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Metagenomic analysis of the gut microbiome of Nile tilapia (*Oreochromis niloticus*) from biofloc and pond aquaculture systems in Kerala, South India

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Abstract

Nile tilapia (*Oreochromis niloticus*) is one of the most commonly cultured fish species in global aquaculture, with production expected to increase further due to its strong culture performance and resistance to various environmental conditions. The gut microbiota significantly affects the host's health and the gut of Nile tilapia harbour a diverse microbial community. An experimental study was conducted to analyse the bacterial composition and functional profile of the gut microbiota of Nile tilapia from two different aquaculture systems, Biofloc and Pond culture systems in Kerala, South India. Bacterial communities in the gut of tilapia collected from two systems were classified down to the genus level using 16S rRNA amplicon sequencing. The most prevalent bacterial phyla in the Pond group were Firmicutes, Proteobacteria and Fusobacteria, while Bacteroidetes, Firmicutes and Proteobacteria dominated the biofloc group. At the genus level, *Streptococcus* and *Pseudomonas* were the most abundant in the Pond group, whereas *Prevotella* and *Blattobacterium* were more prevalent in the Biofloc group. Higher microbial diversity was observed in the gut microbiome of Nile tilapia from the biofloc group. The functional profile of the gut microbiome of Nile tilapia exhibited alterations between the two aquaculture systems. The functional profile of the gut microbiome of Nile tilapia assessed by PiCrust2 revealed that the membrane transport-associated function was predominant in the Pond group and carbohydrate metabolism-associated function in the Biofloc group. The study illustrated how the host environment significantly impacts the composition, diversity and functional profile of the gut microbiome of Nile tilapia.

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Introduction

Aquaculture is the rapidly expanding animal food industry globally and continues its growth to meet the nutritional demands of the increasing human population. Tilapia are the second most widely farmed fish globally, cultivated in more than 100 countries due to their hardiness, rapid growth and adaptability to diverse culture systems. Among them, Nile tilapia (*Oreochromis niloticus*) is the most common species used in aquaculture (Meenakshisundaram *et al.*, 2021; FAO, 2022).

Biofloc technology (BFT) has emerged as an alternative to conventional pond culture, offering both water quality improvement and enhanced nutrition for cultured fish. Unlike normal pond conditions, where excess feed and metabolites accumulate, biofloc systems promote the growth of heterotrophic microbial communities that assimilate nitrogenous wastes, thereby reducing water exchange requirements and improving environmental sustainability. In addition, the microbial flocs serve as a natural protein-rich feed supplement, supporting growth performance and immune responses in fish (Avnimelech, 2009; Emerenciano *et al.,* 2013). Comparative studies have shown that Nile tilapia reared under biofloc systems exhibit improved feed utilisation and resilience to stress compared to those cultured in traditional pond systems (Kuhn *et al.*, 2009).

The gut microbiota plays a crucial role in the overall health and physiological functions, including development, feeding, metabolic activities, growth, health, stress response and immune response of fish species and hence, considered as an extra organ. Gut microbiota composition is influenced by various host factors (host genotype and developmental stage), environmental factors (salinity, season, geographical location, rearing conditions and water quality) and dietary factors (trophic level and dietary constituents) (Johny *et al.*, 2021). These factors could subsequently modify the composition and diversity of the microbiome, along with its function and metabolic activity, which in turn impact the feeding habits, growth, energy storage and overall health of the fish (Butt and Volkoff, 2019). Studies by Navarrete *et al.* (2012) have shown that gut microbiota varied according to the host developmental stage and get adapted to the changing environmental conditions. The gut ecosystem of Nile tilapia harboured a diversified group of microbial populations. The gut microbiota composition of Nile tilapia was assessed through both qualitative and quantitative analysis employing bacterial count techniques (Pakingking *et al.*, 2015; Kaktcham *et al.*, 2017). *Aeromonas hydrophila, Burkholderia cepacia, Chromobacterium violaceum, Aeromonas veronii, Citrobacter freundii, Flavimonas oryzihabitans, Escherichia coli* and *Plesiomonas shigelloides* were the frequently isolated bacterial communities from the gastrointestinal regions of Nile tilapia cultured in the semi-intensive system by the conventional culture-dependent methods (Molinari *et al.*, 2003). As there is advancement in next-generation sequencing technologies, the identification of bacterial communities has become more accessible. The dominant phyla in the gut microbiota of Nile tilapia included *Proteobacteria, Firmicutes, Cyanobacteria, Fusobacteria* and *Actinobacteria* (Bereded *et al.*, 2020). Research into how the environment affects gut microbiota has led to a greater comprehension of what a healthy microbiota looks like in various environmental circumstances (Bereded *et al.*, 2021). Aquatic organisms have a complex and intimate relationship with their external environment. Consequently, any changes in environmental factors could cause variations in fish's gut microbial composition and structure (Tan *et al.*, 2019). The microbial composition in the biofloc of outdoor GIFT Tilapia grow-out culture, as well as the functional attributes of the microbiome in the biofloc system, were investigated in the studies by Meenakshisundaram *et al.* (2021).

This study characterised the gut microbiota of Nile tilapia reared in biofloc and a nearby pond aquaculture system using 16s rRNA amplicon sequencing. We compared the gut microbiota composition, alpha diversity and predicted the functional profiles between the two culture environments hypothesising that gut microbiota structure and function would differ with rearing conditions. Identifying bacterial taxa and predicting their functional profile provides insights for probiotic applications to enhance fish health and growth in aquaculture.

Materials and methods

Sampling sites and fish sampling

The samples were collected in January 2023 from different aquaculture systems: Biofloc and Pond aquaculture systems in the Ernakulam District of Kerala. Table 1 details the specific locations and environmental parameters of the sampling sites.

Five Nile tilapia samples each were obtained from Biofloc and Pond aquaculture systems. On arrival in the laboratory, live fish were anesthetised using higher doses of clove oil. The entire gut was aseptically removed from each fish and stored at -80°C until DNA extraction and sequencing. Each group (Biofloc and Pond) comprised three fish and the gut microbiome analysis was conducted using three distinct biological replicates for each group.

DNA extraction

Microbial DNA extraction from the gut was done using the CTAB/EDTA/Chloroform method, according to McMurtrie *et al.* (2022). The extracted DNA pellets were reconstituted in nuclease-free water and stored at -20^{0}C until used for sequencing.

PCR amplification and Sequencing

Six individual DNA samples underwent amplicon sequencing using the Illumina HiSeq system (2x250bp; Illumina, San Diego, CA). DNA sequencing libraries targeting the V3-V4 hypervariable region of the bacterial 16S rRNA gene were amplified using the Primers: 341F (5'-3'): CCTAYGGGRBGCASCAG and 806R (5'-3'): GGACTACNNGGGTATCTAAT. The KAPA2G™ Robust Hot Start Ready Mix PCR Kit (Kapa Biosystems, Wilmington, MA, USA) was used to conduct PCR amplification of 16S rRNA genes in a total volume of 25 µl. The PCR conditions for amplification were according to Matsuo *et al.* (2021) using the BioRad T-100 PCR instrument. The purification of amplified DNA was done with AMPure® XP (Beckman Coulter) and quantification was done with a NanoDrop® 1000 (Thermo Fischer Scientific, Waltham, MA, USA).

Sequencing data analysis

The raw data quality control (QC) was performed using FASTQC version (0.12.1), followed by adapter and low-quality read trimming using TRIMMAGALORE v. 0.6.5. Paired-end reads were merged and chimeric sequences were removed using the VSEARCH plugin in FROGS to generate operational taxonomic units (OTU) based on an 80% sequence similarity threshold. Taxonomic assignments were made for OTUs using assign-taxonomy from FROGS with SILVA 138 database (SILVA 138 99% OTUs). The feature table, metadata and

Table 1. Location and environmental conditions of the sampling sites

	Biofloc culture system	Pond culture system
Coordinates	9^0 48'21.5"N; 76^0 20'42.5"E	9^0 54'42.5"N; 76^0 18'57.9"E
No. of samples collected	5	5
Weight of fish (g)	50.5 ± 0.351	50.3 ± 0.458
Environmental conditions		
Area (m^2)	20	30
Depth (m)	1.5	1.5
Temperature (^{0}C)	28.2	28.7
pH	7.5	7.2
Salinity (psu)	0.94	0.14
Dissolved oxygen (mg l^{-1})	6.3	6.5
Alkalinity (mg l^{-1})	212.2	136.6
Hardness (mg l^{-1})	148	116
Ammonia (ppm)	0.25	0.20

sequences were brought into Phyloseq to perform alpha diversity calculations, such as Chao1, Shannon, Simpson and ACE and the visualisations were created using ggplot and scales packages within R version 4.2.3. Beta diversity was calculated based on the Bray-Curtis index distance and UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and principal coordinate analysis (PCoA) were performed to visualise differences between sample groups. Permutational multivariate analysis of variance (PERMANOVA) was used to statistically analyse beta diversity. The study used linear discriminant analysis effect size (LEfSe), as described by Dhariwal *et al*. (2017), to identify substantially different levels of bacterial taxa in various samples. ANCOM analysis was also performed to identify taxa that significantly differ in abundance between groups (Mandal *et al.,* 2015). PICRUSt2 analysis was conducted to predict the potential functional profiles of the microbiota using 16S rRNA gene data, following the method described by Langille *et al.* (2013).

Results

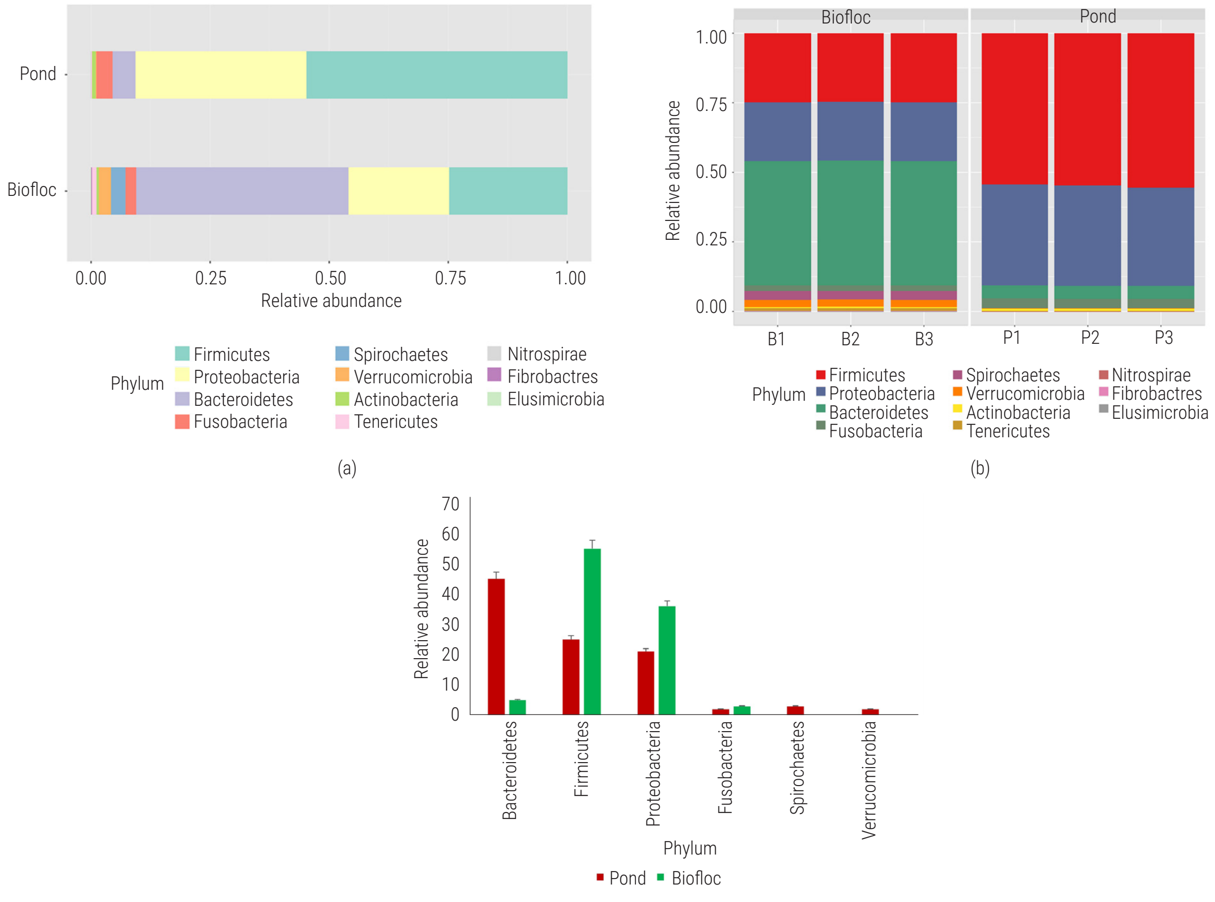
Distribution of bacterial community

The gut microbiota samples of Nile tilapia in Biofloc and Pond culture systems showed the dominance of Firmicutes, Bacteroidetes, Proteobacteria and Fusobacteria at the phylum level. The gut microbiome of Nile tilapia in the Biofloc system harboured a higher percentage of Bacteroidetes (45%), Firmicutes (25%) and Proteobacteria (21%). Fusobacteria, Spirochaetes and Verrucomicrobiota were found in limited abundance. The gut of Nile tilapia from the Pond culture system showed a higher percentage of Firmicutes (55%), Proteobacteria (36%), Bacteroidetes (5%) and Fusobacteria with minimal abundance (Fig. 1a). Bacteroidetes exhibited a significant abundance in the Biofloc group, while the Pond group showed a notable rise in Firmicutes and Proteobacteria (Fig. 1b and c).

At the genus level, *Prevotella* dominated the gut microbiome of Nile tilapia from the Biofloc sample, followed *by Blattabacterium, Oscillibacter, Aeromonas, Tannerella* and *Treponema*. On the other hand, *Streptococcus* and *Pseudomonas* were the dominant genera in the Pond sample (Fig. 2a and b).

Diversity measures

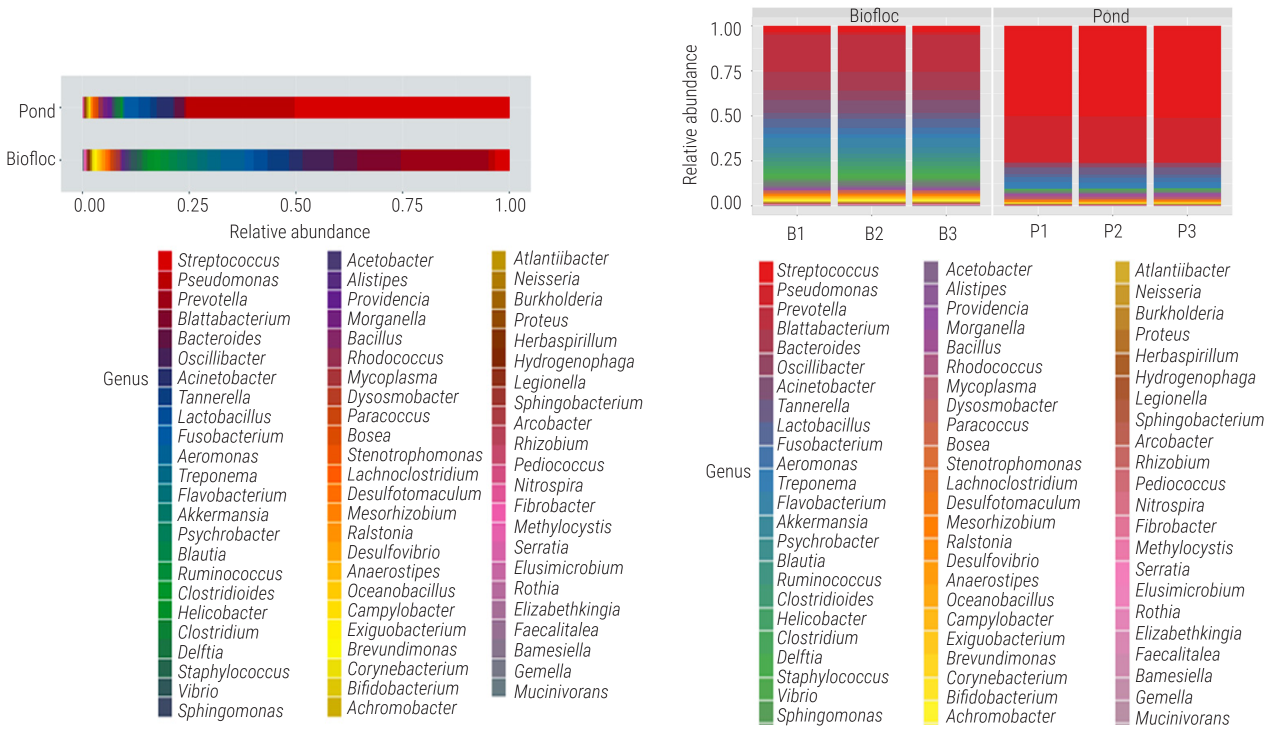
The alpha diversity of the gut microbiota in Nile tilapia from the Pond and the Biofloc culture systems was assessed using Chao1, Shannon, Simpson and Fisher indices. The Biofloc group exhibited higher gut bacterial diversity compared to the Pond samples, with



(a) (b)

(c)

Fig. 1. Gut microbiota composition of Nile tilapia in Biofloc and Pond groups. (a) Abundance of dominant phyla in Biofloc and Pond groups, (b) Abundance of phyla in each sample of Biofloc and Pond groups and (C) Variation in the relative abundance of dominant phyla



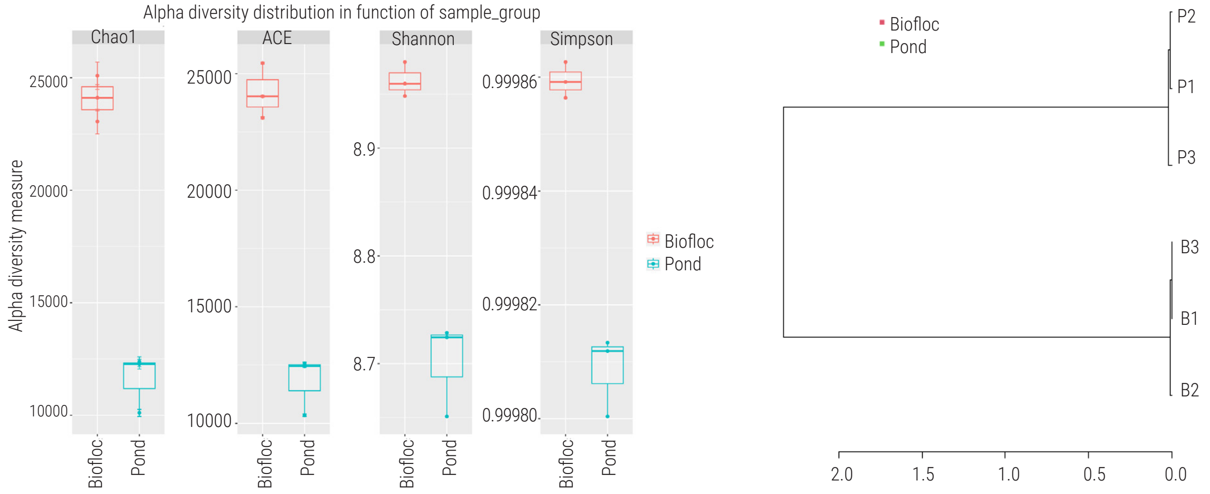
(a) (b)

Fig. 2. Gut microbiota composition of Nile tilapia in Biofloc and Pond groups. (a) Abundance of the dominant genus in Biofloc and Pond groups and (b) Abundance of the genus in each sample

Chao1, Shannon, Simpson and Fisher indices showing significant differrences at ($p<0.05$) (Fig. 3a). Beta diversity analysis using Bray-Curtis distances and PERMANOVA indicated a clear biological distinction between the two groups (F=16484; R2 =0.99976), though the difference was not statistically significant ($p=0.06$). NMDS plot revealed a stress value below 0.1, indicating a good fit, While UPGMA clustering based on unweighted UniFrac distances demonstrated clear separation of the two culture groups (Fig. 3b).

Differential abundance analysis

LEfSe was analysed to assess the variation in bacterial levels within the gut microbiome of Nile tilapia raised in Biofloc and Pond aquaculture systems. The gut microbiota of Nile tilapia in the Biofloc culture system had the dominant bacterial signatures such as *Prevotella*, *Blattabacterium*, *Oscillibacter*, *Aeromonas*, *Tannerella*, *Bacteroides*, *Treponema*, *Akkermansia*, *Psychrobacter*,



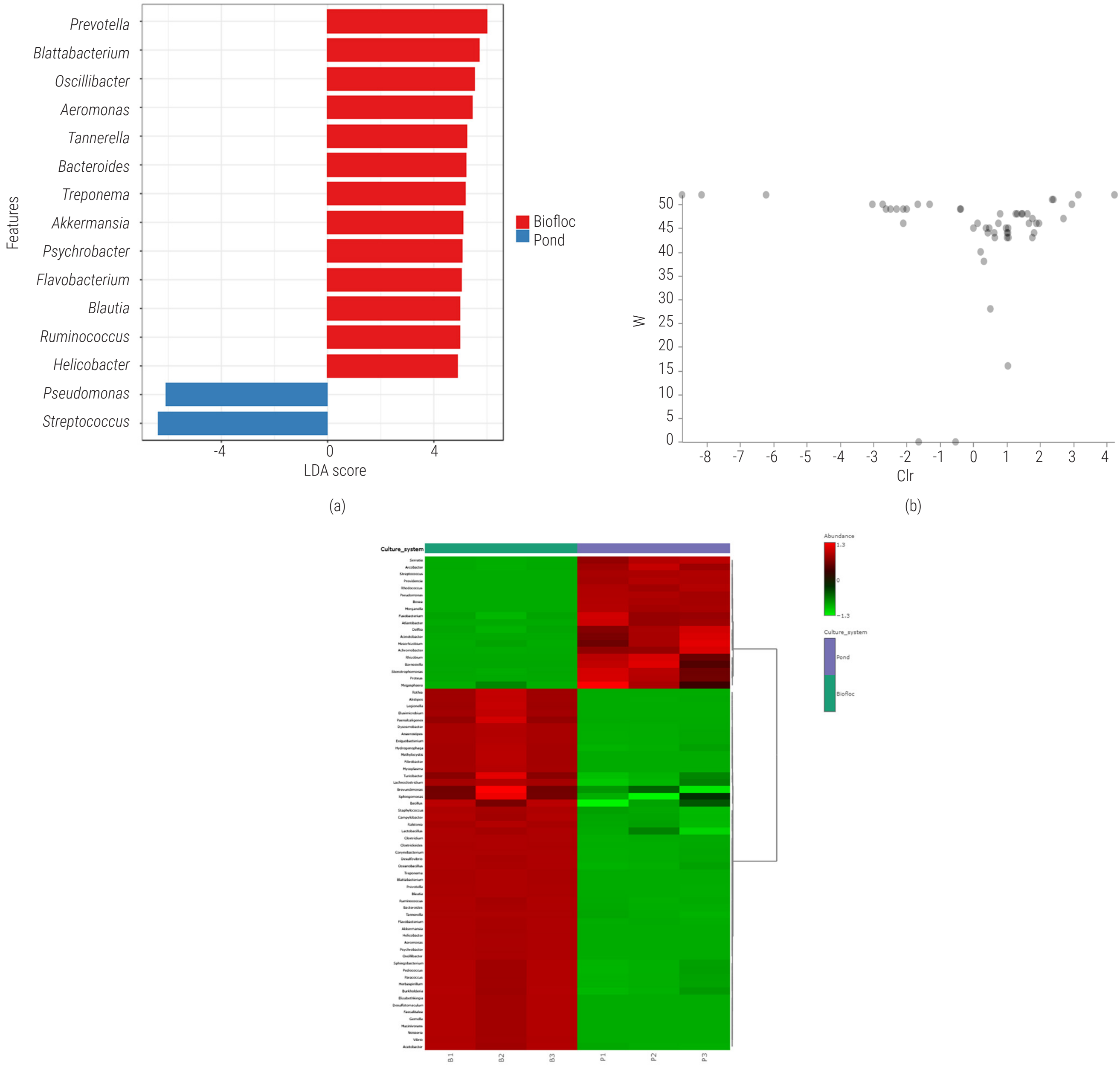
(a) (b)

Fig. 3. (a) Alpha diversity indices of the gut microbiota of Nile Tilapia in Biofloc and Pond groups with significant differences (p-value < 0.05). (b) UPGMA plot of gut microbiota of Nile Tilapia in Biofloc and Pond groups

Flavobacterium, Blautia, Ruminococcus and *Helicobacter*. In contrast, *Streptococcus* and *Pseudomonas* were dominant in the gut microbiota of Nile tilapia in the Pond culture system. (Fig. 4a). The ANCOM analysis produced volcano plots showing a significant and extensive disparity in the microbial populations of the two groups. The abundance of many taxa differed significantly, signalling a noteworthy shift in the microbiome composition (Fig. 4b). A heat map visually displayed the significant difference in the relative abundance of the gut microbiota in two different culture systems at the genus level (Fig. 4c).

Functional analysis

The functional prediction of gut microbiota in Nile tilapia from Biofloc and Pond culture systems was performed using PICRUSt. Analysis of KEGG level 1 pathways revealed significant variations in key categories such as metabolism, cellular process, genetic information processing and environmental information processing between the two culture systems. Within the cellular process category, cellular processes and signaling pathways were upregulated in Nile tilapia from the Biofloc culture system.



(a)

(b)

(c)

Fig. 4. (a) Bacterial genera differentially represented between Biofloc (red) and Pond (blue) groups identified by linear discriminant analysis effect size (LEfSe with LDA score>2, p<0.05). (b) Ancom volcano plot with W and clr values showing the significant difference in the taxa. High W and clr values represent higher significant differences. Points that are close to the top right corner indicate that an ASV is suspected to be truly different across the groups. (c) Heat map showing the abundance of genera and significant variations between the Biofloc and Pond groups

In Genetic Information Processing, fish from the Biofloc system exhibited increased activity in pathways associated with genetic functions such as translation, transcription, replication and repair and folding, sorting and degradation. Environmental Information Processing pathways related to membrane transport and signal transduction were upregulated in Nile tilapia from the Pond culture system. In the metabolism category, bacterial communities in the gut of Nile tilapia from the Pond culture system displayed a higher abundance of amino acid metabolism, lipid metabolism, xenobiotic degradation and metabolism pathways. Conversely, gut microbiota from the Biofloc culture system showed increased activity in pathways related to carbohydrate metabolism, glycan biosynthesis, energy metabolism, nucleotide metabolism and metabolism of cofactors and vitamins, as well as enzyme families at KEGG level 2 (Fig. 5).

Discussion

The bacterial composition in the gut of Nile tilapia of Biofloc and Pond culture system was analysed using 16s rRNA amplicon sequencing. The microbial communities were highly sensitive to the rearing environment (Giatsis *et al.,* 2015). This study examined the gut microbiota of Nile tilapia from two aquaculture systems, Biofloc and Pond and considered potential variations in the culture method. In a previous study by Giatsis *et al.* (2015), it was reported that the bacterial communities would differ among different rearing conditions due to the difference in the aquaculture systems. The gut microbial communities of fish are significantly influenced by factors such as the host's trophic level, habitat and phylogeny (Sullam *et al.,* 2012). Environmental factors, including the water quality parameters of the culture environment, have a crucial role in structuring the bacterial gut composition (Johny *et al.,* 2021). Previous studies have shown that a temperature (28-29°C), dissolved oxygen levels above 4 mg l^{-1} and a pH of 7-8 favoured the growth and activity of heterotrophic bacteria (Summerfelt, 2000; Emerenciano *et al.,* 2017). In our study, the dominant gut microbiota of Nile tilapia from Biofloc and Pond culture systems consisted primarily of Firmicutes, Proteobacteria, Bacteroidetes and Fusobacteria similar to the earlier reports (Giatsis *et al.,* 2015; Ray *et al.,* 2017; Bereded *et al.,* 2021). We observed the abundance of the phylum Bacteroidetes in the gut microbiota of Nile tilapia from Biofloc, followed by Firmicutes and Proteobacteria. Bacteroidetes are responsible for the assimilation of NH4-N, which reduces the ammonia and nitrite build up in the Biofloc system (Cardona *et al.,* 2016). The organic matter in the Biofloc provide necessary support for growth of Bacteroidetes in addition to meeting their nutritional requirements. The gut microbiota of Nile tilapia from the Pond culture system was dominated by Firmicutes and Proteobacteria. Both these groups are involved in the nutrient cycling and organic matter mineralisation (Dworkin, 2006). At the genus level, *Prevotella* was the dominant genus in the gut microbiota of Nile tilapia in Biofloc, followed by *Blattobacterium.* In a study by Precup and Vodnar (2019), the *Prevotella* genus was considered an important biomarker for maintaining balance or indicating disease in humans. *Streptococcus* and *Pseudomonas* were the dominant genus in the gut microbiome of Nile tilapia from the Pond. Similar observations were also made in an earlier study (Wu *et al.,* 2020), which found that *Streptococcus* and *Pseudomonas* dominated the intestinal microbiota of Nile tilapia in Pond culture. These two organisms are the most common opportunistic pathogens of Nile tilapia (Abdel-Razek, 2019; Mu *et al.,* 2019) and disease or increased stress may disrupt the microbial balance in the intestines, causing dysbiosis from multiplication of opportunistic pathogens that would normally be harmless (Li *et al.,* 2016; Tran *et al.,* 2018). Monitoring the density of these opportunistic pathogens in the gut is crucial for assessing the risk of disease occurrences as it has been demonstrated that the increased overall presence of opportunistic pathogens in the gut could be the main reason for fish diseases (Wu *et al.,* 2020). Higher alpha diversity of the gut microbiome of Nile tilapia in the Biofloc compared to that of the Pond group observed in the present study might be due to the opportunistic pathogens like *Streptococcus* and *Pseudomonas* in the gut. Earlier studies have shown that the alpha diversity decreases in fish under dysbiosis conditions (Li *et al.,* 2016; Li *et al.,* 2017; Parshukov *et al.*, 2019; Xiao *et al.,* 2021).

The major KEGG pathways showing alterations in the gut microbiome of Nile tilapia from the two aquaculture systems included metabolism, genetic information processing, cellular processes and environmental information processing. The functional profile of the gut microbiome of Nile tilapia from Biofloc

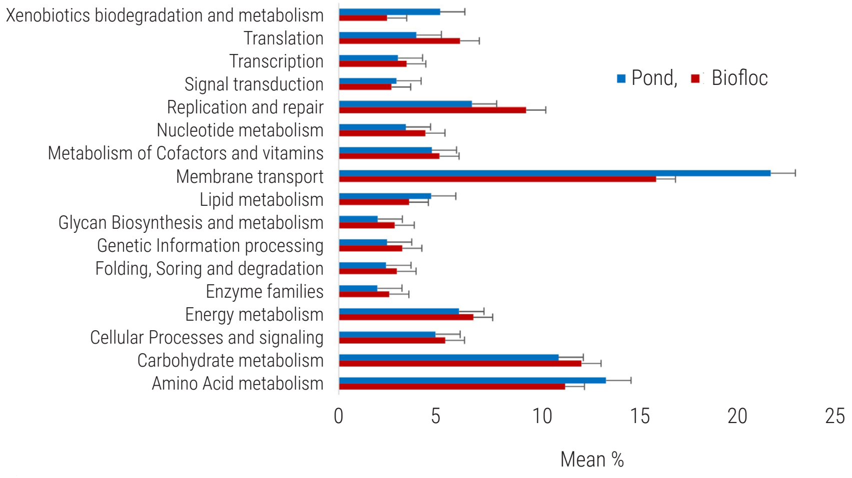


Fig. 5. Predicted functions of gut microbiota of Nile tilapia in Biofloc and Pond groups representing the differences in KEGG pathways at level 2

culture environment revealed an increase in the KEGG pathways, namely translation, transcription, replication and repair and folding, sorting and degradation in genetic information processing, cellular processing and carbohydrate metabolism, glycan biosynthesis and metabolism, energy metabolism, nucleotide metabolism, metabolism of cofactors and vitamins and enzyme families. Similar to the present study, Meenakshisundaram *et al.* (2021) also demonstrated that the genes within the Biofloc played a significant role in carbohydrate metabolism. This was evidenced by the increased proliferation of heterotrophic bacteria due to carbon supplementation. In the pond aquaculture system, the functional profile of the gut microbiome of Nile tilapia showed a higher abundance in membrane transport and signal transduction in environmental information processing and amino acid metabolism, lipid metabolism and xenobiotic degradation and metabolism in the metabolism pathway. This result favourably corresponded with the outcome of an earlier study of Nile tilapia raised in Pond culture, where a functional enrichment was observed in the microbial functions such as membrane transport, carbohydrate metabolism, amino acid metabolism, replication and repair, cellular processes and signalling, translation and signal transduction of the intestinal microbiota (Wu *et al.,* 2020).

The most abundant pathway, membrane transport, was significantly higher in Nile tilapia from the Pond aquaculture system. This might be due to the presence of opportunistic pathogens like *Streptococcus* and *Pseudomonas*. Upregulation of membrane transport of intestinal microbiota of crucian carp and *Penaeus* (*Litopeaneus*) *vannamei* has been reported when they were infected by red operculum disease and WSSV respectively (Li *et al.,* 2017; Wang *et al.,* 2019). A higher abundance of carbohydrate metabolism in Biofloc and amino acid metabolism in Pond culture systems showed the dependence and the presence of carbohydrate and protein-rich diets in the respective systems. Functions of replication and repair, translation, cellular processes and signalling and signal transduction pathways were related to the host immune system modulation and energy metabolism was related to energy provision for digestion and metabolism (Webster, 1980; Parvatiyar *et al.*, 2012; Belkaid and Hand 2014). In our study, the gut microbiome of Nile tilapia in Biofloc was dominated by the pathways of replication and repair, translation, transcription, and cellular processing, which were reported to have an essential role in the modulation of the immune system in tilapia (Wu *et al.,* 2020). These results prove that the performance of Nile tilapia in the Biofloc would be better in terms of strong immune system and disease resistance. This would also support findings that differences in environmental conditions played an important role in shaping the intestinal microbiota of fish (Butt and Volkoff, 2019). Present study revealed that the difference in rearing environment plays an important role in shaping the functional profile of the gut microbiota. The observed divergences in the gut microbiota composition of Nile tilapia from Biofloc and Pond aquaculture systems could be attributed to the differences in the two aquaculture systems, their diet composition, water quality variations and the presence of opportunistic pathogens. Further research needs to be conducted on the functional profile of bacterial communities as well as empirical bacterial composition to predict and prevent disease outbreaks in aquaculture. Identification of opportunistic pathogens among gut microbiota presents a new potential biomarker for assessing the susceptibility to diseases and the ways for development of appropriate probiotics for effective aquaculture management.

In conclusion, this study revealed alterations in the bacterial compositional dynamics, diversity and functional predictions of the gut microbiome of Nile tilapia from two different aquaculture systems (Biofloc and Pond), using 16S rRNA amplicon sequencing. The Biofloc group was dominated by the phylum Bacteroidetes, whereas the Pond group showed dominance of the phyla Proteobacteria and Firmicutes. At the genus level, *Prevotella* and *Blattobacterium* were predominant in the Biofloc group, while *Streptococcus* and *Pseudomonas* were prevalent in the Pond group. The assessment of the gut microbiome functional profile of Nile tilapia using PiCrust2 showed that membrane transport-associated functions were predominantly observed in the Pond group. In contrast, carbohydrate metabolism-associated functions were more prevalent in the Biofloc group. Examining opportunistic pathogens might offer a new potential biomarker for assessing the risk of fish diseases and developing probiotics for effective aquaculture management. The results suggest that the culture environment significantly influences the composition, species richness, evenness and functional profile of the gut microbiota in Nile tilapia (*O. niloticus*).

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