Feed Types Driven Differentiation of Microbial Community and Functionality in Marine Integrated Multitrophic Aquaculture System

Yale Deng [1,2] , **Fan Zhou** [3], **Yunjie Ruan** [1,4,*], **Bin Ma** [5], **Xueyan Ding** [3], **Xiaomei Yue** [6], **Wenjun Ma** [3] **and Xuwang Yin** [7]

1 Institute of Agricultural Bio-Environmental Engineering, College of Bio-Systems Engineering and Food Science, Zhejiang University, Hangzhou 310058, China; yale.deng@wur.nl
2 Aquaculture and Fisheries Group, Department of Animal Sciences, Wageningen University, 6708 WD Wageningen, The Netherlands
3 Zhejiang Fisheries Technical Extension Station, Hangzhou 310023, China; zhoufan0302@126.com (F.Z.); Dingxy_sc@sina.com (X.D.); mwj8890@163.com (W.M.)
4 Academy of Rural Development, Zhejiang University, Hangzhou 310058, China
5 Institute of Soil and Water Resources and Environmental Science, College of Environmental and Resource Sciences, Zhejiang University, Hangzhou 310058, China; bma@zju.edu.cn
6 Business Economics Group, Department of Social Science, Wageningen University, 6706 KN Wageningen, The Netherlands; xiaomei.yue@wur.nl
7 College of Fisheries and Life Science, Dalian Ocean University, Dalian 116023, China; yinxuwang@dlou.edu.cn
* Correspondence: ruanyj@zju.edu.cn; Tel.: +86-571-8898-2373

Abstract: Integrated multi trophic aquaculture (IMTA) improves the production of aquatic animals by promoting nutrient utilization through different tropical levels. Microorganisms play an important role in elements cycling, energy flow and farmed-species health. The aim of this study was to evaluate how feed types, fresh frozen fish diet (FFD) or formulated diet (FD), influence the microbial community diversity and functionality in both water and sediment in a marine IMTA system. Preferable water quality, higher animal yields and higher cost efficiency were achieved in the FD pond. Feed types changed the pond bacterial community distribution, especially in the rearing water. The FFD pond was dominated with Cyanobacteria in the water, which played an important role in nitrogen fixation through photosynthesis due to the high nitrogen input of the frozen fish diet. The high carbohydrate composition in the formulated diet triggered higher metabolic pathways related to carbon and lipid metabolism in the water of the FD pond. Sediment had significantly higher microbial diversity than the rearing water. In sediment, the dominating genus, *Sulfurovum* and *Desulfobulbus*, were found to be positively correlated by network analysis, which had similar functionality in sulfur transformation. The relatively higher rates of antibiotic biosynthesis in the FFD sediment might be related to the pathogenic bacteria introduced by the trash fish diet. The difference in microbial community composition and metabolic pathways may be associated with the different pathways for nutrient cycling and animal growth performance. The formulated diet was determined to be more ecologically and economically sustainable than the frozen fish diet for marine IMTA pond systems.

Keywords: formulated diet; integrated multitrophic aquaculture; microbial community; functionality; sustainability

1. Introduction

Aquaculture is one of the fastest-growing animal food producing sectors worldwide, and occupies more than half the world's fish supply while natural fisheries are facing the risk of overexploitation [1,2]. Coastal aquaculture is expanding due to the large demand for marine products for human consumption [3]. Pond aquaculture, especially polyculture and integrated systems, is widely applied for marine species culture in Southeast China [4]. Specific impacts caused by coastal aquaculture on the environment include excessive water consumption, culture effluents discharge, nutrient pollution, and infection of disease-causing organisms [5]. To reduce the environmental impact of pond aquaculture, it is necessary to increase the feed efficiency and enhance the natural food web availability [6]. Compared to monoculture, integrated multi trophic aquaculture (IMTA) can improve nutrient recovery by providing the feeding niches of different species in one habitant [7]. In IMTA, primary production (e.g., microalgae and seaweed) take up inorganic nitrogen or phosphorous, which will be eaten by filter feeders (e.g., muscles, shellfish), then finally captured by shrimp or fish [8]. IMTA is an ecologically sustainable aquatic system which achieves higher production with lower cost [9,10]. Therefore, pursuit of the maximum sustainability is the main goal of IMTA, which could be achieved by optimizing the nutrient cycling and energy flow.

Environmental microorganisms play an important role in nutrient cycling, water-quality maintenance and farmed-species health in pond systems [11]. Oxygen and ammonia concentration are the two main factors determining the productivity of pond aquaculture. In ponds, autotrophic nitrifier and sulphur bacteria oxidize ammonium, nitrite and sulphite through an aerobic process in the water and denitrifying and sulphate-reducing bacteria perform anaerobic processes in the sediment [12–14]. Through the activity of heterotrophic decomposers, nitrogen and phosphorous are released to stimulate primary production of phytoplankton. Similar microbial community composition was detected between water, sediment and animal gut microbiota [15,16]. Hence, the water and sediment microbiota may serve as a source of the gut microbiota of farmed animals, which influences animal health and growth performance [17,18].

Administrated feed and primary productivity from algae and bacteria are the basic sources of food for all animals in the ponds. In intensive aquaculture ponds, a large amount of feed is used to maximize the animal production. However, a large fraction of the feed administrated to the pond can remain uneaten or released through fecal loss, and ends up in the sediment [19]. Traditionally, low-value trash fish were used to feed farmed animals which consumed a large amount of wild fish from overexploited fisheries. However, using trash fish as feed is not sustainable due to its low feed efficiency, water pollution and limited capture fisheries resources, which is raising increasing concerns in intensive-farming regions [20]. Nowadays, floating pellets are used, which can reduce overfeeding through visual control of farmers. Fish fed with a formulated diet showed better growth performance, owing to its high digestibility compared to fresh frozen fish [21]. Moreover, a formulated diet usually has a higher composition of carbohydrates, but less protein and lipids than fresh frozen fish on dry matter base [21]. The high-carbohydrate input of formulated diets and high-nitrogen input of frozen fish was hypothesized to favor the growth of microorganisms in different trophic levels. The food web in ponds is complex, with feed input and animal-sediment-water interactions. However, how the feed types affect microbial community composition, water quality and ultimately animal growth performance in full-scale marine ponds is not clear.

Environmental and economic sustainability are two main factors to evaluate the sustainability of pond aquaculture [6]. In this study, we aimed to evaluate the impact of two types of frequently used feed, namely a formulated diet and a fresh frozen fish diet, on the environmental and economic development in a marine IMTA pond system. The responses of water quality, bacterial assembling in water and sediment, microbial functionality and economic benefit of the two types of feed were evaluated. Hence, an understanding of the microbial ecology in ponds is crucial to optimize production without sacrificing environmental sustainability.

2. Materials and Methods

2.1. Animal Husbandry Environment

Two farm-scale integrated multitrophic pond systems were used to test the effect of a fresh frozen diet (FFD) and a formulated diet (FD) on the biomass yield and microbial community composition in both water and sediment. The FD pond was 2.1 hectares in area and the FFD pond was 1.2 hectares in area. Each of the two ponds was stocked with mud crabs (*Scylla paramamosain*) as the main species, intercropping with razor clams (*Sinonovacula constricat*), Malaysian cockles (*Tegillarca granosa*) and ridgetail white prawns (*Exopalaemon carinicauda*), for 6 months. The stocking density of the cultivated animals is shown in Table S1. A commercial diet (Tech-bank food Co., Ltd., Ningbo, China) was used as the formulated diet, while fresh frozen fish were collected from a local store. The nutrient composition of the formulated diet was: 42.2% crude protein and 8.2% crude fat. The water quality was measured once a month and maintained at acceptable levels for the cultivated animals. Only limited water (<5% per month) was exchanged during the whole experimental period.

2.2. Water and Sediment Samples Collection

Water and sediment samples were collected at the depth of 25 cm and 10 cm, respectively, using five-point sampling from each pond at the same positions. The five points included four points from the four corners and one point at the center of the pond. For the water samples, 250 mL water was collected and filtered through 0.22 μm membrane filter for DNA extraction [22]. For sediment samples, 50 mL sludge was collected for DNA extraction. After filtration, the water samples were subjected to water quality measurement in three replicates. In details, TAN, nitrite, nitrate, dissolved reactive phosphorus (DRP) and total organic carbon (TOC) were determined according to standard protocol [23]. Moreover, the pH of both water and sediment samples was measured everyday using a pH meter (FE28-Standard, Mettler Toledo, Shanghai, China).

2.3. DNA Extraction and Sequencing

DNA extraction and purification was performed according to our previous protocol [12], using PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA). The V3-V4 region of bacterial 16S rRNA was amplified using primer pairs of 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′) with barcode. The program used for PCR amplification was the same as a previous study [24]. The quality of PCR products was detected on 2% agarose gel, and purified with GeneJET Gel Extraction Kit (Thermo Scientific, Waltham, MA, USA). The quantified DNA was subjected to 16S rDNA amplicon sequencing using Illumina HiSeq platform. Sequence data have been deposited in the National Center for Biotechnology Information (NCBI) database under a BioProject accession number of PRJNA561204.

2.4. Bioinformatic Data Analysis

Raw data was cleaned using UCHIME algorithm [25]. A software named uparse (http://www.drive5.com/uparse/) was used to cluster the effective tags to identify operational taxonomic units (OTUs) at the identity of 97%. The detected OTUs were assigned to a taxonomy according to the Silva database. The microbial diversities, including α-diversity and β-diversity, were analyzed using QIIME [26] and visualized in R. The correlation between water quality parameters and microbial community distribution was analyzed by redundancy analysis (RDA) using *vegan* R package. In addition, the 16S rRNA data was further used to predict the microbial functionality using Tax4Fun software (http://tax4fun.gobics.de) with KEGG database as a reference. The significant difference between two treatments was evaluated by a *t*-test. At genus taxa level, the average relative abundance that was bigger than 0.2% in FFD or FD pond was used to build a co-occurrence network by *igraph* R package [15]. The network was visualized with Gephi software according to our previous study [27,28].

3. Results

3.1. Water Quality and Economic Performance

The water quality parameters of the water samples from the two pond systems at the end of this study are shown in Table 1. No significant difference between total ammonia nitrogen (TAN) and oxidation reduction potential (ORP) was detected between FFD and FD in water samples. FFD treatment had significantly higher concentrations of nitrite, nitrate, dissolved reactive phosphorous (DRP) and total organic carbon (TOC) than FD treatment in the water, indicating the potential of high nutrition levels in the FFD pond. Moreover, relatively higher pH and ORP were detected in the FD pond. Though a significant difference in water quality was detected between the two treatments, the water quality was still acceptable for farmed species in both ponds. Besides, acceptable water quality was observed in the discharge effluent (TAN < 0.9 mg L^{-1}, NO_2-N < 0.3 mg L^{-1}, NO_3-N < 0.6 mg L^{-1}, pH 8.06–8.94) measured weekly during the entire experiment (Table S2).

Table 1. The water quality parameters of water samples from two pond systems. Values are mean ± SD (n = 15 samples treatment^{-1}). ** $p < 0.01$, * $p < 0.05$, ns: not significant.

Water Quality Parameters	FFD	FD	Significance
TAN (mg L^{-1})	0.34 ± 0.06	0.29 ± 0.09	ns
NO_2^--N (mg L^{-1})	0.06 ± 0.01	0.03 ± 0.01	**
NO_3^--N (mg L^{-1})	0.39 ± 0.07	0.24 ± 0.05	**
DRP (mg L^{-1})	0.09 ± 0.01	0.03 ± 0.01	**
TOC (mg L^{-1})	14.8 ± 2.02	10.19 ± 2.76	**
pH	8.02 ± 0.07	8.26 ± 0.17	*
ORP (mV)	264.2 ± 40.67	305.8 ± 28.21	ns
Alkalinity (mg L^{-1})	129.8 ± 16.45	164.2 ± 10.31	*

The harvested biomass and growth of farmed animals are shown in Table S1. Better growth performance was achieved in FD than FFD pond in terms of both main species and intercropping species. It is worth noting that no razor clam was harvested in the FFD pond at the end of the experiment, whereas 1665 kg of razor clams was harvested in the FD pond. The investment, yield and net income analysis of FFD and FD ponds during the experiment is shown in Table S1. A similar expense per hectare was invested in both ponds (FFD, 143,910 CNY/ha; FD, 151,726 CNY/ha). Even though the FD treatment had a slightly lower capital cost on feed, the net income of FD pond (129,974 CNY/ha) was doubled when compared with FFD pond (63,630 CNY/ha). By changing the feed type from FFD to FD, the profit rate increased from 30.7% to 46.1% without any extra expense on feed.

3.2. Microbial Community Diversity and Distribution

On average, 52,444 quantified reads were achieved for all the water and sediment samples. The α-diversity of all samples are shown in Figure 1, and a significant difference between groups was determined by a Wilcoxon test. The sediment samples had significantly higher diversity compared with water samples (Figure 1). No significant difference in microbial diversity between FD and FFD groups was observed in sediment samples. Significantly higher microbial diversity was observed in FD water samples than FFD water samples by Shannon index (Figure 1a), which accounted for phylogenetically similar taxa since no difference in phylogenetic diversity (PD) in the whole tree was detected (Figure 1d).
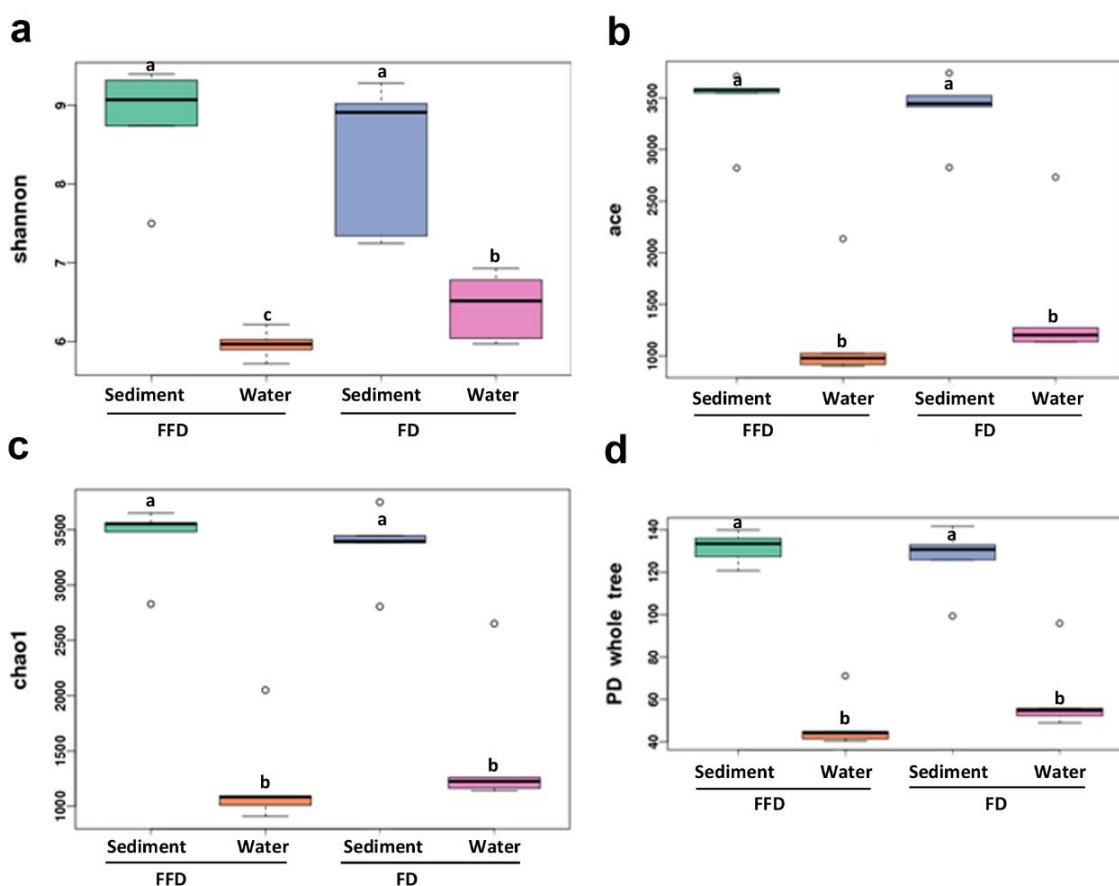
Figure 1. The alpha diversity ((**a**) Shannon; (**b**) ace; (**c**) chao1; (**d**) phylogenetic diversity (PD) whole tree) of sediment and water samples from both fresh frozen diet (FFD) and formulated diet (FD) treatments. The bar with different letters indicates significant difference (*p* < 0.05).

In addition, the microbial distribution of sediment and water samples were analyzed by principal coordinate analysis (PCoA) using a weighted Unifrac distance matrix (Figure 2). Sediment and water samples displayed distinct differences in microbial composition between treatments (Figure 2a). The first axis explained 89.4% of the variations which was associated with the sample type (i.e., sediment and water). Water samples showed a significantly (*p* < 0.001) lower variation than sediment samples, presented by weighted Unifrac distance, in the FFD pond (Figure 2b). The extent to which feed type (i.e., FFD and FD) could influence the microbial community composition in water and sediment samples was similar according to weighted Unifrac distance (Figure 2c). However, analysis of similarity (Anosim) demonstrated that the difference in microbial community between treatments was only significantly (*p* < 0.05) bigger than within the treatments in water samples (Table S3).

3.3. Microbial Composition in Water and Sediment

The top 10 abundant phyla and genera among all samples are shown in Figure 3. Proteobacteria was the most dominating phylum in both water (average 40.1%) and sediment (61.9%) samples, followed by Cyanobacteria (average 21.9%) in the water and Bacteroidetes (average 13.3%) in sediment. On the genus taxa level, *Synechococcus*, *Marivita* and *Owenweeksia* had higher abundance in FFD than FD, whereas *Candidatus_Aquiluna* was higher in abundance in the FD pond. Moreover, the significant difference in relative abundance between the two treatments at the phylum level was analyzed by *t*-test. Results showed that at the phylum taxa level, 5 out of 50 and 7 out of 43 phyla were detected as significantly (*t*-test, *p* < 0.05) different between the two treatments in sediment and water samples, respectively (Table 2). In water samples, the abundance of Cyanobacteria was significantly higher in

the FFD than in the FD pond (Table 2). Bacteroidetes were also found to be significantly higher in the FFD water samples (Table 2). Also, the abundance of Firmicutes, Tenericutes, Cloacimonetes and Lentisphaerae were significantly higher in the water of the FFD pond than the FD.
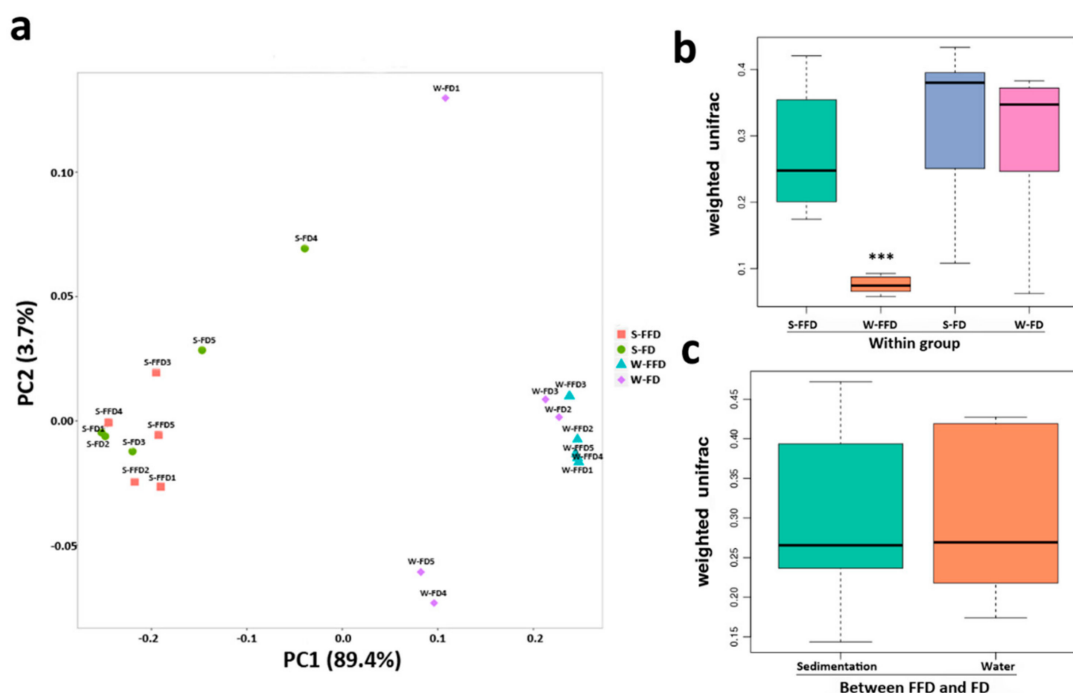
Figure 2. The microbial community distributions of sediment (marked as S) and water (marked as W) samples. (**a**) Principal coordinate analysis (PCoA) of both sediment and water samples; (**b**) weighted Unifrac distance within group; (**c**) weighted Unifrac distance between FFD and FD ponds.

Table 2. The average relative abundance of significantly different phylum taxa in sediment and water samples.

| Phylum		FFD		FD		*p* Value
		Average (%)	Sd	Average (%)	Sd	
Sediment	Chloroflexi	1.13×10^{-2}	2.85×10^{-3}	1.88×10^{-2}	6.06×10^{-3}	0.04698
	Spirochaetae	1.04×10^{-2}	7.33×10^{-4}	6.79×10^{-3}	2.97×10^{-3}	0.04991
	TM6	2.98×10^{-3}	1.04×10^{-3}	1.47×10^{-3}	6.03×10^{-4}	0.02828
	Lentisphaerae	5.86×10^{-4}	3.08×10^{-4}	1.29×10^{-4}	8.44×10^{-5}	0.02700
	WCHB1-60	1.17×10^{-4}	8.55×10^{-5}	5.86×10^{-6}	1.31×10^{-5}	0.04258
Water	Bacteroidetes	2.24×10^{-1}	1.91×10^{-2}	1.36×10^{-1}	4.92×10^{-2}	0.01314
	Cyanobacteria	2.73×10^{-1}	2.99×10^{-2}	1.65×10^{-1}	4.36×10^{-2}	0.00256
	Firmicutes	3.09×10^{-3}	1.71×10^{-3}	1.21×10^{-2}	2.78×10^{-3}	0.00054
	Chlorobi	2.10×10^{-2}	4.23×10^{-3}	3.76×10^{-3}	1.68×10^{-3}	0.00030
	Tenericutes	8.73×10^{-4}	1.08×10^{-4}	4.34×10^{-4}	3.19×10^{-4}	0.03393
	Cloacimonetes	8.97×10^{-4}	3.57×10^{-4}	3.46×10^{-4}	3.08×10^{-4}	0.03147
	Lentisphaerae	3.99×10^{-4}	2.88×10^{-4}	1.17×10^{-5}	1.61×10^{-5}	0.03981
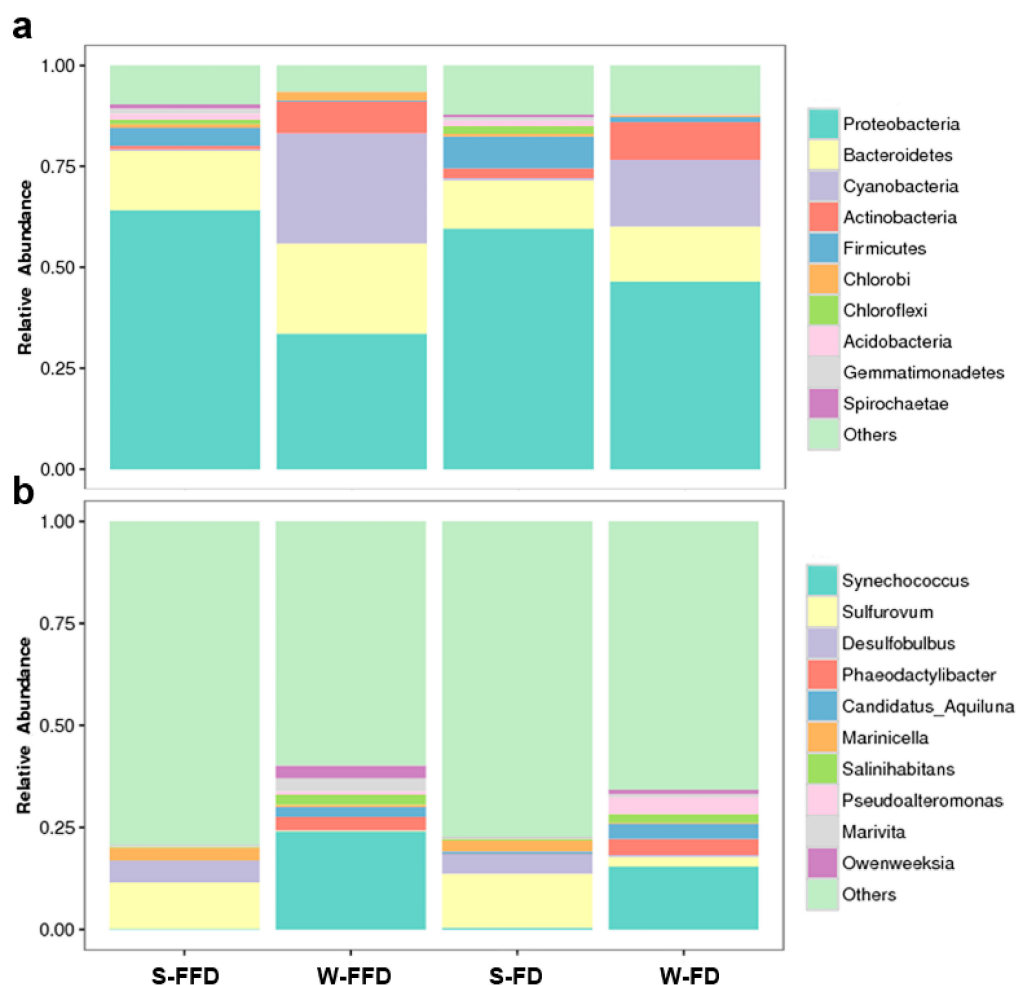
**Figure 3.** The top 10 taxa at both (**a**) phylum and (**b**) genus level in all samples.

3.4. Correlations between Water Quality, Microbial Community and Functionality

RDA analysis was performed to explore the correlation between water quality and water microbial community at the phylum level (Figure 4). Water quality and nutrient concentration were found to be correlated with the microbial community distribution. The best-fitting environmental parameters explaining the microbial community composition were TOC, pH and phosphorous. In our analysis, the water quality parameters explained 94.23% of the variance in water microbial community. Proteobacteria and Bacteroidetes were not significantly correlated with the water quality index, implying that inorganic nutrients did not change the dominating phylum in the water. On the other hand, Cyanobacteria and Chlorobi were significantly correlated with inorganic nitrogen, phosphorous and TOC.

Furthermore, the bacterial interactions within each pond were visualized by co-occurrence network analysis on the genus taxa level (Figure 5). The nodes represent a unique genus and were colored according to their phylum taxa. The size of the nodes was propositional to the number of degree (i.e., interactions). Positive interactions are depicted in green lines and negative interactions are depicted in red lines. In the FFD pond, 41 nodes generated 686 edges of which 56.7% was positive interactions. On the other hand, 51 nodes generated 883 edges in the FD pond which included 52.7% positive interactions. The number of average degrees of FFD and FD treatments were 33.3 and 34.6, respectively. In the FFD pond (Figure 5a), *Sulfurovum* was positively correlated with *Desulfobulbus* and *Sulfurimonas*. *Vibrio* had the highest weighted degree in FFD pond, which was negatively correlated with *Synechococcus* and *Pseudoalteromonas*, and positively correlated with *Propionigenium*, *Alistipes* and *Reichenbachiella*. Moreover, *lactobacillus* was also found to have a strong correlation with other species in the FD pond (Figure 5b).
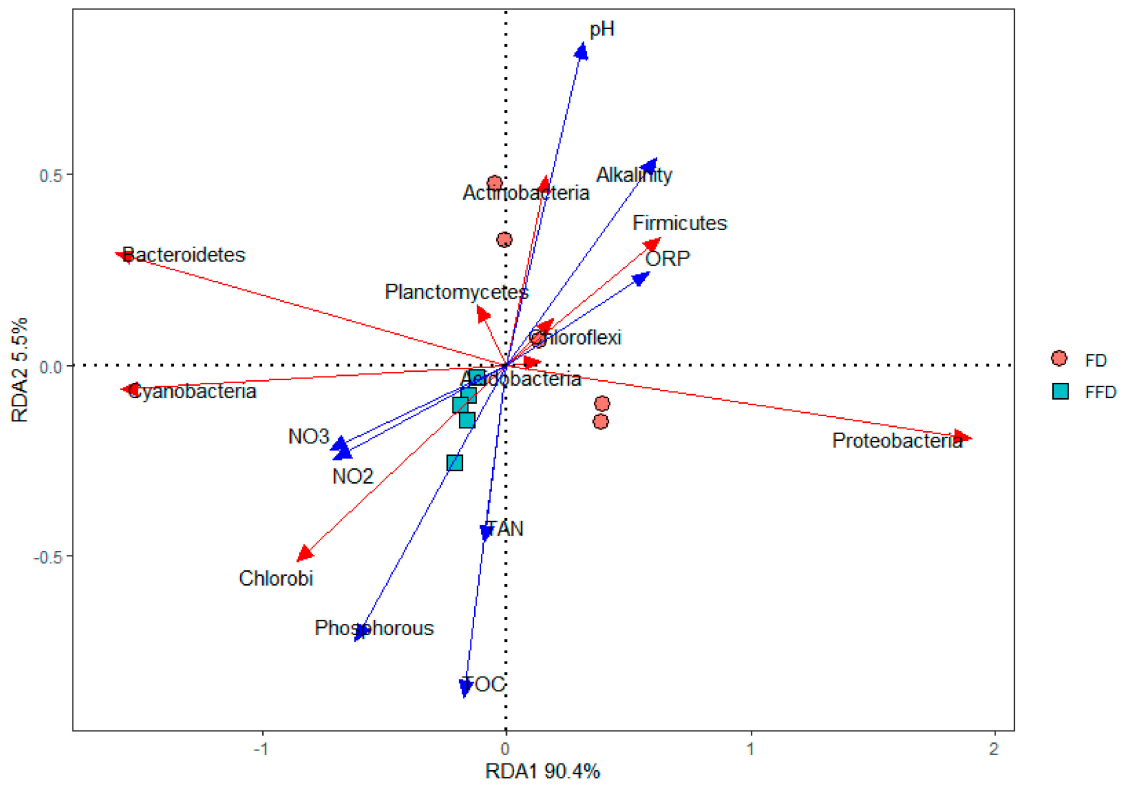
Figure 4. The correlation between water quality and water microbial community by redundancy analysis (RDA).
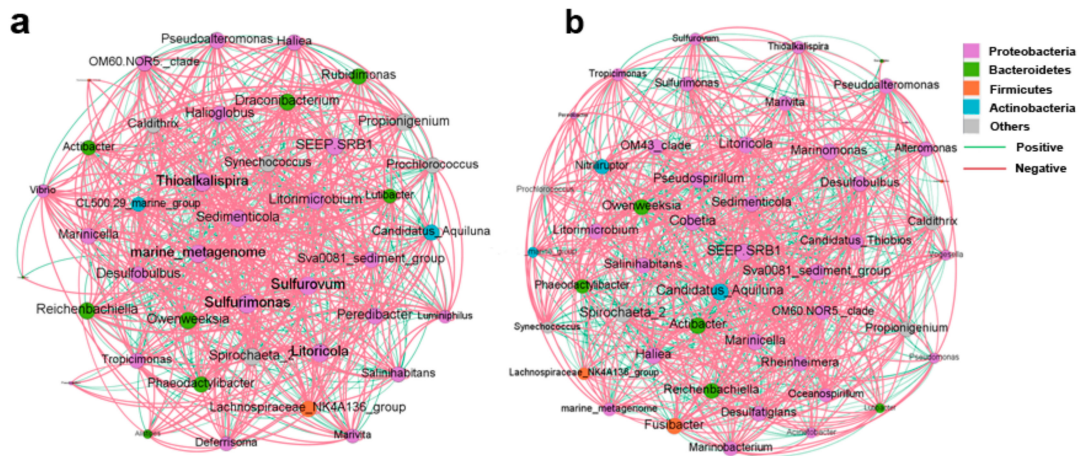
Figure 5. Co-occurrence network of bacterial interactions in both (**a**) FFD and (**b**) FD ponds.

The metabolic pathways in both the FFD and FD pond samples were predicted according to KEGG database. The significantly different ($p < 0.05$) metabolisms between the two treatments are shown in Figure 6. In sediment, the FFD pond had higher abundance of metabolic pathways related to antibiotic synthesis, including tetracycline biosynthesis and novobiocin synthesis (Figure 6a). On the other hand, the FD pond had higher expression of organic carbon degradation and polycyclic aromatic hydrocarbons and ethylbenzene degradation (Figure 6a). In the water community, the FFD pond had high activity in energy metabolism, i.e., photosynthesis (Figure 6b). Moreover, lipid metabolism, such as fatty acid degradation and primary/secondary bile acid biosynthesis in the FD was always higher than the FFD in water samples (Figure 6b).
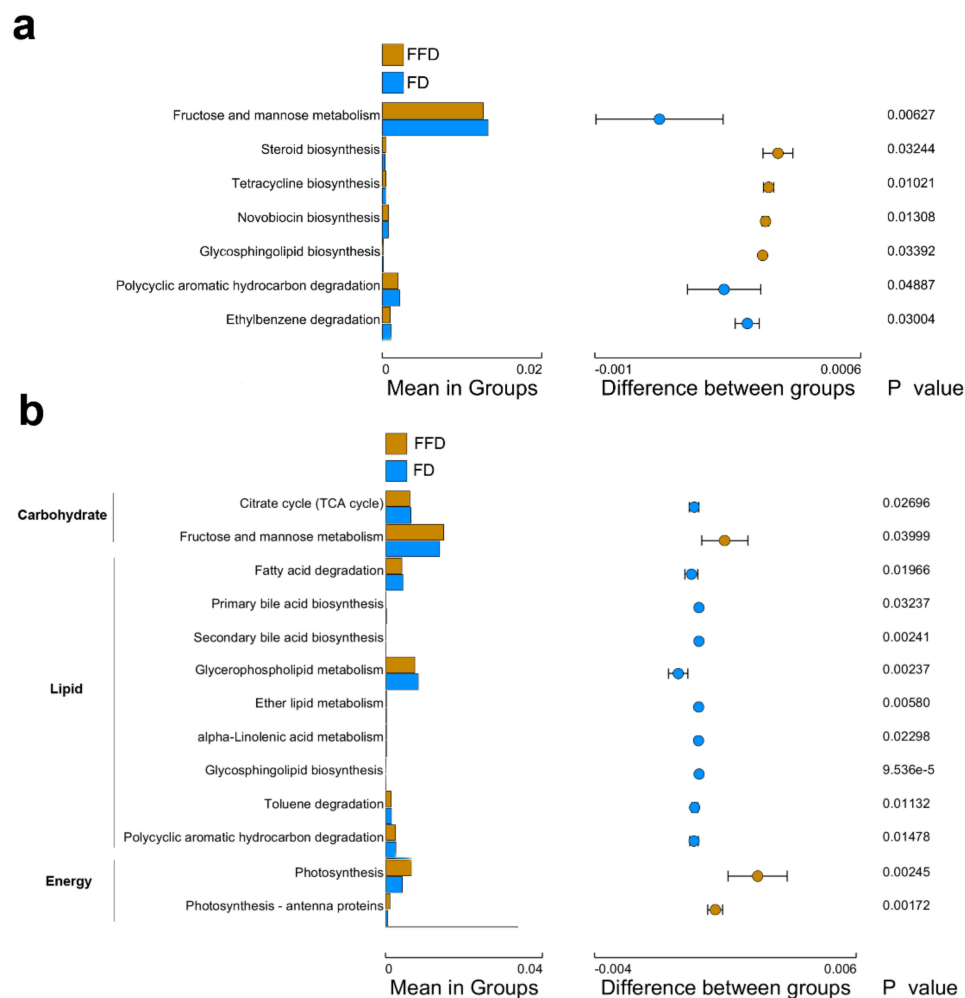
Figure 6. Significantly different metabolism pathways between FFD and FD in (**a**) sediment and (**b**) water samples.

4. Discussion

4.1. Water Quality and Its Effect on Microbial Distribution

The integration of filter-feeding organisms (e.g., razor clams and Malyasian cockles) as well as microalgae and bacteria that are able to uptake particles and nutrients from the water column may decrease disease outbreak and maintain water quality for intensive farming. However, the nutrient concentrations, including nitrogen, TOC and phosphorous, were found to be significantly higher in the FFD pond than the FD pond (Table 1). This could be explained by the higher protein and lower carbohydrate content in frozen fish compared to the formulated diet, which would cause more nitrogen excretion from the cultured animals [21,29]. Meanwhile, the low digestibility of the fresh frozen fish diet and low nitrogen-retention efficiency of raw fish produced more waste into the water and led to water quality deterioration [30]. On the other hand, the pH and alkalinity of the FD was significantly higher than the FFD. The decomposition of uneaten fresh fish may acidify the water body and decrease the alkalinity in the FFD pond. A lower pH was also detected in the sediment of the FFD pond (pH 6.38) than the FD pond sediment (pH 6.85).

On the other hand, the microbial community diversity is largely influenced by the availability of carbon sources and the ecological niche [31]. Sediment has higher accumulation of uneaten feed and feces than the water, providing an abundant carbon source for bacteria growth. Moreover, through the depletion and consumption of oxygen during transportation through the surface of sediment, the microenvironment changes from aerobic to anaerobic, providing more niches for bacteria than

water [32]. Therefore, higher bacterial diversity was observed in the sediment than in the water samples. Moreover, the higher diversity in the FD water than the FFD water could be explained by the highly diverse carbohydrate composition in formulated diet, though the sediment did not show the same trend (Figure 1a).

4.2. Microbial Composition in Water and Sediment

Water samples showed a significantly ($p < 0.001$) lower variation than sediment samples, presented by weighted Unifrac distance, in the FFD pond (Figure 2b). Normally, water is more homogenous than sediment, which could explain the low variation in water. However, a similar result was not detected in the FD pond which could be explained by the bigger area of the FD pond, resulting in less homogeneity in the water. Analysis of similarity (Anosim) demonstrated that the difference in microbial community between treatments was only significantly ($p < 0.05$) bigger than within the treatments in water samples (Table S3). The treatment effect on sediment microbial community was masked by spatial effect in our study, and more sampling points should be collected to explore the spatial effect in the future research. Therefore, we could hardly conclude that feed type could change the microbial community in the sediment due to the large variations detected within sediment samples.

In a natural ecosystem, different microbial community composition has been discovered between water and sediment [33]. This could be explained by the niche difference and nutrient availability, which plays a key role in microbial distribution [34]. In water samples, Proteobacteria and Cyanobacteria were the top two abundant phyla in the pond systems. Proteobacteria were found high abundance in marine environments [35]. Cyanobacteria, also known as blue–green algae, is an oxygenic photoautotroph which is the principle primary producer in ponds [36]. The relatively high inorganic nutrient level of the FFD pond (Table 1) may favor the growth of Cyanobacteria which could provide extra food and oxygen to farmed animals during the daytime. However, the high proportion of Cyanobacteria in the FFD water (average 27.3%) may cause algae bloom, especially during the summer season, introducing toxicity to animals and deoxygenation of bottom water [37]. Bacteroidetes have also shown high relative abundance in FFD water samples, which might be associated with its activity in the degradation of complex organic matters, especially polysaccharides and proteins (e.g., algae and animal compounds) [38]. At the genus level, *Synechococcus*, members of Cyanobacteria, together with *Marivita*, belong to phytoplankton and can perform photosynthesis [39]. *Candidatus_Aquiluna* is also a photoheterotroph, which is a member of Actinobacteria [40].

Sediment served as a sink for ammonia, sulfide and reactive phosphate, and is the location for the most active microbial transformations involving organic matter and nutrient cycling in ponds [41]. Chloroflexi was the only phylum that had a high percentage in the FD sediment (Table 2), which was associated with the inclusion of a plant-derived component in the formulated diet. Species belonging to Chloroflexi were found responsible for the degradation of carbohydrates in wastewater treatment reactors [42,43]. Metagenomics analysis revealed that Chloroflexi played a role in carbon cycling of sediment environment [44]. The undigested complex polysaccharides in formulated diets may accumulate in the sediment and increase the abundance of Choroflexi. At the genus taxa level, *Sulfurovum* and *Desulfobulbus* were the two dominating genera in sediment (Figure 3b), both of which are related to sulfur metabolism [45]. However, no significant difference in the abundance of the top 10 genera was discovered between FFD and FD in sediment samples. The difference in sediment samples was mainly introduced by rear species (data not shown).

Microalgae and bacteria have both stimulatory and inhibitory effects on each other through modification of the chemical microenvironment of the other group through metabolic activities [13]. For instance, bacteria can stimulate phytoplankton growth by regenerating nutrients, and algae can produce polysaccharides that are readily utilized by heterotrophic bacteria. Nevertheless, phytoplankton and chemoautotrophic bacteria also compete for inorganic nutrient substrates, especially ammonia. Besides, algae can produce antibiotics and some bacteria can lyse phytoplankton cells [46]. There are trade-offs associated with dominance by phytoplankton or bacteria in pond aquaculture.

Phytoplankton is an import oxygenator and contributes ammonia removal, however the disadvantages of phytoplankton-dominated ponds (i.e., FFD pond) are the low degree of control over water quality, community composition due to high dependence on solar availability, and undesired metabolism. On the contrary, bacteria-dominated ponds (i.e., FD pond) are more stable in water quality, but require an external oxygen source to support nutrient cycling [13]. Moreover, it was demonstrated feasible to promote the nutrient cycling and recovery by adding organic carbon to increase the bacterial floc activity in aquatic systems [47,48].

4.3. Correlations between Water Quality and Microbial Community

The RDA analysis agreed with the fact that many species belonged to Cyanobacteria and Chlorobi are capable of nitrogen fixation to support primary production [49,50]. The high composition of Cyanobacteria and Chlorobi in the FFD pond suggested that the nitrogen removal was mainly performed through nitrogen fixation. The low carbohydrate concentration in trash fish may hardly support the denitrification in the FFD pond since not enough electron donors were available. It was documented that pond systems rely on natural processes for purification, including ammonia which is converted to less-toxic nitrate through nitrobacteria and nitromonas, while nitrate is taken up by phytoplankton and macroalgae [51]. Actinobacteria and Firmicutes were found to be positively correlated with pH, ORP and alkalinity in our study. The pH is a main factor in shaping the soil microbial community where Actinobacteria was also discovered positively correlated with soil pH at a continental scale [52].

Network-based approaches have been used to investigate the ecological patterns between microorganisms and identify potential interactions of keystone species [27,34]. In the FFD pond (Figure 5a), *Sulfurovum* was positively correlated with *Desulfobulbus* and *Sulfurimonas*, which can be explained by their similar functionality in oxidizing sulfur [53]. *Vibrio* had the highest-weighted degree in the FFD pond, which was found to have a high abundance in fish guts when fed with trash fish [21]. *Vibrio* is one of the most prominent bacteria genera causing disease for marine aquaculture [54]. In the FFD pond, *Vibrio* was negatively correlated with *Synechococcus* and *Pseudoalteromonas*, and positively correlated with *Propionigenium*, *Alistipes* and *Reichenbachiella*. Here, it was reported that *Pseudoalteromonas* could produce beneficial antimicrobial compounds [55], which could explain its negative correlation with the facultative pathogenic *Vibrio*. In the FD pond (Figure 5b), *Prochlorococcus*, a genus of Cyanobacteria, had the highest-weighted degree, which was negatively correlated with *Sulfurimonas*. Moreover, *lactobacillus* was also found to have a strong correlation with other species in the FD pond, which has been identified as a probiotic in aquaculture to increase animal growth and health [56]. Overall, different feed types selected different functional species by network analysis. The high degree of pathogenic *Vibrio* in the FFD pond and probiotic *Lactobacillus* in the FD pond implied the high risk of disease outbreak with fresh frozen diet when the water quality is not optimal in ponds.

4.4. Different Microbial Functionality Triggered by Feed Type

In the sediment community, the FFD pond had higher abundance of metabolic pathways related to antibiotic synthesis (Figure 6a). Unlike the formulated diet, which is processed under heat conditions, the fresh frozen fish diet is unprocessed and might contain potential pathogens and cause enrichment of environmental antibiotics. Moreover, the organic enrichment of uneaten fish in the sediment led to an increased presence of pathogenic bacteria [10]. The presence of high potential pathogenic bacteria might increase the growth of microorganisms producing antibiotics in FFD pond. However, the presence of the antibiotic pathway detected in the FFD pond may increase the possibility of antimicrobial resistance, which can influence human health [57]. The degradations of polycyclic aromatic hydrocarbons and ethylbenzene were found to have high activity in the sediment of the FD pond. This could be explained by the use of a formulated diet which contained agrochemical contaminants including polycyclic aromatic hydrocarbons and ethylbenzene [58].

In the water community, the FFD pond had high activity in energy metabolism, i.e., photosynthesis (Figure 6b). This result was consistent with the presence of relative higher abundance of Cyanobacteria in the water of the FFD pond (Figure 3a). The FFD pond had a relatively low carbon/nitrogen ratio in the water (Table 1), which was favorable for the growth of autotrophic microorganisms. Instead, the formulated diet contains some plant-derived compounds, such as soy protein, starch and cellulose. The metabolic pathway of fish gut microbiota also revealed higher carbohydrate metabolism when fish were fed with formulated diet than a trash fish diet [21]. Thus, the formulated diet pond had high activity related to citrate cycle (Figure 6b). It is worth noting that the lipid metabolism in FD was always higher than FFD in water samples. Possible explanations include the high lipid composition in formulated diet and the low digestibility of plant-based lipids which could leak into the water body, which might increase the lipid metabolism in water.

4.5. Investment and Benefits Analysis

Better growth performance was achieved in the FD than the FFD pond in terms of both main species and intercropping species (Table S1). Razor clams are filter feeders, mostly feeding on microalgae and organic matters in the water and sediment. Here, we speculated that the potential microbial infection (i.e., *Vibrio*), limited feed availability and acidification in the sediment (data not shown) might explain the loss of razor clam in FFD pond. When considering that 11.4 tonne/hectare fresh frozen fish was used in the FFD pond and 2.6 tonne/hectare formulated diet was used in the FD pond, a much lower feed conversion ratio (FCR) was achieved in FD than FFD. Moreover, the substantial demand of fresh fish for the FFD pond is limited since capture fisheries cannot expand further.

With similar expenses per hectare in both FFD and FD ponds, the net income of the FD pond was doubled when compared with the FFD pond (Table S4). This difference in net income was mainly caused by the higher production of razor clams, Malaysian cockles and mud crabs in the FD pond. Because of the higher average body weight, the price of Malaysian cockle in the FD pond (36 CNY/kg) was relatively higher than the FFD pond (30 CNY/kg), which also contributed to the higher revenues in the FD pond. By changing the feed type from FFD to FD, the profit rate increased from 30.7% to 46.1% without any extra expense on feed. Overall, the formulated diet was proved to be more ecologically and economically sustainable than the frozen fish diet when considering the low potential of organic pollution and eutrophication (Table 1), less carbon footprint requirement and better maintenance of biodiversity (Table S1), less dependence on fisheries had high benefit reward (Table S4) and potentially beneficial bacterial interactions (Figure 5).

5. Conclusions

The two types of feed, namely fresh frozen fish diet and formulated diet, shaped the microbial assembly in mainly water samples of IMTA pond systems. The shift in microbial community and functionality changed the pattern of nutrient cycling and farmed-species growth performance. Sediment had significantly higher microbial diversity than the rearing water. The water samples from the FFD pond were dominated with Cyanobacteria which can perform nitrogen fixation through photosynthesis. The formulated diet triggered higher metabolic pathways related to carbon and lipid metabolism in the water of the FD pond. The sediments were dominated with *Sulfurovum* and *Desulfobulbus*, which were found to be positively correlated by network analysis in sulfur metabolism. Relatively higher rates of antibiotic biosynthesis were predicted in FFD, which might be related to the pathogenic bacteria introduced by the trash fish diet. Overall, the preferable water quality, higher cost-efficiency and presence of beneficial bacteria interactions proved that the formulated diet was more ecologically and economically sustainable than the frozen fish diet for a marine IMTA pond system.

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