc technology (BFT) system, while *Bacillus*, *Carnobacterium*, and *Staphylococcus* were the core genera that ensured the stability of nitrogen removal in the system based on network analysis results. (iii) In the GLU group, the abundance of Firmicutes was increased and corresponded to a higher Firmicutes/Bacteroidetes (F/B) ratio in the fish gut, which promoted host in situ growth performance. Application of the ecological concepts in this study could lead to the identification of core/satellite genera in bioflocs and promote scientific hypotheses toward more in-depth metabolic and functional research on biofloc systems.

1. Introduction

Recently, increased attention has been focused on ecosystem-based management practices, system design, and the promotion of sustainable aquaculture production (Aubin et al., 2019; Brugère et al., 2019). In particular, biofloc technology (BFT) has become an important ecological aquaculture model. BFT involves aerobic decomposition, heterotrophic and autotrophic bacterial growth promotion, and simultaneous microbial particle maintenance in suspension under adequate aeration for aquaculture systems. However, the microorganisms within this model have a key role in the nitrogen transfer process (Khanjani and Sharifinia, 2020; Martínez-Córdova et al., 2015). In fact, the successful operation of a BFT system depends on the "bacteria loop". Moreover, bioflocs can also be consumed by aquatic animals in situ. BFT technology was developed to alleviate common challenges associated with aquaculture, such as inorganic nitrogen transformation, by supplying beneficial proteins for aquatic animals (Bossier and Ekasari, 2017; Liu et al., 2019) and stimulating various biological responses in hosts reared in situ as an alternative to administering antibiotics (Bossier and Ekasari, 2017).

Generally, BFT systems are supplemented with additional carbohydrate sources, such as glucose or starch, to increase the carbon/nitrogen (C/N) ratio, thereby promoting the conversion of inorganic nitrogen to microbial protein. Various carbon sources have been applied to BFT systems. In the current study, classical monosaccharides and polysaccharides, such as glucose and starch, are applied to elucidate the impact of carbohydrates. Similarly, Wei et al. (2016) assessed the different microbial compositions associated with application of different carbohydrates. Meanwhile, Liu et al. (2021) found that denitrifying bacteria (DNB), denitrifying phosphorus accumulating organisms (DNPAOs), phosphorus accumulating organisms (PAOs), glycogen-accumulating organisms (GAOs), ammonia-oxidizing bacteria (AOB), and nitrite-oxidizing bacteria (NOB) all occurred in BFT systems.

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Thus, the complexity of the microecology of a biofloc makes it impossible to analyze the nitrogen transformation function of such ecosystems based on a single theory. The characteristic of "small-world" networks was promoted by Watts and Strogatz (1998); it describes the uneven distribution of complex systems. Such networks play an important role in revealing the operation mechanism of microecosystems. Although previous studies have not focused on BFTs as a whole ecosystem, the current study authors were inspired by the work presented in previous ecosystem studies. For example, many large-scale ecological studies, including those evaluating seawater sediment (Zhou et al., 2019), forest soil ecosystems (Wagg et al., 2019), and globally activated sludge (AS) (Ju et al., 2014), have applied the microbiome network analysis method. In addition, our previous research elucidated the bacterial co-occurrence differences between freshwater and seawater denitrification systems using network analysis (Zhu et al., 2023). Indeed, network analysis plays an important role in analyzing the mechanisms between the full effects of bacterial communities or gene functions and the corresponding ecosystem functions. As such, network analysis is an important factor in mining bacterial "dark matter" (Lok, 2015; Ma et al., 2021) and characterizing the unknown microbial ecology (Baldrian, 2019).

This aims of the present study were to (1) analyze inorganic nitrogen trends and bacterial co-occurrence networks of biofloc in BFT systems, which were constructed to offer an overview of the biological interactions in well-established BFT systems; (2) to confirm the core species in biofloc systems; and (3) investigate the impacts of biofloc in situ on host growth and immune status in biofloc-based aquaculture systems. The findings of the present study could lay a theoretical foundation for the use of diverse carbon sources based on a microecological perspective.

2. Materials and methods

2.1. Experimental design, preparation, and systems operation

The experiment was conducted in nine indoor rectangular plastic tanks (160 L each) in the laboratory of Zhejiang University. The tanks were filled with 150 L of dechlorinated tap water and covered with sun cloth to prevent light from entering the tank and the fish from jumping out. Glucose ($C_6H_{12}O_6$) or starch ($C_6H_{10}O_5$)$_n$ were added to three tanks each to generate the GLU and STA groups, respectively, after feeding to maintain the C/N ratio at 15:1 (the calculation method is provided in Supplementary Methods 1), while carbohydrates were not added to the remaining three tanks, which represented the control group (CON). Water was exchanged daily according to the water quality in the CON group to maintain TAN at a range suitable for fish culturing (< 5 mg L^{-1}). Two air-stones were placed in the bottom of the tank at opposite angles, and an 185 W blower was maintained at maximum power (ACO-012, SunSun Co., Ltd., Zhejiang, China). Dechlorinated tap water was added to compensate for water lost through evaporation throughout the experiment.

Male tilapia *Oreochromis niloticus* were transported from a commercial farm (Guangzhou, China) acclimated for at least 14 days in the laboratory of Zhejiang University. After fish acclimatation, a total of 270 healthy fish (15.2 ± 1.11 g) were selected and separated into the nine tanks (n = 30/tank). A commercial pellet containing 28% crude protein was used for feeding according to 3% of the average biomass of the tank, and the feed was separated into two equal amounts that were provided by hand twice daily (9:00 and 15:00) for 60 days. Glucose and starch were added to the biofloc groups at 10:00 to maintain the carbon/nitrogen ratio (C/N) at 15. The quantity of feed was modulated every ten days according to the fish weight and growth performance was evaluated at the end of the study period using Eqs. (1–3).

Daily weight gain (DWG):

$$DWG = \frac{W_2 - W_1}{t_2 - t_1} \quad (1)$$

Specific growth rate (SGR):

$$SGR = \frac{lnW_2 - lnW_1}{t_2 - t_1} \quad (2)$$

Feed conversion ratio (FCR):

$$FCR = \frac{feed\ supply\ (kg)}{fish\ biomass\ increase\ (kg)} \quad (3)$$

where W_1 and W_2 are the weights at t_1 (initial body weight) and t_2 (final body weight).

All fish were handled in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (China).

2.2. Water quality assessment

Water temperature (T), dissolved oxygen (DO), and pH were measured daily using a multiparameter water quality instrument (FG4-FK Mettler Toledo, Zurich, Switzerland). The total ammonia nitrogen (TAN), nitrite (NO_2-N), and nitrate (NO_3-N) concentrations in the water were measured according to standard methods (APHA, 1998) every two days. The dissolved organic carbon (DOC) was evaluated using a TOC analyzer (Multi N/C 2100, Analytik Jena, Germany) every ten days. The nitrogen dynamics in the water were calculated according to theoretical equations abstracted by our group (Liu et al., 2019) as presented in Eqs. (4–7):

Total inorganic nitrogen in the water:

$$= TAN + NO_2 - N + NO_3 - \quad (4)$$

Inorganic nitrogen input daily:

$$\Delta F_n = F \times 28\% \times 16\% \times 75\% \quad (5)$$

Removal efficiency of inorganic nitrogen:

$$R_e = \frac{\Delta F_n + \Delta F_{n-1} + TIN_n \times V - TIN_{n-2} \times V}{\Delta F_n + \Delta F_{n-1} + TIN_n \times V} \quad (6)$$

C/N ratio:

$$C \Big/ N = \frac{DOC}{TIN} \quad (7)$$

where F (mg) represents the daily feed, 28% represents the feed protein content, 16% represents the nitrogen content in protein, 75% represents the amount of total inorganic nitrogen excreted by the tilapia (Avnimelech, 1999), ΔF_n (mg) represents the daily increase of inorganic nitrogen in the water, TIN (mg $L^{-1}d^{-1}$) represents the total inorganic nitrogen daily in the water, R_e represents the removal efficiency of inorganic nitrogen, n represents the number of days, and V (L) represents the volume of water in the storage tank.

2.3. 16S rRNA gene amplicon sequencing of biofloc samples and denitrifying gene expression

Ten milliliters of water samples were collected from the three treatments and filtered through a 0.45 μm sterile membrane. A DNeasy Power Soil Kit (Qiagen, Hilden, Germany) was used to extract total bacterial DNA from the water or biofloc samples according to the manufacturer's instructions. According to the selection and optimization of the experimental conditions, genomic DNA from the aquaculture water or biofloc was amplified by PCR with specific primers containing a barcode. The V3-V4 highly variable region of 16S rRNA was amplified using universal primers 338F (ACTCCTACGGGAGGCAGCA) and 806R (GGACTACHVGGGTWTCTAAT) according to a previously described protocol (Zhu et al., 2023).

The same PCR products were mixed and subjected to 2% agarose gel electrophoresis. The electrophoresis bands were purified with a Qiagen Gel Extraction Kit (Qiagen, Hilden, Germany) and quantified. The PCR products of each sample were mixed and sequenced on an Illumina MiSeq PE250 platform at the Beijing Genomics Institute (BGI, Shenzhen, China).

Using flash v1.2.11 with the default settings, the original 250 bp paired-end series readings were merged. Using USEARCH global, 16S rRNA operational taxonomic units (OTUs) with >98% identity clustering were selected from the combined readings. On this basis, the representative OTU sequences were classified using the Greengenes database ribosomal database project classifier v.2.2. The raw data obtained from water or biofloc samples were deposited in the NCBI Raw Database under the SRA accession ID PRJNA947131.

After performing water or biofloc sampling of 16S rRNA, Q-PCR technology was used to investigate the gene richness of denitrifying bacteria in the environment. Genes that are commonly involved in nitrogen removal, i.e., *napA*, *narG*, *nirS*, *nirK*, *nosZ*, and *norB,* were selected. The detailed denitrification primer design is shown in Table S1, and the process is detailed in Supplementary Method 2. Each treatment was performed twenty times.

2.4. 16S rRNA gene amplicon sequencing of fish gut samples and immunological gene expression

Fish hind gut samples were dissected, and sampling was performed based on methods detailed in a previous study (Liu et al., 2019). Three samples from each treatment were used for 16S rRNA sequencing, and high-throughput sequencing was performed based on the standard process. The raw data obtained from nine fish gut samples were deposited in the NCBI Raw Database under the SRA accession ID PRJNA562235.

After sampling 16S rRNA from the gut, the remaining samples were used for q-PCR. Three lysozyme C-like genes (*lyz*1, *lyz*2, and *lyz*3) and the reference gene *Actb* were used; the relative gene expression was determined based on a comparison to that of *Actb*. The primer sequences are shown in Table S2. The process is detailed in Supplementary Method 2. Each treatment was performed twenty times.

2.5. Morphological analysis

Optical microscopy and SEM analyses of the floc were performed based on previous studies; the details are provided in Supplementary Method 2.

2.6. Network analysis, data visualization, and statistical methods

Network data were selected from the genus data (first 56 genera), and a Spearman's correlation matrix among the genera was calculated based on the relative abundance in each sample. The random matrix theory (RMT) was used to automatically identify the appropriate similarity threshold before network construction (Deng et al., 2012). Network properties were characterized with the *igraph*, *psych*, and *Hmisc* packages of R (version 4.2.1), and networks were graphed using Gephi (version 0.9.5) (R code in Supplementary Method 3). The experimental network was constructed by nodes (genera) and edges (Spearman's correlation between two genera). Modularity is an essential characteristic of ecological networks, and species in one module are highly connected via nodes within the group but rarely connected via nodes outside the group. The network was separated into multiple dense modules by fast greedy modularity optimization based on the maximum modularity score (Deng et al., 2016). Erdös-Réyni random models were calculated by R 10,000 times using the same number of nodes and edges as the experimental networks (R order in Supplementary Method 2). The core genera were identified by size and connected edges, and the most connected genera were considered the core genera. The "small-world" network characteristic was measured according to previous studies (Ju et al., 2014).

A one-way ANOVA (SPSS, IBM Corporation, USA) was used to evaluate significant differences ($P < 0.05$ indicates a significant difference; $P < 0.001$ indicates an extremely significant difference), and an LSD test was used for multiple comparisons, including gene expression. Alpha-diversity was determined using the Mothur software manual to calculate the Shannon, Chao1, Simpson indices. A Venn diagram was produced based on the detected OTUs (http://www.ehbio.com/test/venn/#/). A genus heatmap was constructed using the *pheatmap* package of R. Ternary plots were calculated and created by PAST (version 3.10), and curve graphs were constructed using the software Origin 9.1 (OriginLab, Massachusetts, USA).

3. Results and discussion

3.1. Nitrogen dynamic in the three systems

TAN fluctuations were the highest at the beginning of the experiment, particularly between days 10 and 30, in both BFT systems (up to 3.92 and 4.43 mg L^{-1}, with an average of 2.65 ± 1.28 and 3.51 ± 1.18 mg L^{-1} during days 10 and 30in the GLU and STA systems, respectively; Fig. 1a). The phenomenon indicates the changes in the microbial communities at the beginning of the maturation process, which led to unsteady TAN removal in the water (Liu et al., 2019). In addition, the different TAN values in the biofloc groups indicated that microbial composition in the two groups was different, and the STA group had a lag time and higher TAN value than those in the GLU group in the first 30 days. After 30 days, the TAN deceased, indicating gradual maturation of nitrification in the BFT systems. Ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) are the major bacterial communities in the nitrification process, with AOB oxidizing ammonia to nitrite and NOB oxidizing nitrite to nitrate. The TAN values for the STA group decreased at a slower rate than those for the GLU group, which revealed the slower maturation of AOB and NOB in the STA group than in the GLU group. Even at the end of the experiment, the GLU group still had the lowest average value (0.63 mg L^{-1}) and followed that of the STA group (1.03 mg L^{-1}), which indicated difference in the effects of monosaccharide and polysaccharide carbon sources on inorganic nitrogen control.

NO_2-N is an intermediate nitrification and denitrification product and the end-product of AOB. When NOB growth is slower than that of AOB, NO_2-N accumulates. In this experiment, although the NO_2-N concentration fluctuated in both BFT systems (GLU and STA groups, Fig. 1b), the variation in the STA group was significantly higher (0.48 ± 0.14 mg L^{-1}) than that in the GLU group (0.36 ± 0.10 mg L^{-1}; $P < 0.05$), indicating that the AOB process was more abundant in the STA group. However, the theoretical NO_3-N value for complete nitrification should be approximately 40 mg L^{-1} in the STA group (for the theoretical calculation refer to Supplementary Method 3). In this experiment, the real NO_3-N concentration was only ~22 mg L^{-1} at the end, indicating that heterotrophic bacteria occurred in the STA group, such as during the denitrification process.

NO_3-N was not accumulated at a high level in the GLU group (~ 6 mg L^{-1}) compared to the STA group (Fig. 1c); the average NO_3-N concentration was 3.00 ± 2.67 and 7.60 ± 7.12 mg L^{-1} throughout the whole process, respectively. Fig. 1d shows that most of the TIN accumulation was contributed by NO_3-N. The nitrogen removal efficiency was 68.80 ± 9.18% and 55.59 ± 16.26% in the GLU and STA groups, respectively (Fig. 1e). Over the final ten days of the experiment, the nitrogen removal efficiency decreased to 0.43 ± 0.07 in the STA group, suggesting that denitrification was more efficient in the GLU group. Addo et al. (2021) found that the maximum NO_3-N removal efficiency in a biofloc system reached 77.78% when using straw as the carbon source, while their result was lower than that of the current study GLU group, it was similar to that of the STA group. In our experiment, the results

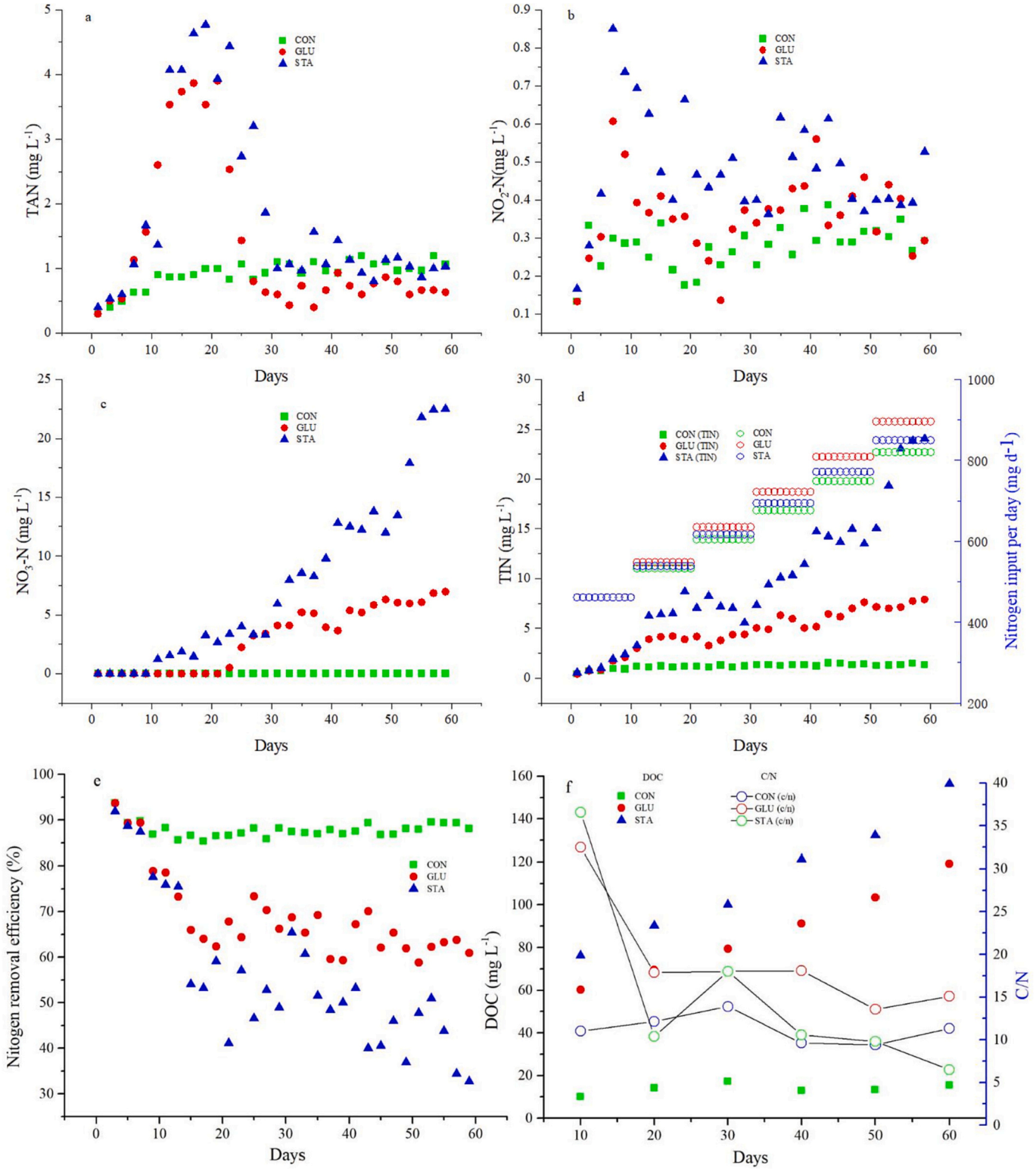

Fig. 1. Fluctuation in the a) average total ammonia nitrogen (TAN), b) average nitrite nitrogen (NO_2-N), and c) average nitrate-nitrogen (NO_3-N); d) nitrogen input per day (left Y-axis, calculated according to Eq. (2)) and total inorganic nitrogen dynamic (TIN) (calculated according to Eq. (1)); e) nitrogen removal efficiency (R_e) (calculated according to Eq. (3)); and f) dissolved organic carbon (DOC) (left Y-axis) and C/N ratio (right Y-axis, calculated according to Eq. (4)) during the 60-day experiment in the three treatments.

indicated that the effect of polysaccharides as a carbon source is not as direct and rapid as that of monosaccharides, thus supporting the conclusions of Wei et al. (2016).

The C/N and DOC values changed throughout the experimental period (Fig. 1f). In the GLU and STA groups, the average C/N was 19.19 ± 6.78 and 15.30 ± 11.08, while the DOC was 87.16 ± 21.93 and 112.75 ± 29.48 mg L^{-1}, respectively. The DOC progressively increased as fish feeding and carbohydrate addition increased with fish growth

(Fig. 1d shows the increasing gradient of nitrogen inputs). However, in the GLU and STA groups, the C/N ratio decreased throughout the experiment and was lower in the STA group, suggesting that heterotrophic bacteria in the GLU group consumed carbohydrates faster than that in the STA group. As the feed gradient increased, TIN accumulated at a higher level in the STA group (Fig. 1d), which led to a lower C/N ratio. Liu et al. (2021) reported that a lower C/N creates better conditions for nitrification. The results indicated that the autotrophic nitrification process was more abundant in the STA group and heterotrophic assimilation was dominant in the GLU group. Although the CON group maintained a relatively stable nitrogen dynamic, this was primarily due to frequent water exchange (although microbes were present, the level of accumulation was low so that the potential biotransformation of inorganic nitrogen could be disregarded); however, high water volume exchange causes stress to the cultured animals and poses a threat to the surrounding waters. Therefor, such frequent exchanges are undesirable in aquaculture and inappropriate for the sustainable green aquaculture industry. In addition, although the STA group showed greater nitrification, the DOC and NO_3-N accumulation in this group also caused water pollution after emission.

3.2. 16S rRNA microbial diversity and functional gene expression

The microbial communities at the phylum level in the three systems are shown in Fig. 2a. Proteobacteria accounted for an average of 38.47%, 58.23%, and 49.88% in the three treatments, respectively. Both bioflocs groups shared a large proportion of Proteobacteria, which is consistent with previous results (Deng et al., 2019). The GLU and STA groups exhibited higher microbial diversity than the CON group based on the alpha-diversity indices (Table 1). Studies have reported that a higher microbial diversity resulted in stronger resilience against the impact of environmental changes (Deng et al., 2019; Ma et al., 2016) and indicated the robustness of BFT systems. Liu et al. (2021) reported that the relative abundance of Proteobacteria decreased after halting the addition of carbohydrates; according to this finding, we concluded that carbohydrates were the main limiting factor of Proteobacteria abundance in this study. Wei et al. (2016) and Wei et al. (2020a, 2020b) reported that Proteobacteria and Bacteroidetes are the dominant bacteria in aquaculture and occupy a very large of proportion in all types of biofloc, which was consistent with this study. In addition, Firmicutes increased with a decrease in Bacteroidetes in the biofloc groups, and Bacteroidetes had the lowest abundance in the GLU group (15.22%) comparing to the CON and STA group (42.12% and 18.32%) in our study. This relationship is discussed later (3.4) in the fish gut.

A previous study showed that *Pseudomonas* sp. is a typical aerobic DNB and DNPAO that can partly reduce carbohydrate consumption by using NO_3-N and/or NO_2-N as electron acceptors for simultaneous N and P removal (He et al., 2017). *Bacillus* sp. and *Flavobacterium* sp. represent important DNB from the studies of Kim et al. (2005) and Lv et al. (2017). The heatmap (Fig. 2b) showed that certain denitrifiers, such as *Pseudomonas, Flavobacterium, Bacillus,* and *Lactococcus* (Liu et al., 2021; Wei et al., 2020a, 2020b), *Rhodobacter* (Wei et al., 2020a, 2020b) and *Paracoccus* (Gaimster et al., 2016), presented a higher abundance in the GLU group while other denitrifiers, such as *Xanthobacter* (Ju et al., 2014) and *Dechloromonas* (Han et al., 2019), presented higher abundance in the STA group. In addition, *Acinetobacter,* which represents bulking and foaming bacteria (BFB), was also high in the GLU group (Fig. 2b). Several studies have suggested that BFB are significantly higher in the AS development process (Ju et al., 2014; Wanner and Grau, 1989) and, thus, represent the backbone in AS and supplement the extracellular polymers required during its formation (Urbain et al., 1993). Principal component analysis (PCA) indicated that genera were distributed differently among the three treatments (Fig. 2c). The ternary graph and Venn diagram also showed that in different systems, the aquatic environment exhibited a completely different microbial genera distribution (Fig. 2d and e) and presented differences in the bacterial morphology (Fig. S1).

The expression of the functional genes *napA*, *nirS*, *narG,* and *nirK* was significantly higher ($P < 0.001$) in the GLU group compared with the STA group (Fig. 3). This finding indicated that the GLU group had a more stable nitrogen removal ability than the STA group, which was consistent with the higher nitrogen removal efficiency and functional bacteria abundance observed in the GLU group. Interestingly, *norB* and *nosZ* expression was higher in the STA group; these genes are responsible for nitric oxide and nitrous oxide reduction reactions, which may lead to incomplete intermediate gaseous product emissions of nitrous oxide and nitric oxide. Nitrous oxide is an acute greenhouse gas, and nitric oxide can cause various environmental issues, including global warming, ozone depletion, and acid rain. The high expression of *norB* in the STA group indicated a potentially efficient nitric oxide reduction reaction, which is a prerequisite for subsequent nitrous oxide reduction and *nosZ* gene expression by providing nitrous oxide as a substrate. Therefore, the high expression level of *norB* in the STA group might have resulted in the high amount of available nitrous oxide; however, the lower expression of *nosZ*, which is responsible for the reduction of nitrous oxide, may have led to incomplete denitrification. These changes are consistent with nitrate accumulation in the STA group.

In summary, all regulatory factors, including T, DO, and pH, were relatively consistent (Table S3), with the exception of carbohydrates. Thus, carbohydrate addition represented the primary factor influencing the differences in bacterial diversity.

3.3. Correlation-based ecological network analysis

In this study, 56 nodes and 531 links were produced in the visual network structure, in which 54.24% of the links between each node were positively correlated (red edge) and 45.76% were negatively correlated (green edge; Fig. 4). Positive connections in the correlation network generally indicated shared functions and associations, while negative connections reflected regulatory and inhibitory interactions (Yang et al., 2014). The two most related phyla were Proteobacteria and Firmicutes (Table S4), which accounted for the giant components (i.e., a connected component of a network that contains a significant proportion of the entire nodes in the network) in the overall network (Fig. 4). Most of the functional bacteria responsible for nitrification and denitrification were distributed in these two phyla. For example, *Rhodobacter* and *Paracoccus* belonged to Proteobacteria, while *Bacillus, Carnobacterium*, and *Staphylococcus* belonged to Firmicutes; all of these organisms represent important nitrification and denitrification genera.

To verify the characteristics of the network, an Erdös-Réyni random network was visualized (Fig. S2), which was established and compared with the existing experimental network. In this network, the observed APL (1.38), CC (0.94), and modularity (MD) (3.38; values >0.4) (Newman, 2006) were all greater than the APLr (1.65), CCr (0.34), and MDr (0.31), which were the respective Erdös-Réyni random networks (Table 2). The CC/CCr ratio was 2.76, which indicated the 'small world' characteristic of the experimental network (Ju et al., 2014; Zhou et al., 2019).

Overall, the bacterial genera in this experiment had a stronger co-occurrence pattern (30.11%) than random pattern (26.93%), and their degree of intra-phylum co-occurrence varied (Table S4). The different co-occurrence patterns of phyla in the experimental network (O) and random network (R_{ER}/R_{Theo}) were significant for exploring the non-random assembly patterns in microecology systems (Barberán et al., 2012). The phyla (Firmicutes-affiliated nodes are blue) showed a co-occurring incidence of 2.82%, while that of the random model was 1.88% (Table S4). Firmicutes is a highly diverse phylum that is also ubiquitous in AS (Ju et al., 2014). The co-occurrence incidence of Proteobacteria (23.16%) was slightly lower than the theoretical incidence determined by the Erdös-Réyni random network model (27.12%), and its members were widely distributed in different modules, which may indicate that carbon supplementation limits the co-occurrence pattern

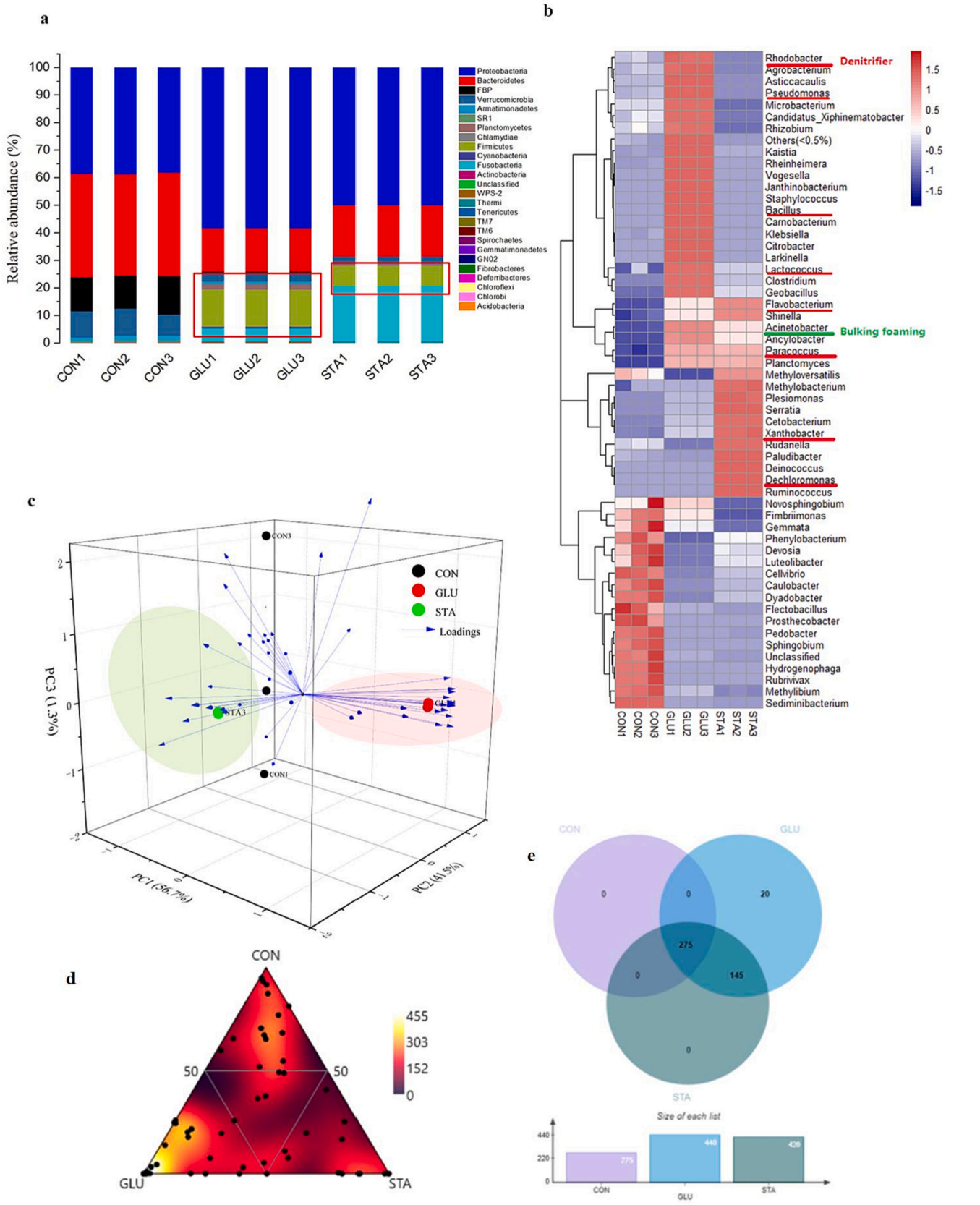

Fig. 2. Relative abundance of a) phyla and b) genera detected in the three treatments; c) principal component analysis (PCA) of genera in the three treatments according to the variance-covariance matrix; d) ternary graph; and e) Venn diagram.

Table 1
Number of OTUs and alpha-diversity indices of water or floc in the three treatments.

Sample name		OTUs (3% cut-off)	Chao	Ace	Shannon	Simpson
CON	water_A	226	257.54	259.16	3.29	0.08
	water_B	231	278.04	280.30	3.36	0.08
	water_C	224	273.79	262.98	3.38	0.08
GLU	glucose_flocA	438	728.70	795.85	3.89	0.04
	glucose_flocB	440	738.38	806.84	3.89	0.04
	glucose_flocC	440	738.38	806.28	3.89	0.04
STA	starch_flocA	419	686.69	713.11	3.48	0.08
	starch_flocB	419	686.69	710.43	3.48	0.08
	starch_flocC	418	682	701.30	3.48	0.08

Note: CON: control group, GLU: glucose group, STA: starch group.

within Proteobacteria. This finding also suggests that carbohydrate regulation interfered with the bacterial assembly in the three treatments. Barberán et al. (2012) revealed that a significant co-occurrence rate of operational taxonomical units (OTUs) from the same phylum was much higher in a soil microorganism network than in random expectations. Ju et al. (2014) found that Alphaproteobacteria in AS had a higher co-occurring incidence than the corresponding Erdös-Réyni random theoretical value. These co-occurring trends in the same phylum can be explained by the influence of the bacterial assembly. For example, taxonomically closely related bacterial genera are also closely related ecologically, thus reflecting their common niche preference or synergistic relationship. In macro-organisms, such as plants (Losos, 2008), this ecological characteristic explains the importance of habitat filtering on bacterial assembly. In this study, the different regulatory factors, such as carbohydrates, would provide the role of habitat filtering and thus impact the bacterial co-occurrence patterns.

In addition to the high co-occurrence occasionally observed among different phyla, a non-random pattern was also found between different phyla (inter-phylum differences). A typical example is that members of Firmicutes and Proteobacteria showed a significantly higher than expected inter-phylum co-occurrence incidence (19.58%) compared with the random incidence (R_{theo}, 12.8%). The second highest incidence was found between Bacteroidetes and Proteobacteria (12.24%; Table S4).

In nature, many ecosystems are consistent with the phylogenetic dispersion, such as schoenoid sedge and mammals (Cooper et al., 2008; Slingsby and Verboom, 2006), suggesting that these complicated interactions among bacteria influence particular environmental conditions. This is a very interesting result that connects the bacterial communities in the micro-world of a biofloc with the macroecosystem.

To elucidate the relationship between the microbial interaction groups and environmental variables, modularity separation for microbial co-occurrence was conducted (Newman, 2006). Five modules that primarily constituted the ecological network were illustrated (Fig. 4b). These modules were composed of various bacterial phyla (Table S5), suggesting that the microbial phylum interactions occurred concurrently among different functional genera. Firmicutes represents an important phylum associated with the nitrogen removal process and was observed primarily in module 3 (Fig. 4b and Table S5). This finding highlights that microbial associations tended to be responsible for the biochemical function of nitrogen transfer under the addition of glucose or starch.

Lactococcus, Geobacillus, and *Clostridium* belong to the phylum Firmicutes and were associated with module 3, which was connected closely with modules 0 and 4. In addition, three modules (0, 3 and 4) formed the giant component in the entire network. *Ruminococcus* was the only Firmicutes genera in module 2. Modules 1 and 2 were connected, although they were disassociated from the other modules. This finding suggested that this genus, which presented a low abundance and fragile relationship with the largest functional group, may play a satellite role in terms of overall function (Hanski, 1982; Van Der Gast et al., 2011). This result provides a hypothesis that could be extrapolated in future research and has important theoretical significance for clarifying the functional species of the BFT ecosystem.

3.4. Host gut microbial composition, growth, and immune gene expression

The microbial community composition of the intestinal tract at the phylum level is presented in Fig. 5a. The average relative abundance of Proteobacteria in the fish gut was 38.89%, 31.64%, and 66.86% in the CON, GLU, and STA treatments, respectively. The proportion of Fusobacteria in the three treatments was 13.51%, 42.03%, and 29.96%,

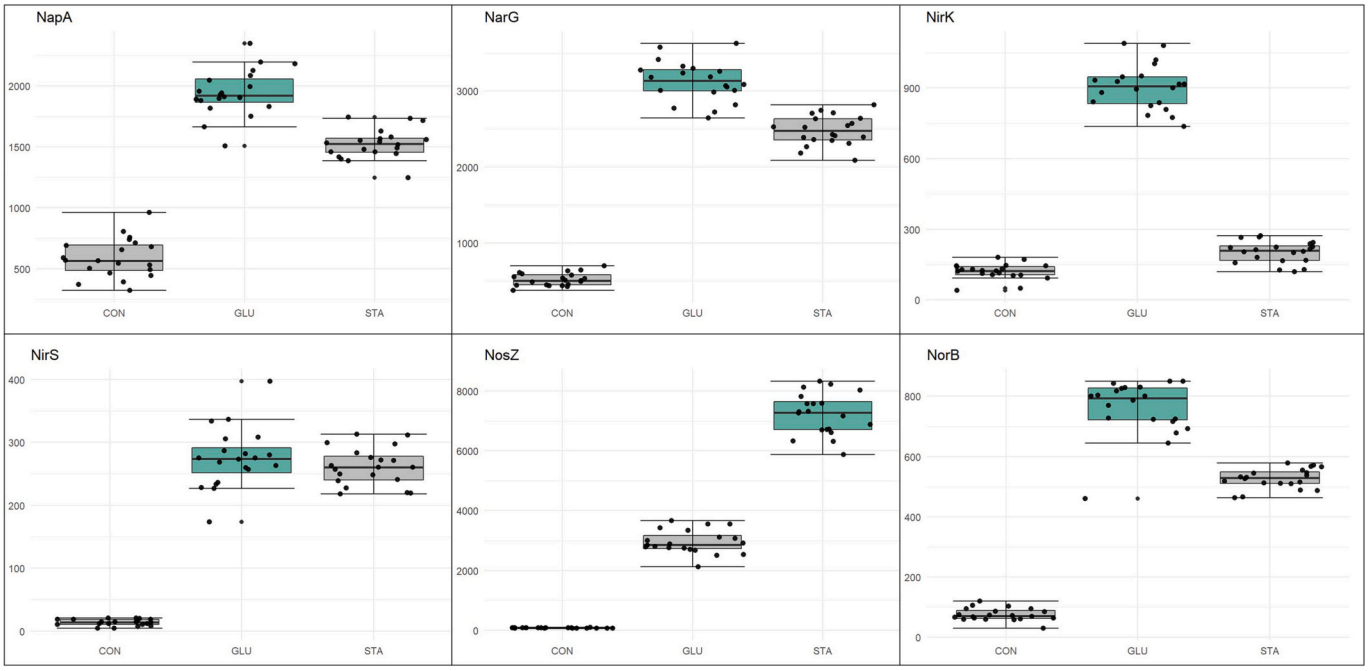

Fig. 3. Denitrification function gene expression (unit: copies uL^{-1}). Extremely significant differences were observed in the GLU group compared to the other groups ($P < 0.001$).

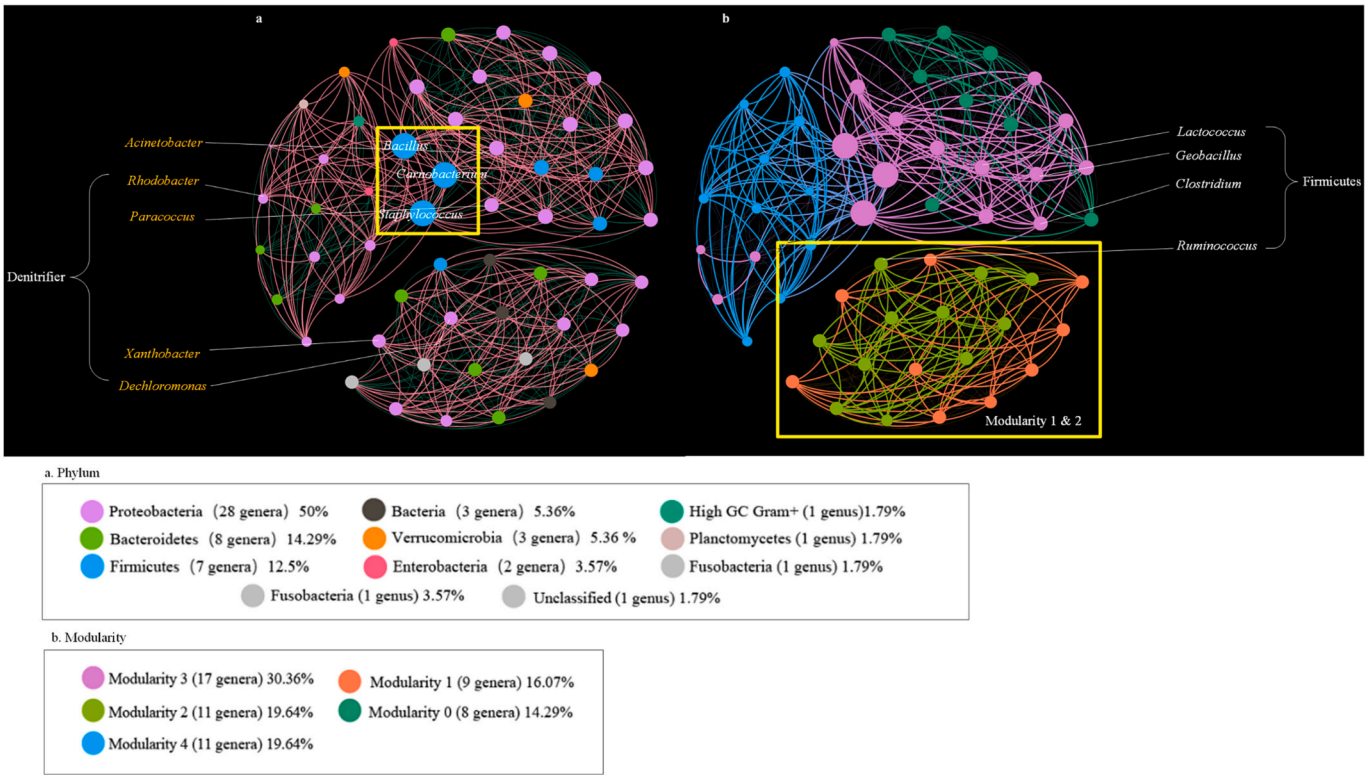

Fig. 4. Network analysis of the 56 genera in the water samples of the three treatments. Only the results after filtering the connection are shown, and they were fit based on Spearman's $\rho > 0.6$ and *P*-value <0.01. The size of each node is proportional to the number of connections (i.e., degree) and colored by the phylum. Red edge, positive correlation; green edge, negative correlation. Colored based on the a) phylum and b) module. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Topological parameters of the experimental networks of floc.

	Modularity (MD)	Clustering coefficient (CC)	Average path length (APL)	Network diameter (ND)	Average degree (AD)	Graph density (GD)
whole experimental network (56 nodes, 531 edges)	3.38	0.94	1.38	3	18.96	0.34
The corresponding random network[a]	0.31 ± 0.01	0.34 ± 0.007	1.65 ± 0.0007	2.57 ± 0.49	18.96	0.34

[a] the average value of Erdös-Réyni random model after 10,000 times calculation.

while that of Firmicutes was 6.15%, 60.20%, and 1.59%, respectively. In a previous study of BFT systems based on a biodegradable polymer carbohydrate, the dominant phyla of the fish gut were also Proteobacteria, Bacteroidetes, Firmicutes, and Fusobacteria (Liu et al., 2019). These results indicated that the GLU group had a different gut microbial colonization, which could be influenced by the surrounding aquatic environment (Liu et al., 2019). The gut bacterial composition differed overtly from the corresponding biofloc bacterial composition in this study, indicating that gut has a certain regulatory effect on the biofloc microorganisms entering the intestinal tract. However, relatively higher abundance levels of Firmicutes were observed in the GLU groups (Fig. 5a), which is consistent with the environmental samples, which had higher Firmicutes abundance and lower Bacteroidetes abundance (Fig. 2a). Firmicutes are a classical probiotic phylum that includes *Bacillus*, *Lactobacillus*, *Bifidobacterium*, etc. that corresponds to better growth performance in the GLU group.

In a previous study of the fish gut, the Firmicutes/Bacteroidetes (F/B) ratio in the gut was used as an index for host body weight (Kan et al., 2015; Liu et al., 2019). In the present study, the CON treatment clearly had the lowest F/B ratio of the three treatments. In a previous challenge test, a reduction in the F/B ratio within *Carassius auratus* gut in an aquatic environment with pentachlorophenol (PCP) possibly corresponded to a decrease in fish body weight (Kan et al., 2015). The fish in the CON treatment had the lowest final body weight (Table 3), which is consistent with this hypothesis.

A reciprocal interaction exists between the gut microbiota and the immune system (Kehrmann et al., 2020). Regulatory secretion of antimicrobial peptides, such as lysozymes, directly affect the microbiota in the intestinal lumen (Ayabe et al., 2004). In the present study, the expression profiles of the three immune-related genes exhibited an identical pattern, and the value in the GLU group was significantly higher than that in the other two groups ($P < 0.001$; Fig. 5b). This finding is reasonable given that the health status responded to the higher growth performance (Liu et al., 2019).

In short, this experiment studies BFT systems as a micro-ecosystem. Under different carbohydrate regulation, the bacterial distribution was not random but rather showed different co-occurrence patterns, thus resulting in heterogenous nitrogen transformation and host growth performance.

4. Conclusion

This study explored the microbial co-occurrence characteristics in biofloc by network analysis and the inorganic nitrogen removal

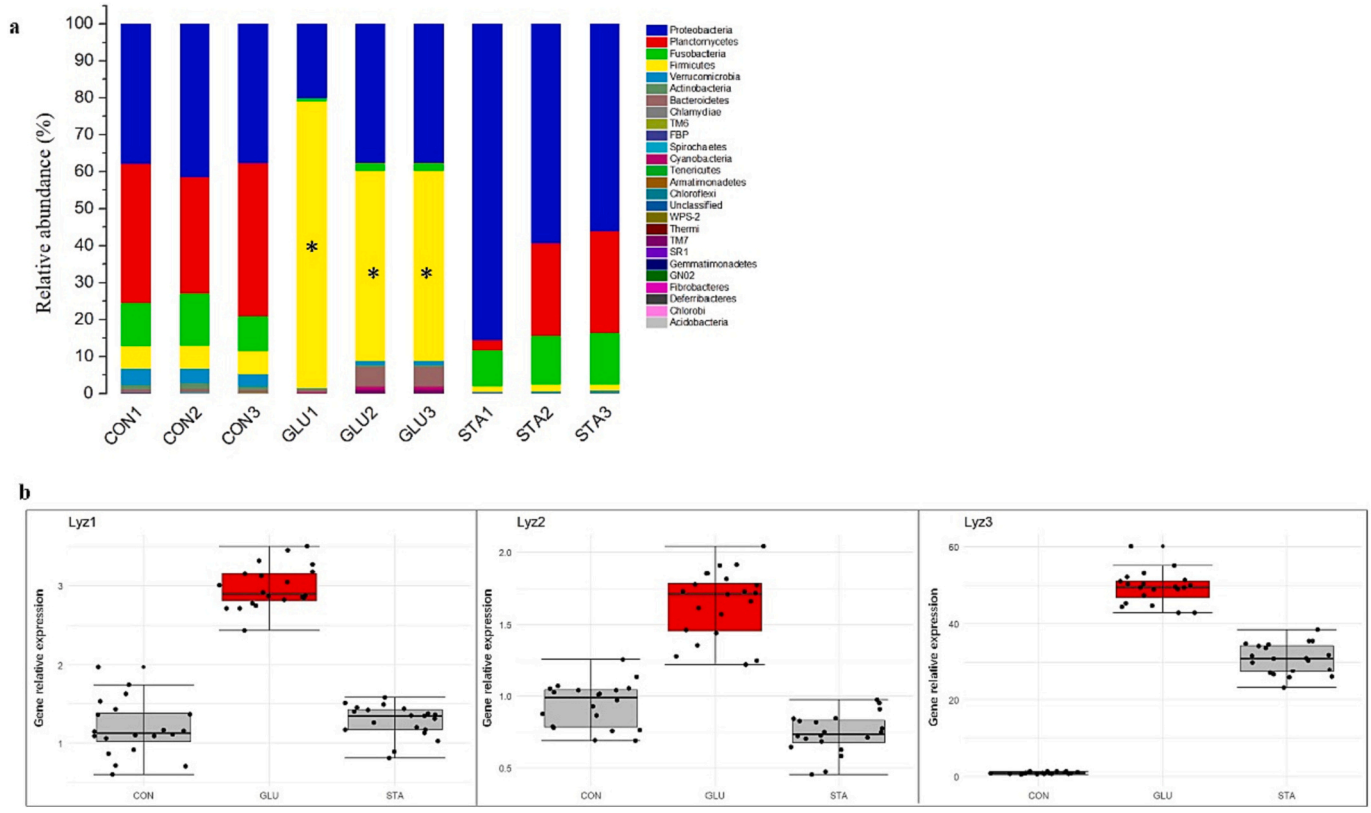

Fig. 5. a) Bacterial composition at the phylum level in the fish hind gut, and b) relative expression of immunological genes among the fish gut microorganisms. The values in the GLU group are significantly higher compared with that of the other groups ($P < 0.001$).

Table 3
Growth parameters of the reared fish in different experimental groups during the 60-day experiment.

	CON	GLU	STA	Related reference
IBW	15.25 ± 1.11			
FBW	29.50 ± 3.03[a]	32.51 ± 1.93[b]	30.67 ± 1.4[ab]	
DWG	0.18 ± 0.04[a]	0.22 ± 0[b]	0.19 ± 0.01[a]	
SGR	0.83 ± 0.12[a]	0.95 ± 0.01[b]	0.87 ± 0.04[ab]	2.13 ± 0.29[1]; 2.18–2.33[2]
FCR	1.3 ± 0.05[a]	1.16 ± 0.03[b]	1.28 ± 0.02[a]	3.51–4.97[3]; 1.9[4]; 0.78–0.92[1]; 1.20–1.47[2]

Each value represents mean ± S.D. Means in the same row with different superscripts are significantly different at $P < 0.05$.
[1](Luo et al., 2014), [2](Zhang et al., 2016), [3](Azim et al., 2008), [4](Crab et al., 2009).

efficiency of different carbohydrates. In addition, the biofloc in situ could impact gut microbial community structure distribution and in turn distinct growth and immune status in BFT systems. The findings of the study may facilitate the use of genomic and transcriptomic experimental designs in future. Indeed, the results have prompted the authors to further investigate the interactions of microorganisms within BFT systems with the goal of revealing novel unreported pathways of nitrogen metabolism.

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Author contributions

Gang Liu conceived, planned, designed, and performed the data analyses and the experiments. Songming Zhu, Zhangying Ye and Ying Liu conceptualized, designed, and supervised the study. Gang Liu wrote the manuscript. All authors contributed to the final paper.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

We have submitted the 16S data to NCBI.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2023.739964.

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