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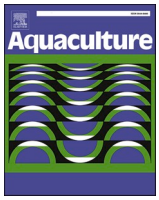

Prokaryotic and eukaryotic microbial community dynamics in biofloc systems supplemented with non-starch polysaccharides

Apriana Vinasyiam [a,b], Marc C.J. Verdegem [a], Julie Ekasari [b], Johan W. Schrama [a], Fotini Kokou [a,*]

[a] Aquaculture and Fisheries Group, Wageningen Institute of Animal Sciences, Wageningen University, the Netherlands
[b] Department of Aquaculture, Faculty of Fisheries and Marine Science, IPB University, Indonesia

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ABSTRACT

Biofloc technology has been developed as a sustainable system in aquaculture, which uses microbial processes to remove nutrients from the water and convert them into microbial biomass. For optimal performance, the addition of carbohydrates is essential, while their type can regulate microbial activity and composition. Specifically, we hypothesize that dietary supplementation of wheat bran, containing indigestible carbohydrates such as non-starch polysaccharides, can provide complex substrates supporting diverse microbial communities within the biofloc. In this study, we investigated how higher dietary wheat bran input affected the biofloc prokaryotic and eukaryotic microbial communities in Pacific white shrimp (*Litopenaeus vannamei*) culture. Two pelleted diets were made: a control diet (CONdiet) with a composition similar to a commercial shrimp diet, and a diet rich in non-starch polysaccharides (WBdiet), which was created by adding wheat bran to the control diet ingredient mix, before pelleting. These two diets were fed isonitrogenous to the shrimp for 42 days, and sampling of the biofloc was performed every three weeks. The results showed shifts in both the alpha and beta diversity in biofloc during the experiment. Diet had a significant effect on the prokaryotic community composition in the biofloc at the end of the culture period, with several genera being enriched in biofloc tanks fed the WBdiet such as *Muricauda*, *Pirellula*, and *Cyanobacteriaceae*. Regarding the eukaryotic communities, overall, only a few taxa were significantly affected within the WBdiet, belonging to the *Trebouxiophyceae* and *Suillus* groups. Interestingly, when feeding the WBdiet, the biofloc microbial communities exhibited predicted functionalities that were more abundant in carbohydrate metabolism, and specifically related to pentose, fructose, mannose and galactose metabolism. These results provide a basis for the control of biofloc microbial communities by using ingredients rich in plant-derived non-starch polysaccharides which shrimp cannot digest but are good energy source for the microbiota in the biofloc.

1. Introduction

Biofloc technology (BFT) has been developed as an efficient, eco-friendly, and sustainable aquaculture system (De Schryver et al., 2008; Emerenciano et al., 2017). The principle of BFT involves the recycling and conversion of waste excreted by the culture animals into microbial biomass. Biofloc are aggregates of suspended particles and microorganisms held together by extracellular polymeric substances (EPS) produced *in situ* by the microbiota (Bossier and Ekasari, 2017). Biofloc form a complex system in which various nitrogen metabolic pathways are present; photoautotrophic uptake by algae, heterotrophic and autotrophic bacterial respiration and assimilation, as well as fungal assimilation (Ebeling et al., 2006; Xu et al., 2016). So far, studies about the microbiota in biofloc systems mostly focused on the prokaryotic group, since heterotrophic bacteria play an important role in the biofloc system (Addo et al., 2021; Cardona et al., 2016; Chakrapani et al., 2021; El-Husseiny et al., 2018; Ferreira et al., 2021; Guo et al., 2022; Jiang et al., 2020; Panigrahi et al., 2018; Tinh et al., 2021; Xu et al., 2022; Zhu et al., 2022). Studies investigating eukaryotic communities, mainly focused on the composition of microalgae communities and their abundance as indicated by the chlorophyll-a concentration (Chakrapani et al., 2021; Jiang et al., 2020; Kabir et al., 2020; Rajkumar et al., 2016;

* Corresponding author at: Aquaculture and Fisheries Group, Wageningen Institute of Animal Sciences, Wageningen University, Zodiac Building, De Elst 1, 6708 WD Wageningen, the Netherlands.
E-mail address: fotini.kokou@wur.nl (F. Kokou).

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Tinh et al., 2021). Despite their importance on biofloc functionality, the number of studies looking at eukaryotic community taxonomic composition is much lower compared to the ones focusing on the prokaryotic communities, while for the fungal community in biofloc, studies are nearly non-existent.

It is well established that the microbial composition in the biofloc can be steered by increasing the carbon (C) to nitrogen (N) ratio by adding organic carbon to the culture system (Addo et al., 2021; Deng et al., 2018; Panigrahi et al., 2018). Not only the quantity but also the types and complexity levels of carbon sources can affect the microbial community composition and functionality (Chakrapani et al., 2021; El-Husseiny et al., 2018; Kabir et al., 2020; Serra et al., 2015). Typically, carbon addition following feeding is the driving force in biofloc management which enters usually in the form of rich in carbohydrate (CHO) sources such as starch, molasses, or cassava; however, such conventional application is labour-intensive under farming conditions, as the right quantity, quality and timing of carbohydrates addition require daily attention from the farmer (Tinh et al., 2021). A proposed solution is to incorporate ingredients in the diet rich in carbohydrates which are less digestible by the animals such as non-starch polysaccharides (NSP), thus resulting in NSP-rich and carbon-rich faeces, ending up in the system, providing a carbon and energy source to the biofloc microbiota. In turn, the biofloc can maintain the water quality and provide an additional feed source for the culture organisms (Hargreaves, 2013; Rajkumar et al., 2016; Ray et al., 2017). Wheat bran is a carbon source commonly used as a feed ingredient for shrimp, containing 56% NSP (CVB, 2022). Therefore, we hypothesize that dietary wheat bran addition will increase the carbon to nitrogen (C:N) ratio of the shrimp faeces, providing extra energy to the microbiota in the biofloc.

Opposing the conventional practice of biofloc management which is providing the carbon supply directly to the culture system, in this study, we aimed to incorporate the extra CHO as input to the pelleted diet. The experiment investigated the effect of a high dietary wheat bran input to a biofloc system for Pacific white shrimp, *Litopenaeus vannamei,* on the prokaryotic and eukaryotic microbial community composition, and how its composition evolved. Furthermore, we also looked specifically at the fungal community, considering that fungi contribute significantly to the metabolic processes in biofloc (Grossart et al., 2019). Our study outcomes on eukaryotes and fungal communities shed novel insights on their complementary roles in biofloc systems besides the prokaryotic community, which has been so far mostly studied.

2. Materials and methods

2.1. Experimental design

Two pelleted diets were prepared: a control diet (CONdiet) with a composition similar to a commercial shrimp diet, and a diet rich in non-starch polysaccharides (WBdiet), which was created by adding wheat bran to the CONdiet mix, before pelleting. To meet the nutritional needs of Pacific white shrimp, the concentrations of essential amino acids, monocalcium phosphate, chalk, cholesterol, vitamin, premix, and yttrium in both diets were kept constant (Table S1). The additional carbon source in this study, wheat bran, was included in the diet rather than administered separately as in the conventional approach of biofloc management. This resulted in a higher amount of carbon input in the WBdiet tank, while the protein amount was comparable (isonitrogenous feeding). The experiment used 1000-L indoor mesocosm tanks as a biological unit, equipped with a 600-watt Schego stick heater, an aeration ring placed at the bottom of the tanks, an aeration stone hanging in the center of the tanks, and a net cover (mesh size ~1,5 × 1,5 cm). To mimic the sunlight and to stimulate the photosynthesis of algae, a pair of lights were set in between 2 tanks, namely Light Emitting Plasma Grow (LEP, Gavita Pro 270e), High-Intensity Discharge (HID) High-pressure Sodium (HPS) bulbs (400 W) with reflector (Gavita HortiStar 600 SE EU, 96% efficiency). To compare the two diets, three replicate tanks were used per diet. The study was carried out in the Carus Animal Research Facilities of Wageningen University and Research (WUR).

2.2. Biofloc and shrimp stocking

The experiment started with mature biofloc in order to prevent water quality from deteriorating if no CHO was added, as in the case of the CONdiet group. It has been reported in previous studies that once the biofloc system is mature (30–50 days), its functionality will not be affected by no CHO addition for a few weeks (Martins et al., 2020; Samocha et al., 2007; Xu et al., 2016). A volume of 450-L biofloc inoculum water from the previous culture cycle was mixed with both freshwater and saltwater water until reaching a volume of 750 L and a salinity of 23 ± 1.0 ppt. The biofloc water was continuously aerated before and after the water stocking into six mesocosm tanks. Pacific white shrimp (0.05 ± 0.02 g/ind) were stocked at a density of 100 individuals per tank the next day. The shrimp were supplied by Crevetec in Ternat, Belgium.

2.3. Shrimp culture and system maintenance

Mechanical belt feeders were used to feed shrimp continuously for 10–12 h per day starting at 16.00. The daily feeding rate in the WBdiet gradually decreased from 11.9 to 3.3% BW, assuming the FCR increased from 0.6 to 1.1 throughout the experiment. The diets were fed isonitrogenous, assuring that shrimp in each diet got the same amount of protein.

Each tank received freshwater weekly to compensate for the evaporation and water droplet loss from aeration (84 ± 7 L / week). Water quality was monitored daily and maintained to reach temperature range of 27.2 ± 0.6 °C, DO 7.0 ± 0.1 mg O_2 L^{-1}, pH 8.**0**, salinity 23.2 ± 1.0 ppt and conductivity 36.7 ± 1.5 μS cm^{-1}, measured using a multi-parameter electronic meter (WTW Multi3630IDSTM). Furthermore, the concentrations of ammonium (NH_4^+, mg/L) and nitrite (NO_2^-, mg/L) were measured using MColortestTM, EMD Millipore, while the concentration of nitrate (NO_3^-, mg/L) was checked using MQuant®, EMD Millipore).

2.4. Sample collection and preparation

Biofloc was sampled on day 1, 21 and 42. Water containing biofloc was sampled from the center of each tank using a 2-L jar for 10 times. A total volume of 20 L of shrimp culture water was then pooled in a 50 L plastic bucket. Water was then mixed using an IKA T-25 Ultra-Turrax dispersing instrument at a speed of 300 rpm for 30 s. A 2-L of stirred water was then transferred to four 2-L jars.

To collect biofloc, a total of 50-ml biofloc water was filtered through a 0.45 μm pore-size filter using a vacuum pump and a glass filtration chamber (Tinh et al., 2021). Prior to the filtration, water containing biofloc in the jar was continually mixed with a magnetic stirrer at 300 rpm (Heidolph Mr. Hei-Mix L, Heidolph Instruments, Germany), to ensure a homogenous biofloc sample across filtration batches. After the filtration, twice 50-ml demi water was allowed to pass the filter to rinse the remaining salt. The filters containing biofloc particles were folded and stored in a sterile tube. The samples were then temporarily stored in the liquid nitrogen, before being stored at -80 °C.

2.5. Biofloc microbial DNA extraction

For microbiota analysis, DNA was extracted from the biofloc samples. The samples were extracted using the DNeasy PowerSoil kit (Qiagen, Valencia, CA) according to Tihn et al. (Tinh et al., 2021). The harvested DNA was quantified using the Nanodrop spectrophotometer. Sequencing of the PCR-amplified V4 region of the 16S rRNA (prokaryotic microbial communities), using primers 515 F (GTGCCAGCMGCCGCGGTAA) and 806 R

(GGACTACHVGGGTWTCTAAT), of the 18S SSU (eukaryotic microbial communities), using primers 3NDf-CS1F (GGCAAGTCTGGTGCCAG) and V4-Euk-CS2R (ACGGTATCTRATCRTCTTCG), and of the ITS (eukaryotic fungal communities), using primers ITS1F (CTTGGTCATTTAGAGGAAGTAA) and ITS2 (GCTGCGTTCTTCATCGATGC), was performed using a MiSeq PE300 Next Generation system (Illumina) by Genome Quebec, following the company's protocol. Sequencing data can be found at the NCBI (SRA) database under the study accession code PRJNA953186.

2.6. Microbial communities data analysis

For the microbial community analysis, an open-source software package, DADA2 (Callahan et al., 2016), was applied to model and correct Illumina-sequenced amplicon errors. Data were demultiplexed into forward and reverse reads according to the barcode sequence into sample identity, and trimming was performed, according to Kokou et al. (Kokou et al., 2020). DADA2 resolves differences at the single-nucleotide level and the end product is an amplicon sequence variant table, recording the number of times each exact amplicon sequence variant (ASV) was observed in each sample (100% sequence identity). Taxonomy was assigned using the *assignTaxonomy* function of DADA2 package, which provides a native implementation of the naive Bayesian classifier method for this purpose (Wang et al., 2007). Silva reference databases against the 16S gene [138 version; (McLaren, 2020)] for the prokaryotes and the 18S gene [128 version; (Glöckner et al., 2017; Morien and Parfrey, 2018)] for the eukaryotes were used, while for the fungi the UNITE ITS database was used (Põlme et al., 2020). Owing to the variation in sequence depths between samples, all samples were normalized to the lowest depth by subsampling (18,052 reads for 16S; 14,080 for the 18S; and 2000 for the ITS).

For the alpha-diversity analysis, richness (observed taxa) and Shannon H′ diversity were calculated for all microbial communities using *microbiome* package (Lahti et al., 2017). Non-parametric tests (Wilcoxon test) and linear mixed-effect models (*nlme* R package, (Pinheiro et al., 2012)] were used to assess alpha diversity. Non-parametric permutational multivariate analysis of variance [PERMANOVA; (Anderson, 2001)] was used to assess beta diversity, along with Principal coordinate analysis (PCoA) using Bray Curtis as a similarity metric for visualization. To investigate how the variation in the microbial community on day 42 is explained by the biofloc composition, distance-based redundancy analysis (dbRDA) was performed (Legendre and Anderson, 1999), using PRIMER 6.0 software (ecological research application for Windows OS by Informer Technologies). For the analysis, the relative abundance of ASVs was transformed using square root and biofloc composition variables were added as explanatory variables for the distance-based analysis, such as total carbon (TC); Biological Oxygen Demand (BOD); Carbon to Nitrogen ratio (C:N); Microbial Activity (MicAct+); Chlorophyll in the water, (ChlWat); Chlorophyll in the biofloc (ChlBiof); Total Nitrogen (TN); Crude Protein (CP) composition of the biofloc. Moreover, ASVs which were differentially enriched between diets were detected using linear discriminant analysis effect size (LEfSe tool) (Segata et al., 2011). To examine the unique and shared ASVs of prokaryotic, eukaryotic, or fungi, we defined the presence of an ASV in the diet group when its prevalence was 100%, meaning that it is present in all three tanks per diet. Core genera were defined as taxa that had higher than 95% prevalence and >1% in relative abundance.

To predict the functional content of the biofloc prokaryotes, we used the PICRUSt2 tool (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; (Douglas et al., 2020). The unique amplicon sequences were aligned to available reference sequences and the Nearest Sequenced Taxon Index (NSTI) score was used to evaluate the availability of reference genomes that are closely related to the most abundant microorganisms in the samples. Sequences with NSTI scores >2 were removed from the dataset (103 of 7056 ASVs), as predictions would be of low accuracy. The functional profile results were then analyzed using the DESeq2 tool (R package (Love et al., 2014)).

3. Results

3.1. Microbial diversity dynamics of prokaryotic and eukaryotic biofloc communities

Alpha diversity analysis (richness and Shannon H′ index) of the prokaryotic, eukaryotic, and fungal communities in the biofloc samples, did not reveal significant differences between dietary diets (Fig. 1, Table S2). However, richness significantly changed over time ($P < 0.05$), as indicated by linear mixed-effects (LME) analysis (Table S2), showing a decrease in all microbial groups (Fig. 1A-C.i). Time also showed a significant effect ($P < 0.05$) on the Shannon H′ diversity index (Table S2), increasing for the prokaryotic community (Fig. 1A.ii) while decreasing for eukaryotic and fungal communities (Fig. 1B.ii, and Fig. 1C.ii).

To investigate the structure of the biofloc microbial community in the different diets, principal coordinate analysis (PCoA) using the Bray-Curtis metric was performed based on the relative abundance of ASVs for prokaryotic (Fig. 2A), eukaryotic (Fig. 2B) and fungal communities (Fig. 2C). In all microbial groups tested, the communities became more separate by day 42 (Fig. 2), which was more evident for prokaryotes showing a distinct cluster between diets (Fig. 2A). Time and interaction effects between time and diet showed a significant effect on community structure for prokaryotic and eukaryotic groups as indicated by the Permanova analysis ($P < 0.05$; Table 1). Meanwhile in the fungal community, only time showed a significant effect ($P < 0.05$; Table 1). These results were further supported by comparing the similarity index using Bray-Curtis analysis within and between diets for prokaryotic, eukaryotic, and fungal microbial communities (Fig. 3). The similarity for all microbiota groups in both diets decreased significantly over time, with the CONdiet showing a greater change compared to the WBdiet (Dunn test; $P < 0.05$). Notably, the similarity within and between diets in fungal microbiota was lower compared to the rest of the eukaryotic and the prokaryotic groups (Fig. 3C).

Distance-based linear model (DistLM) analysis revealed that dissolved concentration of total nitrogen (TN) and chlorophyll-a concentration in filtered water (ChlWat) together explained 58% (R^2 sequential) of the observed total variation in the profiles of the prokaryotic community (Table S3). In the eukaryotic community, 84% of the total variation was explained by TN, ChlWat and ChlBiof, microbial activity (MicAct), crude protein content of biofloc (CP), C:N ratio (C:N ratio), biological oxygen demand (BOD), and dissolved concentration of total carbon (TC) (Table S3). Specifically in fungal community, TN, ChlWat, BOD and C:Nratio explained 56% of the total variation (Table S3). In all measured communities, ChlWat and TN were correlated with differences in microbial profiles (Fig. 4).

Venn diagram analysis indicated that in total 208 ASVs (41.3% of total ASVs) were shared between the CONdiet and WBdiet during day 21 and 42 (Fig. S1A). On day 42, two core genera namely *Pirellula* and *OM27 clade* were found solely in biofloc of the WBdiet, and *Calorithrix* was the only core genus present in biofloc of the CONdiet. The shared core genera in biofloc between the two diets were *Woeseia*, *Pseudoalteromonas*, *Pleurocapsa*, *Maricauda*, *Candidatus nitrosopumilus* and *Bythopirellula*, besides one non-assigned genus (Fig. S1B).

3.2. Microbial composition of prokaryotic and eukaryotic biofloc communities

When looking at the prokaryotic communities, the most abundant phyla on all sampling days were *Proteobacteria* and *Chloroflexi*, followed by *Planctomycetota*, *Bacteroidota*, and *Cyanobacteria* (Fig. 5A). In both diet groups, the relative abundance of *Proteobacteria* increased, while the *Chlorofexi* decreased over time (Fig. 5A). About genera (Fig. 5B), a large portion of the prokaryotic community could not be assigned to a

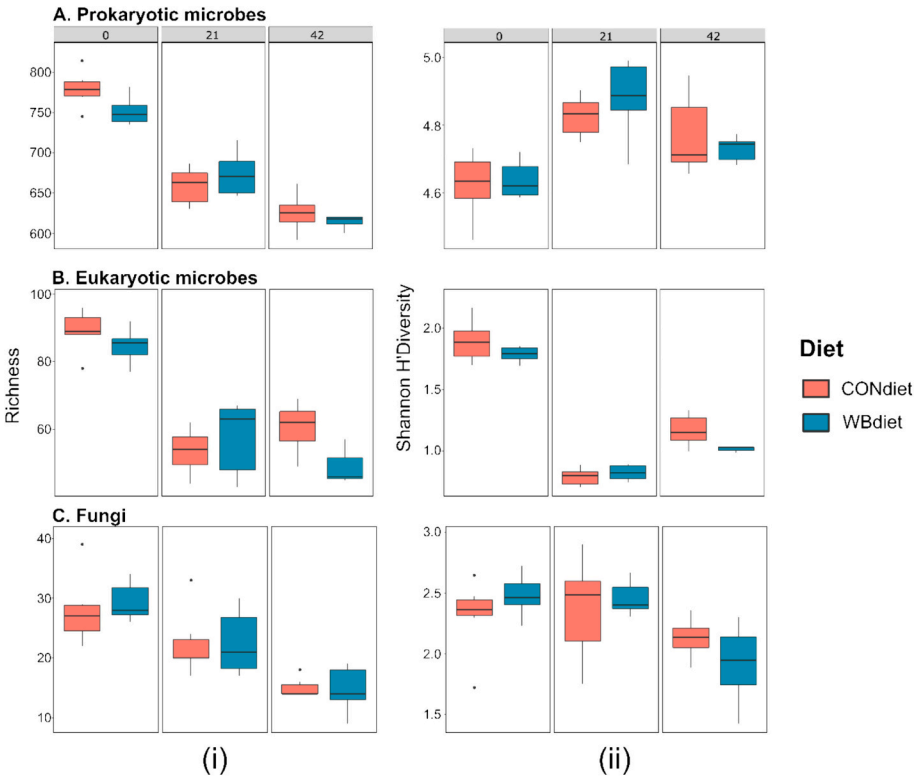

Fig. 1. Alpha diversity of the Prokaryotic (16S rRNA), (A) Eukaryotic (18S rRNA) (B) and (C) Fungal (ITS) microbial communities, coming from the CONdiet or WBdiet over time, including (i) richness and (ii) Shannon diversity index. CONdiet = control diet, WBdiet = wheat bran diet, 0 = day 1, 21 = day 21, 42 = day 42.

genus (approximately 60% of the relative abundance). *Candidatus nitrosopumilus*, *Muricauda*, *Calorithrix*, *Pleurocapsa, Pseudoalteromonas, Woeseia, and Bythopirellula* had a relative abundance >1% in both diets across the three sampling days (Fig. 5B). In both diets, the relative abundance of *Calorithrix* decreased, while in contrast, *Muricauda* and *Pleurocapsa* increased over time (Fig. 5B).

At the phylum level of the eukaryotic communities, *Chlorophyta* occupied the highest proportion (>75% of the relative abundance) and showed an increasing trend over time in both diets (Fig. 6A). Besides, *Nematoda* and *Rotifera* were also found relatively abundant regardless of diet (Fig. 6A). A large proportion of the eukaryotic genera was not assigned to genus level (up to 75%). *Nannochloris* and *Limnias* were found dominant on day 42, and their relative abundance was higher in tanks fed the CONdiet than in tanks fed the WBdiet (Fig. 6B). In contrast, the relative abundance of *Anurofeca*, *Rhabdolaimus*, and *Picochlorum* genera decreased over time (Fig. 6B).

In the fungal community, the main phyla were *Ascomycota* and *Basidiomycota*, while a large portion of the community was not assigned to any taxonomic composition (Fig. 7A). Throughout the experiment, *Ascomycota* increased in relative abundance, while the *Basidiomycota* group decreased, as seen in both diets (Fig. 7A). At genus level (Fig. 7B), *Trichosporon, Engydontium, Apiotrichum*, and *Didymella* were initially amongst the most abundant groups, while on day 42, *Penicillium* became more abundant, followed by *Phoma*, *Fusarium*, *Hortaea,* and *Aspergillus*. The proportion of *Penicillium* and *Aspergillus* increased throughout the experiment, with the latter having a higher relative abundance in tanks fed the CONdiet compared to the WBdiet (Fig. 7B).

The differentially enriched taxa on day 42 between diets were determined using LEfSe analysis (Fig. 8). Biofloc in tanks fed the WBdiet were enriched with the prokaryotes *Caldilineaceae*, *Muricauda*, *Pirellula*, and *Cyanobacteriaceae*, eukaryotes *Trebouxiophyceae*, and fungi *Suillus*. Meanwhile, in tanks fed the CONdiet the biofloc was enriched with the prokaryotes *Pleurocapsa*, *Candidatus Nitrosopumilus*, *Chlorofexi*, *Motilimonas* and *Bdellovibrionaceae*, the eukaryote *Nannochloris*, and the fungus *Debaromyces*.

3.3. Predictive functionality of prokaryotic microbial community

We showed that the prokaryotic microbial composition is affected by diet, which was more evident on day 42 (Fig. 2). Therefore, we evaluated the microbial community predictive functionalities of this group. Specifically, we used the PICRUSt2 tool to understand the impact of dietary NSP supplementation on the functional diversity of the biofloc prokaryotic communities (Fig. 9; the detailed pathways are listed in Table S4).

The supplementation of NSP promoted functionalities related to CHO metabolism, which became more evident with time, and more specifically, a higher abundance of pathways related to the metabolism of pentose, fructose, mannose and galactose was found. In tanks fed the WBdiet, a higher abundance of genes associated with the synthesis of fucI; L-fucose/D-arabinose isomerase, algL; poly(beta-D-man urinate) lyase and GMPP; mannose-1-phosphate guanylyltransferase was observed compared to tanks fed with the CONdiet. Moreover, in the CONdiet, an enrichment of various microbial functionalities related to the metabolism of energy (via photosynthesis and methane metabolism), nucleotide (purine), amino acid (cysteine, methionine, alanine, aspartate, glutamate), cofactors and vitamins (porphyrin, pantothenate, and CoA) and CHO (fructose and mannose) was found.

4. Discussion

Our study explored the microbial dynamics within biofloc aggregates when supplemented with dietary NSP-containing ingredients in the form of wheat bran (WBdiet). Growth and water quality data were also assessed (discussed by Vinasyiam et al., 2023; Figs. S2 and S4), which indicated that Pacific white shrimp growth was not significantly affected by the wheat bran inclusion, but its overall nutrient digestibility decreased when fed with WBdiet (Fig. S2). This led to an increase in the

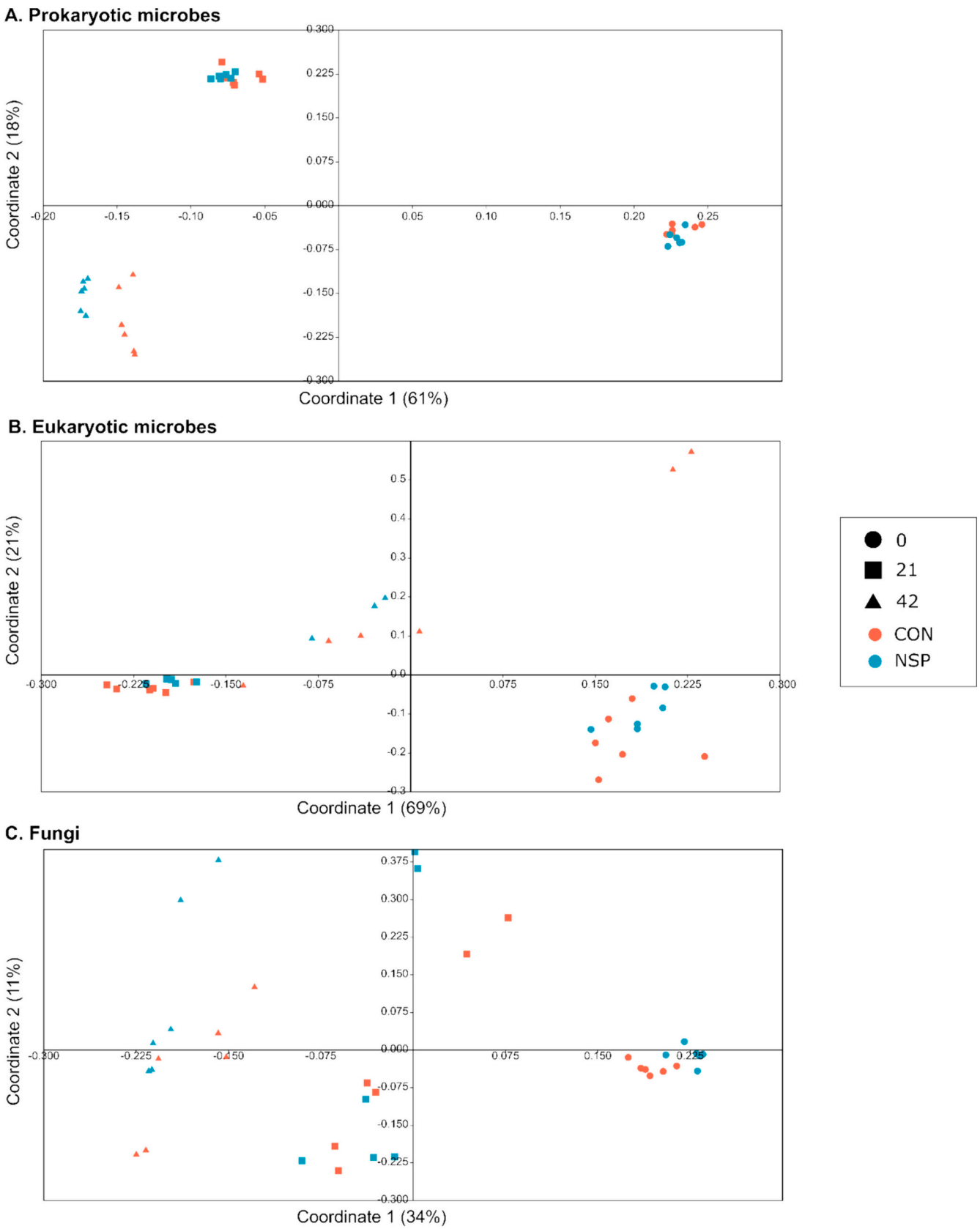

Fig. 2. Principal coordinate analysis (PCoA) using Bray-Curtis metric at the ASV level for prokaryotic (16S rRNA) (A), eukaryotic (18S rRNA) (B) and (C) fungal (ITS) microbial communities. CONdiet = control diet, WBdiet = wheat bran diet, 0 = day 1, 21 = day 21, 42 = day 42.

Table 1
Two-way Permanova analysis for biofloc microbial communities based on Bray–Curtis distances.

Permanova main effects						
Effect	Degrees freedom	Sum of Squares	Mean sum of squares	Pseudo-F	P (perm)	perms
Prokaryotic						
Diet	1	407	407	2.53	0.124	10
Tank (Diet)	4	643	161	1.23	0.143	999
Time	2	7483	3742	29	**0.001**	997
Diet x time	2	467	234	1.8	**0.013**	998
Residuals	8	1048	131			
Total	17	10,048				
Eukaryotic						
Diet	1	212	212	1.95	0.308	10
Tank (Diet)	4	435	109	1.48	0.074	999
Time	2	5403	2701	37	**0.001**	998
Diet x time	2	231	115	1.57	0.133	997
Residuals	8	586	73			
Total	17	6866				
Fungi						
Diet	1	1104	1104	0.62	1.000	10
Tank (Diet)	4	7142	1785	2.02	**0.004**	997
Time	2	14,979	7490	8.46	**0.001**	999
Diet x time	2	2597	1298	1.47	0.111	999
Residuals	8	7084	886			
Total	17	32,905				

Pairwise comparisons on Time (day)			
Groups	t	P(perm)	Unique perms
Prokaryotic			
D1, D21	5.60	0.002	968
D1, D42	6.47	0.005	985
D21, D42	3.76	0.002	974
Eukaryotic			
D1, D21	7.98	0.002	974
D1, D42	7.44	0.004	974
D21, D42	2.90	0.005	983
Fungi			
D1, D21	2.64	0.011	972
D1, D42	3.35	0.004	978
D21, D42	2.59	0.007	968

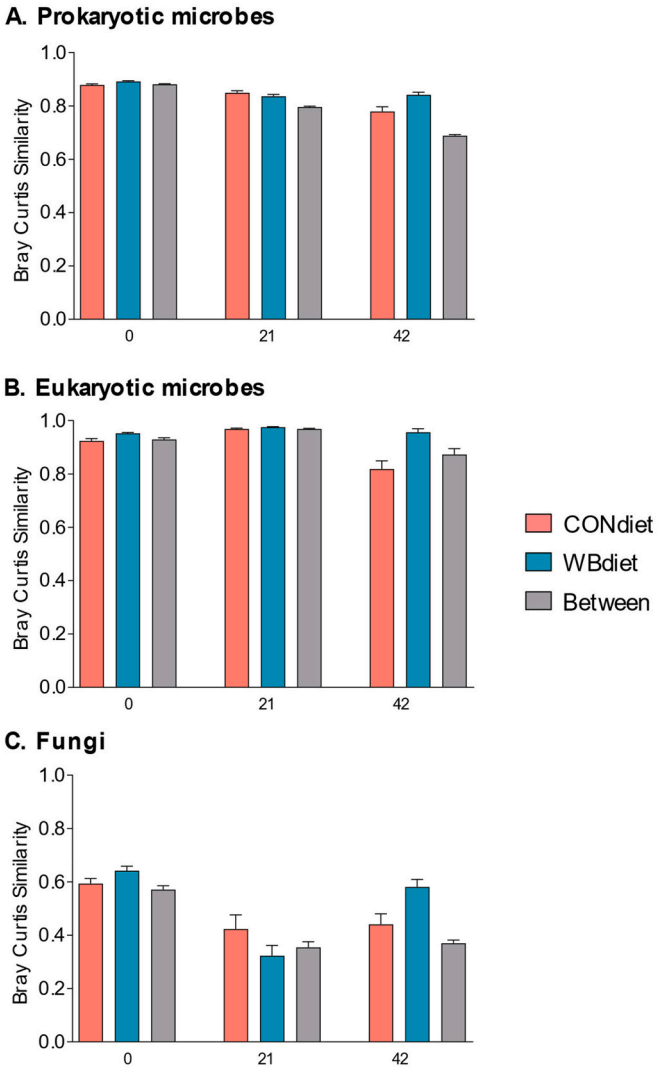

Fig. 3. Similarity index based on Bray-Curtis distance analysis on prokaryotic (A), eukaryotic (B) and fungal (C) microbial communities. CONdiet = control diet, WBdiet = wheat bran diet, Between = pooled data of CONdiet and WBdiet, 0 = day 1, 21 = day 21, 42 = day 42. Each bar showed the means and standard error of the means of the Bray Curtis similarity index of a diet per sampling time.

faecal C:N ratio, which we expected to positively affect biofloc formation in the system (Vinasyiam et al., 2023). Despite that, no effects were observed on biofloc formation during the 42 days of the experimental trial, but interestingly, we observed an increase in the organic matter accumulation in the biofloc coming from the WBdiet (Fig. S3A,D), suggesting a potential long-term effect on biofloc formation. Such outcome also explains the shifts we observed in the microbial composition and functionality. Specifically, we showed that non-starch polysaccharide supplementation affected both the biofloc prokaryotic and eukaryotic microbial composition. In addition, the predictive prokaryotic functionalities were also altered, indicating that a shift towards utilization of NSP-containing ingredients such as wheat bran may be possible.

4.1. Microbial diversity dynamics

We examined the microbial communities of biofloc in shrimp culture fed different amounts of dietary carbon input for 42 days. Time significantly affected both the richness and Shannon diversity of the prokaryotes and eukaryotes (Table S2), as in agreement with Tinh et al. (2021). The alpha diversity indices decreased over time in all tested communities, except for the Shannon diversity index of the prokaryotes (Fig. 1), suggesting that microbial diversity is dynamic across the culture stages. These findings contradict earlier studies looking at bacterial dynamics in the biofloc for L. *vannamei* shrimp culture (Ferreira et al., 2021; Xu et al., 2022). Xu et al. (2022) demonstrated that in the early stages of biofloc formation, a rise in bacterial diversity derives from constant carbon addition which indicates a time of adaptation and maturation for bacterial growth and development. However, an increasing trend was also demonstrated even when the biofloc was mature (Ferreira et al., 2021). Mature is defined as biofloc that has been developing for 30–50 days and the suspended solid concentration reaches at least 5 mL/L (Emerenciano et al., 2017), which fits the biofloc condition that we used in our experiment. Our results suggest that changes in the microbial diversity could still occur in a biofloc system when carbon is added, regardless of its maturity status, indicating the dynamic of these communities. However, higher dietary carbon input via the WBdiet did not significantly affect the alpha diversity indices in all tested microbial communities ($P > 0.05$; Table S2), opposing previous studies (Chakrapani et al., 2021; Gou et al., 2019; Guo et al., 2022;

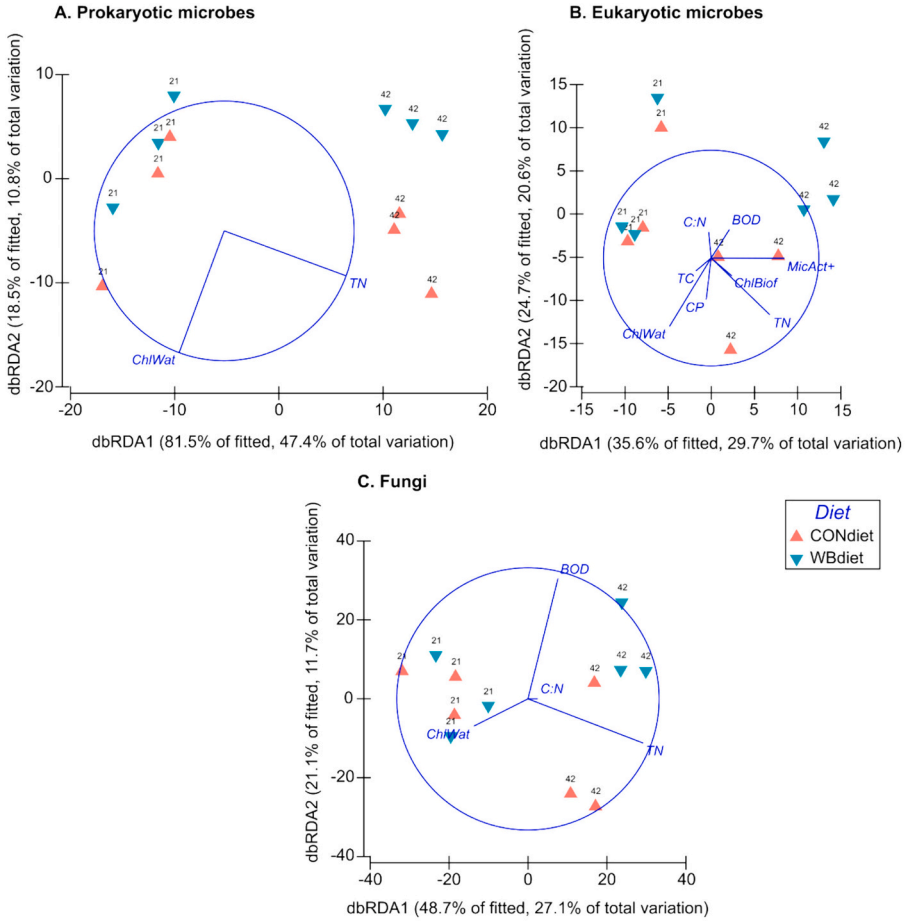

Fig. 4. Distance based Redundancy Analysis (dbRDA) of biofloc prokaryotic (A), eukaryotic (B) and fungal (C) microbial communities. Relative position of biofloc samples in the biplot is based on Bray Curtis similarity of square root transformed relative abundance at the ASV level. Vectors indicate the weight and direction of the different biofloc composition parameters that were best predictors of the microbial composition as suggested by the results of the distance-based linear model (distLM). The dbRDA axes describe the percentage of the fitted or total variation explained by each axis while being constrained to account for group differences. Sample IDs indicate the sampling day (21 and 42) and the diet (WBdiet and CONdiet). TC: total carbon; BOD: Biological Oxygen Demand; C:N: Carbon to Nitrogen ration; MicAct+: Microbial Activity; ChlWat: Chlorophyl in the water; ChlBiof: Chlorophyl in the biofloc; TN: Total Nitrogen; CP: Crude Protein.

Martins et al., 2020; Michaud et al., 2014; Tinh et al., 2021; Xu et al., 2022; Zheng et al., 2018). The decreasing trend of alpha diversity from the beginning to the end in both diets demonstrated that the constant dietary carbon addition could not contribute to the stabilization of the microbial diversity and that create positive selection pressure on certain microbial groups could still occur (Martins et al., 2020), leaving only those that are more adapted to prevail in time.

Looking at the beta diversity of the prokaryotic community, the significant effect of time and interaction with the diet ($P < 0.05$; Table 1) demonstrated that the bacterial profiles developed differently across culture stages and the discrepancy between diets distanced further at the end of the culture period (Fig. 2A). At the beginning of the experiment, the *in situ* carbon input from decaying microbiota in the mature biofloc was higher than the carbon from shrimp faeces in the WBdiet. This, therefore, concealed the effect of higher dietary wheat bran input via the WBdiet. Previous studies revealed mature biofloc provides an internal carbon source from the decayed microbiota and might require less external carbon to maintain water quality (Martins et al., 2020; Samocha et al., 2007; Xu et al., 2016). As the shrimp grew and the feeding level increased, so did the faecal load; the WBdiet started to differentiate the bacterial profiles from those in the CONdiet, explaining the significant interaction between diet and time ($P < 0.05$, Table 1). In the eukaryotic community, diet did not affect the beta diversity, although a trend was found($P < 0.01$; Table 1), supporting earlier findings by Tinh et al. (2021). In our view, this study still supported the idea that the quantity of carbon input is a pivotal factor in steering the bacterial community structure in a biofloc system (Gou et al., 2019; Martins et al., 2020; Zheng et al., 2018); however, a longer period of culture may be required to see a significant effect of the diet. In fact, we observed a trend in the water quality parameters of our system, as detailed in Vinasyiam et al. (2023) and presented in Fig. S4, which supports our premise. Specifically, we found a consistently lower concentration of total organic nitrogen (TON), total inorganic nitrogen (TIN), and total nitrogen (TN) in the system fed with the WBdiet compared to the CONdiet ($P < 0.05$; Fig. S4A—D), and those differences diverged overtime. This demonstrates that the microbial nitrogen utilization between diets became more pronounced towards the end of the experiment. A potentially explanation would be that such effects could be attributed to changes in carbon availability within the system over time: carbon availability ws more influence by *in situ* sources (mature biofloc) at the start, which then shifted to carbon source coming from the feed input towards the end of the experiment. This also explains the shifts in taxonomic composition between the two diets over time, as discussed below.

When comparing the different microbial groups, the prokaryotes and fungi had more distinct microbial clusters between diets than the general eukaryotes (mainly consisting of algae) (Fig. 2). Supporting this finding, the decrease in the similarity index between diet during the experiment was higher in the prokaryotes and the fungi, than in the eukaryotes (Fig. 3). This indicates that higher dietary carbon and NSP

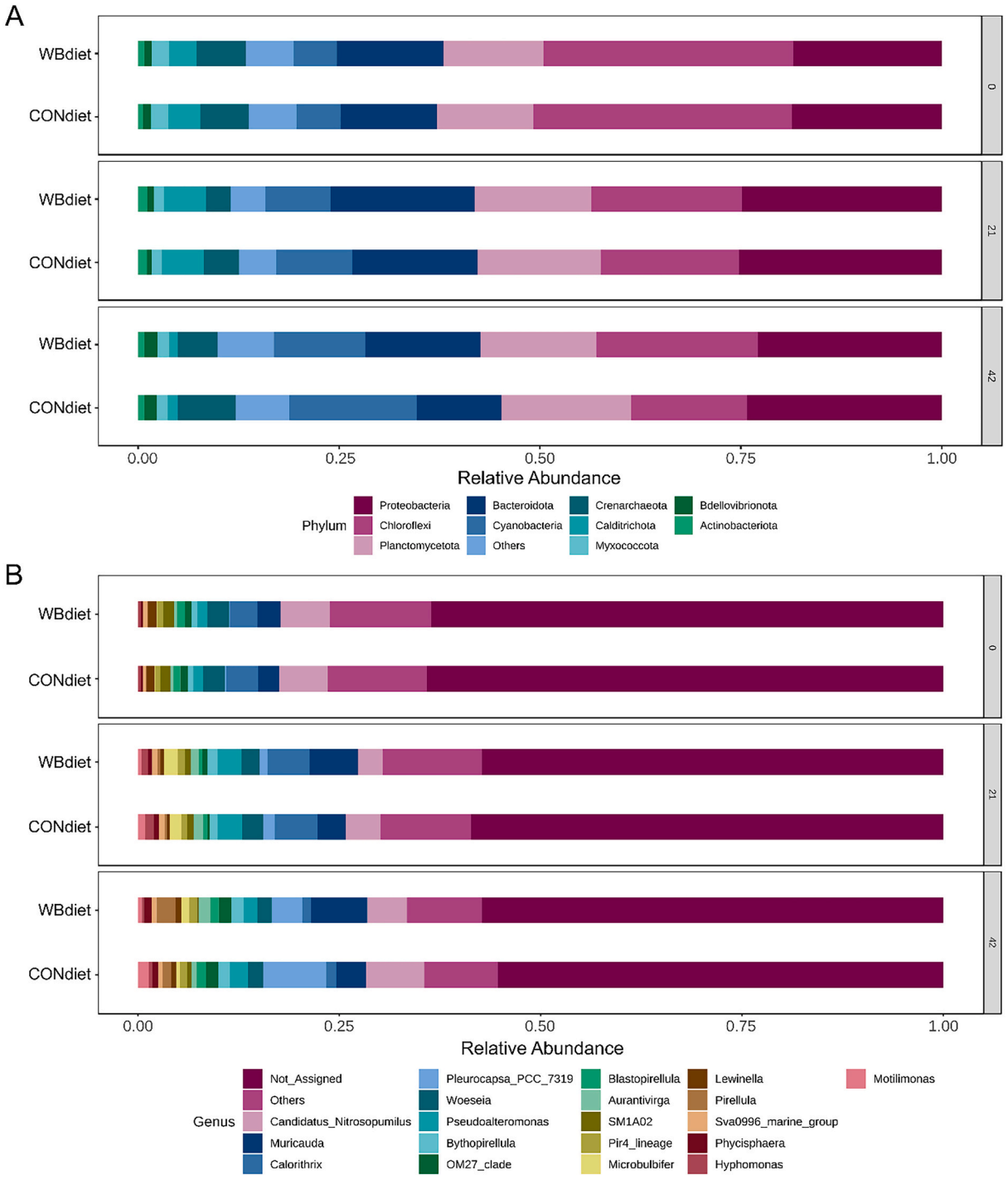

Fig. 5. Prokaryotic microbial composition of the biofloc samples between the CONdiet and WBdiet over time at phylum (A) and genus level (B). CONdiet = control diet, WBdiet = wheat bran diet, 0 = day 1, 21 = day 21, 42 = day 42.

content given to the system via WBdiet has a greater influence on the prokaryotes and the fungal groups, compared to the general eukaryotes. This could be associated with the ability of bacteria and fungi to directly utilize organic carbon via heterotrophic and saprophytic pathways (Grossart et al., 2019; Manan et al., 2016), as well as to act as intermediate degraders for NSP. As such, bacteria and fungi may have the capacity to break down the complex structure of shrimp faecal NSP into simple sugars, which can be more easily degraded by other microbes. Our nutrient digestibility data (Fig. S3A) showed a lower carbon digestibility in the WBdiet compared to the CONdiet; thus, we expected a higher carbon load and type in the WBdiet tanks coming from the faeces, inducing potentially a higher microbial diversity (Chakrapani et al., 2021; El-Husseiny et al., 2018; Tinh et al., 2021). However, this was not observed in the current study, indicating that carbon load itself may not be always directly related to a higher microbial diversity in biofloc. Further research may look closer into how different carbon levels and types influence the microbial diversity and profiles in biofloc systems.

Time significantly altered the alpha and beta diversity of prokaryotic and eukaryotic communities, supporting the earlier findings by Tinh et al. (2021). Time might be related to the dynamic factors within the system, such as the water quality and microbial biomass (Cardona et al., 2016; Pekkoh et al., 2022; Tinh et al., 2021; Xu et al., 2022). Similarly,

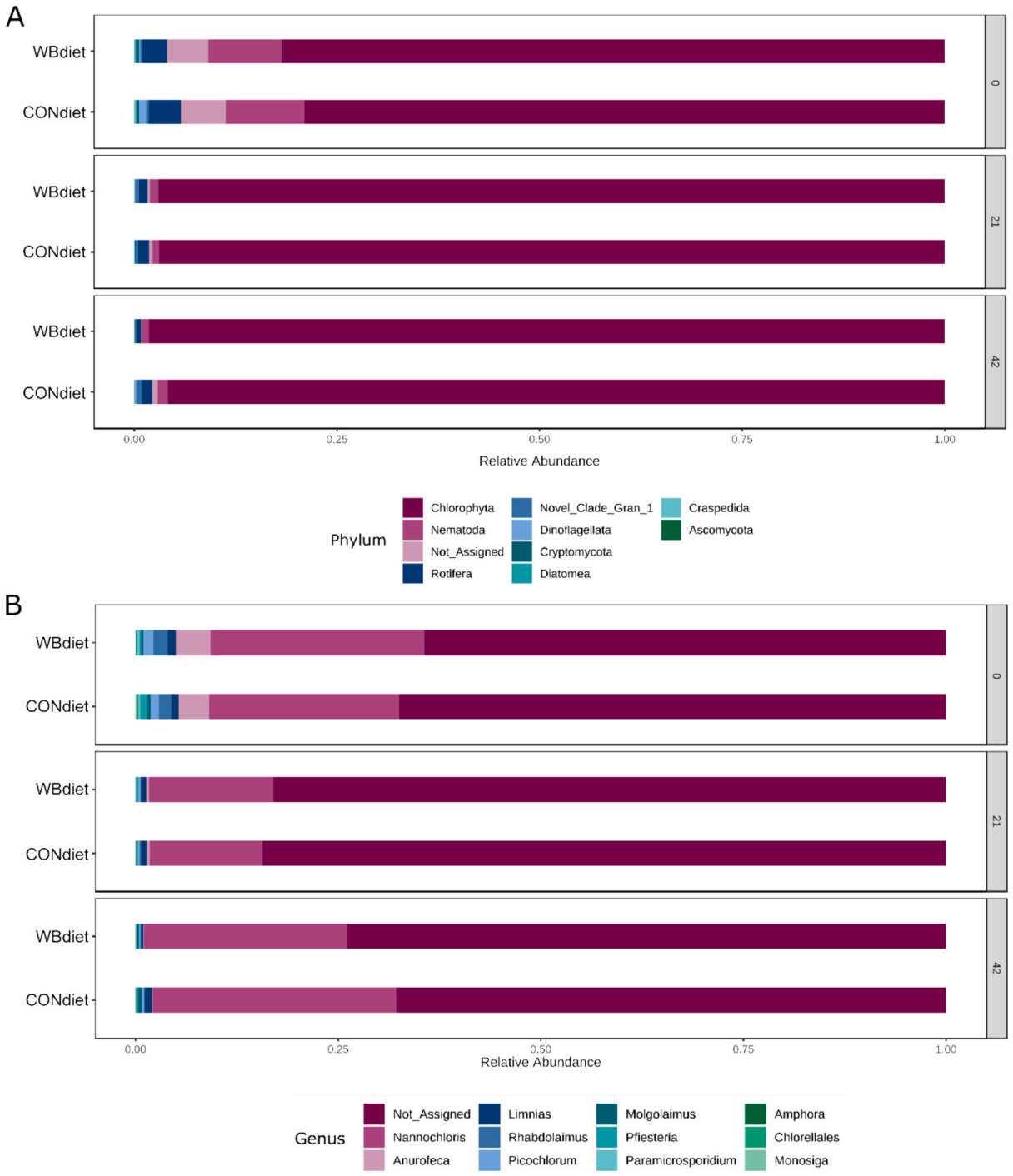

Fig. 6. Eukaryotic microbial composition of the biofloc samples between the CONdiet and WBdiet over time at phylum (A) and genus level (B). CONdiet = control diet, WBdiet = wheat bran diet, 0 = day 1, 21 = day 21, 42 = day 42.

our study also showed that environmental factors such as the dissolved concentrations of total nitrogen (TN) and Chlorophyll-a explained the variations in the microbial profiles in all tested communities (Fig. 4). Other factors were also found to be explanatory specifically for the eukaryotic communities and this was the microbial activity (Fig. 4B). Yun et al. (2022) reported that the proportion of biofloc mainly consisted of eukaryotes (49–73%), followed by prokaryotes (27–51%). Thus, as this microbial group consists of higher organic mass, it is also expected to have a significant contribution to the level of BOD and the system's microbial activity. The shifts of the microbial communities between day 21 and day 42 in all observed communities were associated with the increasing concentration of dissolved TN over time, due to an increasing dietary nitrogen input and the accumulation of nitrogen from microbiota. Nitrogen seemed overall to have a greater influence compared to carbon in the system, potentially because carbon is partially lost via microbial respiration, while nitrogen, when no denitrification is taking place, may be converted into biomass and ammonia (Gou et al., 2019) and thus remain in the system in the form of microbial biomass. In our system, we did not expect nitrogen loss due to denitrification as the bioflocs in the tanks were fully suspended and continuously aerated.

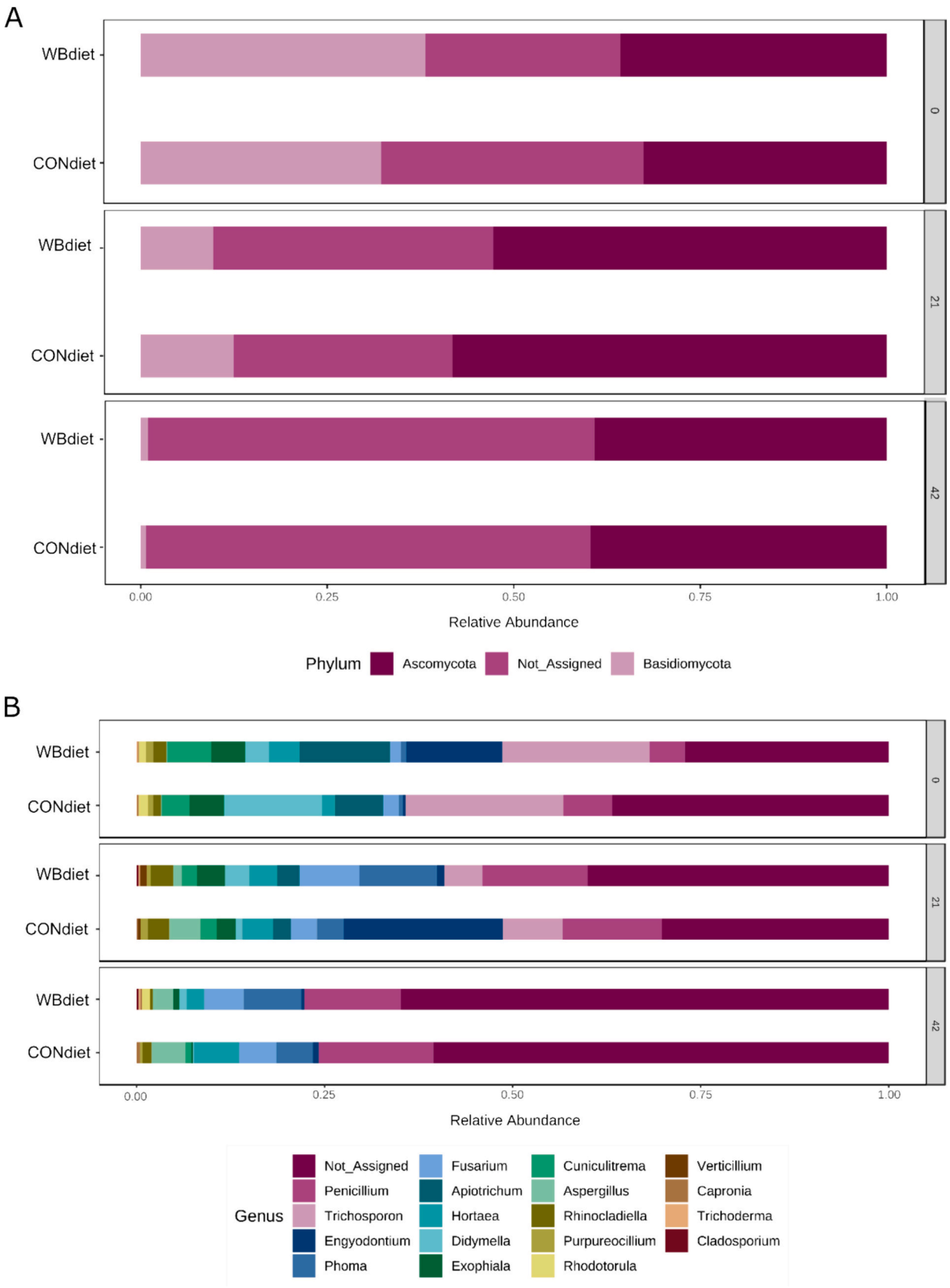

Fig. 7. Fungal microbial composition of the biofloc samples between the CONdiet and WBdiet over time at phylum (A) and genus level (B). CONdiet = control diet, WBdiet = wheat bran diet, 0 = day 1, 21 = day 21, 42 = day 42.

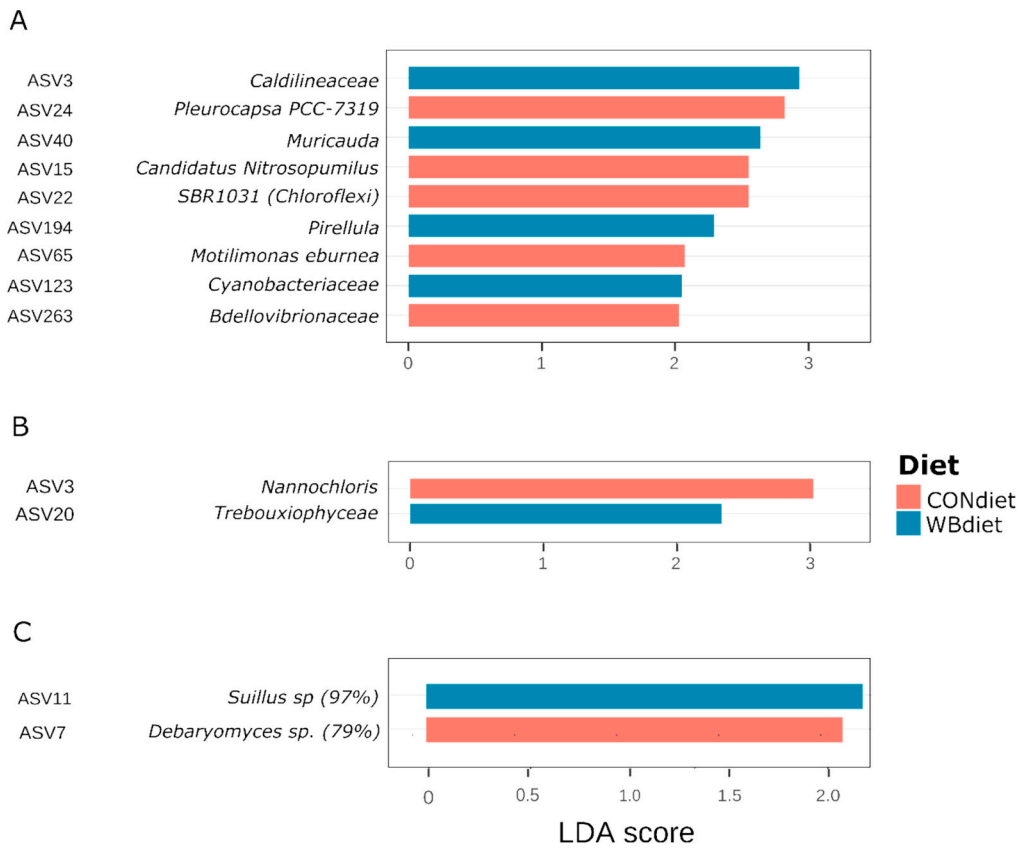

Fig. 8. LEfSe analysis (Linear discriminant analysis Effect Size) on prokaryotic (A), eukaryotic (B) and fungal (C) taxa, showing the linear discriminant analysis (LDA) score of the differentially enriched taxa affected by diet on day 42. For the fungal ASV, manual BLAST was performed in order to identify the closest genus; similarity percentage is included in brackets. CONdiet = control diet, WBdiet = wheat bran diet.

4.2. Microbial taxonomic composition

The relative abundance of the most abundant prokaryotic phyla (*Proteobacteria*, *Chloroflexi*, *Planctomycetota*, *Bacteroidota*, and *Cyanobacteria*) fluctuated over time but still remained independent of diet (Fig. 5A, Table 1). This suggested that the differences between the WBdiet and CONdiet in terms of carbon input quantity and complexity could not drastically alter the diversity of the most abundant phyla in the biofloc. Such finding was consistent with previous studies observing that these phyla as dominant across a wide range of C:N ratios, ranging from 10 to 20 (Cardona et al., 2016; Deng et al., 2019; Gou et al., 2019). Moreover, similar prokaryotic phyla have been reported with various carbon sources differing in structure complexities such as sucrose (Chen et al., 2021), molasses (Cardona et al., 2016; Jiang et al., 2020; Panigrahi et al., 2018), rice straws (Addo et al., 2021), corn starch (Tinh et al., 2021), wheat (Martínez-Córdova et al., 2018) and vegetal floating substrates, such as amaranth, oat and wheat (Peiro-Alcantar et al., 2022), indicating those as core prokaryotic phyla within the biofloc.

Biofloc from WBdiet showed a higher abundance of *Caldilineaceae*, *Muricauda*, *Pirellula*, and *Cyanobacteriaceae* classes compared to the CONdiet (Fig. 8). *Caldilineaceae* is a filamentous bacteria from the *Chloroflexi* phylum (Yu et al., 2018). *Chloroflexi* species form the floc skeleton by producing extracellular polysaccharides (EPS) (Yu et al., 2018; Zhu et al., 2022). Moreover, *Muricauda* has been reported as a dominant biofloc-associated bacteria, which increased with higher carbon availability (Tinh et al., 2021; Zhu et al., 2022). These taxa were thought to contribute to the yellow-brownish color of biofloc (Bruns et al., 2001; Oh et al., 2015). Regarding the *Pirellula* genus that was also shown more abundant in the WBdiet, it has been reported to play an important role in global carbon and nitrogen cycles, for example, as the nitrate reducer (Fraser, 2004; Op den Camp et al., 2007). Cyanobacteria is related to algal blooms development due to the nutrient-rich condition (Cordeiro et al., 2020). The high abundance of Cyanobacteriaceae in WB diet groups could be associated with the addition of phosphorous from wheat bran. Overall, similar to the phyla observations, our study reported prokaryotic genera which have been previously commonly found in biofloc systems (Deng et al., 2019; Tinh et al., 2021; Xu et al., 2022).

Concerning the eukaryotic communities, the dominant phyla found in this study were the algae *Chlorophyta*, followed by the zooplankton groups *Nematoda* and *Rotifera* (Fig. 6A), which have been also commonly found in biofloc systems (Chakrapani et al., 2021; Maicá et al., 2012; Manan et al., 2016; Ray et al., 2010). *Chlorophyta* had higher relative abundance in WBdiet on day 42 (Fig. 6A), which was associated due to the higher organic carbon availability within the system as also stated by Gao et al. (2019). However, some species of *Chlorophyta* are considered an obligate autotroph that could not utilize organic carbon because of the absence of genes encoding organic carbon transporters (Celente et al., 2022; Zaslavskaia et al., 2001). *Nematoda* and *Rotifer* are classified as algae-grazing zooplanktons whose abundance increased along with the abundance of algae in the biofloc system (Hargreaves, 2013). Moreover, *Nematode* and *Rotifer* are considered nutritious live prey for shrimp (Silva et al., 2021). In our study, we found an opposite pattern between the abundance of *Rotifer* and *Nematode* groups which were decreasing with time, with the *Chlorophyta* group which was increasing (Fig. 6), possibly indicating the shrimp's grazing activities on zooplankton increased over time.

Inorganic carbon availability is a determining factor in algal productivity and their photosynthesis activity (Tamburic et al., 2015; Zavrel et al., 2018). Therefore, such results indicated that these nutrients were not limiting. Previous studies showed that addition of organic carbon such as ethanol and glucose did not affect the net photosynthetic rate, but increased *Nannochloris* biomass (Fang et al., 2004). However, in our

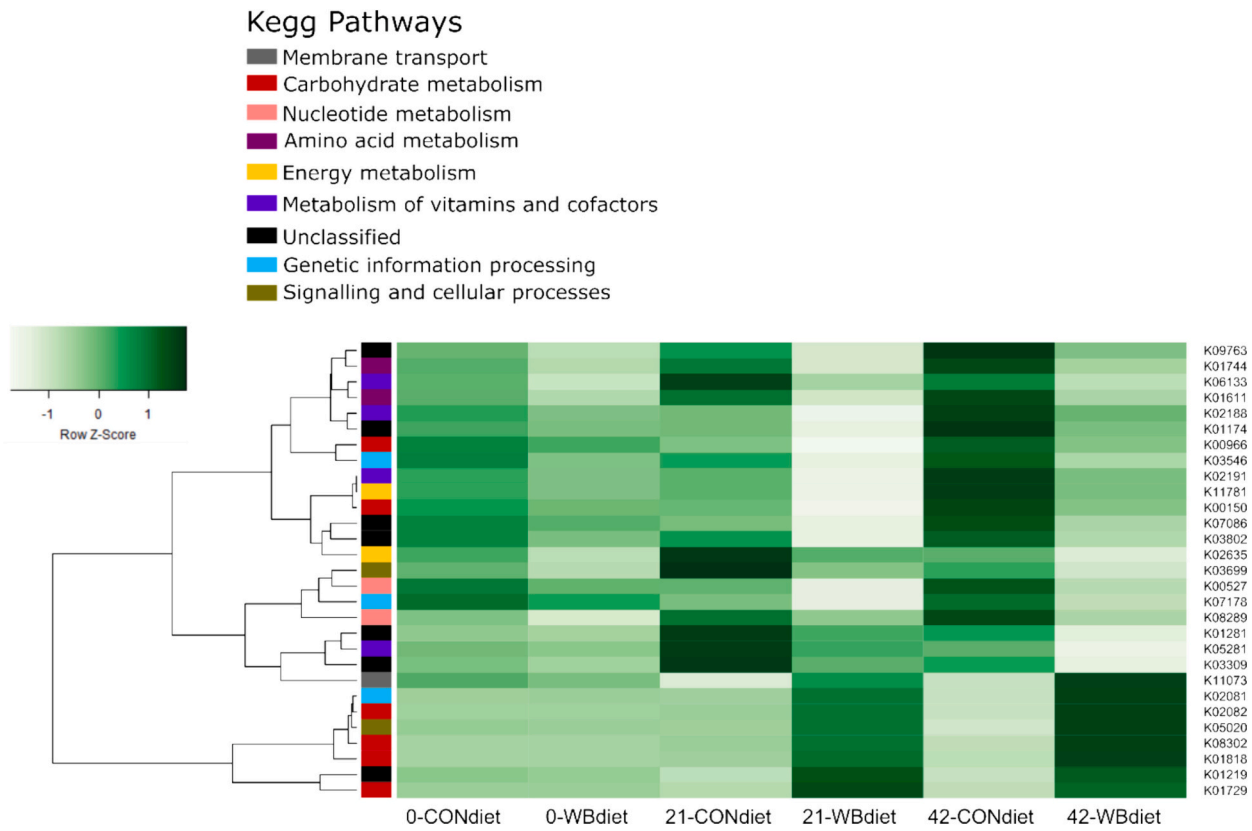

Fig. 9. Predictive functionality analysis using PICRUSt2 based on KEGG (Kyoto Encyclopaedia of Genes and Genomes) orthologs between the CONdiet and WBdiet biofloc groups. Significant pathway enrichment was tested using DESEq analysis. 0-CONdiet = control diet at D1, 0-WBdiet = wheat bran diet at day 1, 21-CONdiet = control diet at day 21, 21-WBdiet = wheat bran diet at day 21, 42-CONdiet = control diet at day 42, 42-WBdiet = wheat bran diet at day 42.

study, *Nannochloris* had a higher relative abundance in the CONdiet on day 42 (Fig. 6A) and not the WBdiet, where a higher organic carbon availability within the system was present. Some species of *Chlorophyta* were reported as obligate autotrophs that could not utilize organic carbon because of the absence of genes encoding organic carbon transporters (Celente et al., 2022; Zaslavskaia et al., 2001); whether this was the case for *Nannochloris* or other factors contributed to this result is not clear from this study.

In the fungal community, the dominant phyla observed were *Ascomycota* and *Basidiomycota,* while a high proportion of taxa were not assigned by the ITS database (Fig. 7A). Diet showed a comparable effect on the dynamics in the relative abundances of these two phyla. *Phoma* and *Rhodoturola* were genera showing higher relative abundances with the WBdiet than with the CONdiet (Fig. 7B). In an in vivo culture, *Phoma* species were reported to grow faster with simple sugars like sucrose compared to NSP-containing carbon sources such as cellulose and rice bran media (Cao and Li, 2022; Luft et al., 2021). The higher relative abundance of the *Phoma* genus in WBdiet tanks on day 21 and day 42 suggests a potentially higher availability of simple sugars in the system, which may have originated from the degradation of complex carbon in the shrimp's faeces by other microbiota groups. Regarding *Rhodoturola*, this group has been reported to be able to utilize lignin-containing ingredients (Gupta et al., 1990; Hainal et al., 2012; Martins et al., 2021), which can be categorized as NSPs. Moreover, both *Phoma* and *Rhodoturola* genera can produce biopolymers composed of CHO synthesized via extracellular pathways during the process of growth and metabolism, creating an extracellular polysaccharides-enriched fraction also known as exopolysaccharides (EPSs) (Luft et al., 2021; Mahapatra and Banerjee, 2013; Osemwegie et al., 2020). This fraction was hypothesized to have a similar function as the EPS matrix secreted by heterotrophic bacteria due to its roles in cellular aggregation, nutrient attachment, and substrate adhesion (Breitenbach et al., 2022; More et al., 2014). However, unlike bacterial EPS production in a biofloc system via the heterotrophic pathway, fungal EPS is commonly produced via fermentation (Mahapatra and Banerjee, 2013). Microbial fermentation was thought to be hardly present in our fully aerated biofloc system. However, a follow-up study may investigate the occurrence and role of fungal EPS in biofloc systems.

4.3. *Predictive functionality of prokaryotic microbial communities*

In our study, dietary wheat bran input via the pelleted feed affected the predicted microbial functionalities of the prokaryotic community. Specifically, predicted functionalities related to CHO metabolism were increased in the WBdiet (Fig. 9), potentially due to the higher absolute amount of carbon input compared to the CONdiet. The total CHO input from the WBdiet was 380 g, which was higher than the 244 g input from the CONdiet. Concerning the CHO metabolism, a significant enrichment of pathways related to pentose, fructose, mannose, and galactose was observed in the WBdiet (Table S4). Wheat bran contains 56% of NSP, mainly in the form of arabinoxylans (70%) and cellulose (24%) (CVB, 2022; Maes and Delcour, 2002; Stevenson et al., 2012). Thus, the inclusion of 40% wheat bran in the WBdiet increased the content of NSP, arabinoxylan, and cellulose by 22%, 16%, and 5.4% respectively, as well as an increase in the NSP content of the shrimp faeces. Such an increase in metabolic pathways related to sugars could have been linked to an intermediate degradation of NSPs by bacteria, breaking them down into simpler substances which can be more easily degraded by microbes. Biofloc consists of metabolic networks where cross-feeding occurs between microbes with by-products of one organism serving as essential nutrient substrates for another. Some bacteria act as generalists, capable of degrading a wide range of polysaccharides, while others are more

specialized groups which target a few types of carbon substances (Tepaamorndech et al., 2020). Gradual degradation of NSP done by various groups of microbiota resulted in similar overall biodegradability indices and microbial activity levels in both diets (Vinasyiam et al., 2023), which is in agreement with previous studies (Chakrapani et al., 2021; Ekasari et al., 2014; Thomsen, 2005), confirming that microbes can utilize and adapt to a wide carbon source, from simple to complex sugars and NSP. It is important to note that in our study, we did not measure the NSP content of the faeces, while the functionalities are only based on prediction, therefore, further studies are required to confirm these outcomes.

Finally, the overall microbial functionalities which involve the conversion of potential toxic nitrogen substances were enriched in the WBdiet compared to the CONdiet. This was confirmed by the lower dissolved total organic nitrogen (TON), nitrate+nitrite, and total nitrogen (TN) found in the WBdiet at the end of the culture period (Vinasyiam et al., 2023). This suggested that the NSP-containing wheat bran as a carbon source was effectively promoting the growth of microbes responsible for controlling inorganic toxic N-species in the biofloc system. Previous studies demonstrated that NSP-containing carbon sources, when given directly to the system after feeding (Chakrapani et al., 2021), were as effective as molasses (El-Husseiny et al., 2018) and maintained animal performance and ammonia levels. This provides a good starting point for further research on microbiota functionality in biofloc systems fed different types of CHO, as also stated by Tinh et al. (2021). In addition, future studies should focus on the functionality of specific microbial groups in relation to environmental parameters such as water quality.

5. Conclusion

Our study evaluated the effect of higher dietary wheat bran input on the microbial composition dynamics and functionalities of the biofloc in L. *vannamei* culture. Until today, most studies focused on prokaryotic and only a few looked into the eukaryotic and fungal microorganisms in biofloc systems, thus our study provides novel insights on the taxonomic composition and dynamics of these communities. Overall, we showed that the dynamics of all microbial communities in response to wheat bran addition differed and fluctuated significantly over time. Wheat bran addition markedly affected prokaryotic communities to become more distinct from the control group, while it also modulated their potential functionalities to shift towards carbohydrate metabolism (pentose, fructose, mannose, and galactose). Eukaryotic community composition was less affected by the wheat bran addition compared to prokaryotic communities but was mainly shaped by the culture period, while fungal communities were more variable and responsive to carbon input in the system. More research is needed to understand the role of the different microbial communities and their interactions to optimize biofloc management.

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Ethics declaration

The work described has been carried out in accordance with the European Communities Council Directive 2010/63/EU, which does not require an animal experiment approval as it involved invertebrates.

CRediT authorship contribution statement

Apriana Vinasyiam: Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Formal analysis, Conceptualization. **Marc C.J. Verdegem:** Writing – review & editing, Supervision, Project administration, Formal analysis, Conceptualization. **Julie Ekasari:** Writing – review & editing. **Johan W. Schrama:** Writing – review & editing, Methodology, Formal analysis. **Fotini Kokou:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Formal analysis, Data curation.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.741396.

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