Bacterial composition and inferring function profiles in the biofloc system rearing *Litopenaeus vannamei* postlarvae at a low salinity

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Abstract

This study investigated the bacterial composition and inferring function profiles in the biofloc system rearing *Litopenaeus vannamei* postlarvae (PL) at a low salinity condition. PL (~ stage 15) were stocked in four parallel tanks filled in water with a salinity of 5.0‰ at a density of 4000 individuals per m^3 for a 28-days culture experiment, during which glucose was added as a carbon source with a C:N of 20:1. At the end of the experiment, water was sampled from each tank and pooled to extract microbial DNA for high-throughput sequencing of the V3-V4 region of the 16S rRNA gene. Results showed that the bacterial community at 28 d was dominated by phyla of *Proteobacteria* (45.8%), *Bacteroidetes* (21.1%), *Planctomycetes* (13.5%), *Chlamydiae* (10.3%), and *Firmicutes* (6.8%). A proportion of 81% inferring KEGG functions of this bacterial community is associated with metabolism. Among functions relating to nitrogen metabolism, 48.5% were involved in converting ammonia to glutamate. Still, the proportion of those engaged in transformation among ammonia, nitrite, and nitrate was 18.0%, inferring higher protein-synthesis but lower inorganic nitrogen-transformation capacities of the bacterial community. At the same time (28 d), high levels of total nitrogen (231.3±6.0 mg L^{-1}) and biofloc (127.0±63.0 mL L^{-1}) but low concentrations of ammonia (0.04±0.01 mg L^{-1}), nitrite (0.2±0.1 mg L^{-1}) and nitrate (12.9±2.5 mg L^{-1}) were observed. The results supply a novel insight for understanding bacterial community function in the biofloc system nursing *L. vannamei* PL at low salinity.

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Introduction

Litopenaeus vannamei is the most important cultured crustacean species globally, whose culture production accounted for 52.9% of the total crustaceans production in the 2018 (FAO, 2018). During the culture of *L. vannamei*, after the larvae period and before the grow-out period, postlarvae (PL) are sequentially nursed for an intermediate period called the prenursery phase (Mishra et al., 2008; Rezende et al., 2018). With prenursery, larger PLs are delivered for the subsequent grow-out culture to increase the survival and growth performance due to the higher resistance of larger PLs to environmental conditions. However, in the conventional prenursery system, due to intensively high stocking density ($\sim$ 60 PL L^{-1}), controlling toxic ammonia and nitrite is a big problem, as well as the biosecurity followed by operations such as water exchange for the management of those nitrogen compounds (Samocha, 2010). Recently, biofloc technology (BFT) has been tried to be used to nurse *L. vannamei* PL (Khanjani et al., 2017; Rezende et al., 2018; Schveitzer et al., 2017) due to the advantages of this technology on nitrogen assimilation *in situ* and pathogen control under minimal or zero water exchange conditions (Avnimelech, 2015; Huang, 2020). Besides, biofloc is also rich in nutrients, immunostimulants, and bioactive compounds, such as essential amino acids (Li et al., 2018), unsaturated fatty acids (Ray et al., 2019), lipopolysaccharide (LPS), and carotenoids (Ju et al., 2008; Vargas-Albores et al., 2019), contributing to growth-improvement, immune-enhancement and probiotic effects for shrimp (Panigrahi et al., 2019c).

Biofloc is an aggregate of bacteria, protozoan, feces, and organic detritus (Avnimelech, 2015; Schryver et al., 2008), among which the bacterial community is considered to play an essential role in the advantages of biofloc mentioned above (Cardona et al., 2016; Ju et al., 2008). Previous studies show that the bacterial community in the marine water biofloc systems rearing *L. vannamei* is dominated by Proteobacteria, Bacteroidetes, Planctomycetes, and Firmicutes (Huerta-Rabago et al., 2019; Martínez-Córdova et al., 2018; Vargas-Albores et al., 2019; Xu et al., 2019). Those phyla are essential to maintaining good water quality for the biofloc system because many species belonging to them use organic matter and nitrogen compounds for growth, such as *Thiotrichaceae*, *Rhodobacteraceae,* and *Saprospiraceae* (Cardona et al., 2016). Additionally, the inferring functions of the bacterial community in the biofloc system, including nitrogen metabolism, biosynthesis of nutrients, immunostimulants, and bioactive compounds, indicate associations of bacterial community with shrimp growth and water quality (Hargreaves, 2013; Ju et al., 2008; Vargas-Albores et al., 2019). From this point of view, studies on bacterial composition and its inferring function profiles would supply profound insights into understanding the role of the bacterial community in the water of the biofloc system.

L. vannamei is an euryhaline species. It could be cultured at low salinity conditions, less than 1.0‰, which is a trend that will continue to grow globally (Roy et al., 2010). *L. vannamei* develops gradually in the postlarvae stages and can quickly be acclimated to salinities as low as 0.5‰ by PL12 (Van Wyk et al., 1999), indicating that PL after stage 12 could be reared at a low salinity condition for a prenursery phase. And recently, in some studies, biofloc technology has been used for prenursery of *L. vannamei* PL under low salinity conditions (8-16‰) (Esparza-Leal, et al., 2016; Luo et al., 2019). However, there is little information about the bacterial community and its inferring functions in the low-salinity biofloc system until now.

This study aimed to investigate the bacterial composition and inferring function profiles in a biofloc system rearing *L. vannamei* PL at a salinity of 5.0‰, to deeply understand the function of the bacterial community in the biofloc system.

Material and methods

Ethics statement

The experiments were carried out at a local farm of Bifuteng eco-agriculture Development Co., Ltd. (BEAD Co., Lat. 28°53'57.88" N, Long. 111°38'3.08" E) and Hunan

University of Arts and Science (HUAS, Lat. 29°3'0.12" N, Long. 111°40'11.43" E), both of which locate in Changde, China, under principles in good laboratory animal care, according to the national standard of China (GB/T 35892-2018), 'Laboratory animal-Guideline for ethical review of animal welfare. The manuscript does not require ethical approval.

Preparation of culture water

Water with a salinity of 5.0‰ for the culture experiment was prepared according to Ray and Lotz (2017), with some modifications. Briefly, artificial sea salt powder (Qianglong corporation, Tianjin, China) and food-grade chemical reagents of KCl, $MgCl_2$, and $CaCl_2$ were added to tap water to make a final salinity of 4.96‰ (~5.0‰, detected with an electric salinity analyzer, AZ8371, Hengxin technological Co., Ltd., China), with K^+, Mg^{2+} and Ca^{2+} concentrations of 292, 934 and 318 mg L^{-1}, respectively (measured according to the standard methods, APHA, 1995). Then, the water pH value was adjusted to near 8.0 using food-grade $NaHCO_3$. After that, sterilization (10.0 mg L^{-1} chlorine dioxide) followed by neutralization (1.0 mg L^{-1} ascorbic acid) was executed according to the processes of previous studies (Gaona et al., 2017; Lara et al., 2017).

Experimental design and operations

Four indoor tanks (width × length × depth = 2 × 2.5 × 1.3 m) of BEAD Co., each of which fixed five porous tubes (2.4 meters in length) in the bottom connecting with a 750-w whirl charging aerator (HG-750S, Sensen Group Co., Ltd., Zhoushan, China), were filled with 5.0 m^3 culture water prepared above. *L. vannamei* PL (~PL15, 2.5±0.5 mg), which had been treated with a desalinating and acclimation procedure to adapt to the experimental conditions (Luo et al., 2019; Van Wyk et al., 1999), were kindly supplied from BEAD Co. and randomly assigned to the four experimental tanks with a stocking density of 4000 individuals per m^3 for a 28-days culture. During the whole culture period, PL was fed with a commercial formulated shrimp diet (crude protein 40.0%, crude lipid 5.0%, crude fiber 5.0%, crude ash 15.0%, moisture 12.0%, Alpha corporation, Jiangmen, Guangdong, China), with a frequency of four times equally a day (6:00, 12:00, 18:00, 24:00), at feeding rates of 25-35% and 20-25% corresponding to the average shrimp weight of < 0.1 g and > 0.1 g, respectively, basing on the estimated total biomass and operations of Van Wyk et al. (1999). Besides, glucose (food grade, carbohydrate content 90.0%, Fufeng Biotechnology Co., Ltd., Hohhot, Inner Mongolia Autonomous, China) was added as an exogenous carbon source, according to carbon to an inputted nitrogen ratio (C:N) of 20:1 (Ebeling, Timmons, & Bisogni, 2006), based on the conception that 25% is theoretically converted as shrimp biomass and 75% would be lost to water body (Piedrahita, 2003). The inputted C:N was the C:N contained in the inputted materials (feed and carbon source). Briefly, 0.9 g carbohydrate or 0.36 g carbon (40% carbon in carbohydrate) is included in 1.0 g glucose with a carbohydrate content of 90.0%. Meanwhile, 0.4 g protein or 0.064 g nitrogen (16% nitrogen in protein) is contained in 1.0 g formulated feed due to the crude protein content of 40.0% (Avnimelech, 1999; Ebeling et al., 2006). Furthermore, 0.384 g carbon is contained in 1.0 g feed according to the calculating method of (Kumar et al., 2017). Thus, in the present study, 1.6 g glucose needed to be inputted when 1.0 g feed was fed to shrimp. No water exchange was operated throughout the experimental period, but the evaporating loss was complemented with dechlorinated tap water per week.

Water sampling and high-throughput sequencing of 16S rRNA gene

At 28 d, 50 ml water was collected from each tank and pooled as one sample according to Martínez-Córdova et al. (2018); and a 200 ml sample was obtained in total. The water sample was filtered through a 0.22-µm pore size membrane. After that, the membrane was collected to extract the bacterial DNA genome with an E.Z.N.A™ Mag-Bind Soil DNA Kit (OMEGA Bio-Tek, Inc., GA, USA), according to the manufacturers' instructions. The

genome was taken as a template to amplify the V3-V4 region of the 16S rRNA gene with the universal primers, 341F: 5'-CCTACGGGNGGCWGCAG-3' and 805R: 5'-GACTACHVGGGTATCTAATCC-3', in a 30-μl mixture containing microbial DNA (10 ng/μl) 2 μl, forward primer (10 μM) 1 μl, reverse primer (10 μM) 1 μl, and 2X KAPA HiFi Hot Start Ready Mix 15 μl (TaKaRa Bio Inc., Japan), via a two-stage PCR procedure with a thermal instrument (Applied Biosystems 9700, USA): 1 cycle of denaturing at 95°C for 3 min, first five cycles of denaturing at 95°C for 30 s, annealing at 45°C for 30 s, elongation at 72°C for 30 s, then 20 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s and a final extension at 72°C for 5 min. Then, the PCR product was purified and recovered with MagicPure Size Selection DNA Beads (TransGen Biotech Co., Ltd., Beijing, China) and quantified and normalized with a Qubit ssDNA Assay Kit (Life Technologies, USA). After that, high-throughput sequencing was conducted on the Miseq platform (Illumina, USA) according to the standard procedure by Sangon Biotech (Shanghai) Co., Ltd.

Bacterial composition analysis

Analyses for the high-throughput sequencing data were carried out under the QIIME 2 (Quantitative Insights Into Microbial Ecology, Version 2019.10) framework (Bolyen et al., 2019). In brief, ambiguous nucleotides, adapter sequences and primers contained in reads, and short reads with lengths less than 30 bp were removed with the cutadapt plugin (Martin, 2011). After that, bases in the two ends of reads with a quality score lower than 25 were trimmed. And then, reads were truncated to the same length from both ends. Reads with too low length to be subjected to the truncated operation were discarded. After that, chimeras were filtered, and pair-ended reads were joined, dereplicated, and clustered to operational taxonomic units (OTU) with an identity of 0.97 using the Vsearch tool (Rognes, Flouri, Nichols, Quince, & Mahé, 2016). then, Coverage, Chao1 index, Berger-Parker index, Shannon index, and Simpson index were computed with the diversity plugin of QIIME 2. Chao 1 is an index measure the theoretical counts of OTUs in a sample and represents the richness of OTUs; Berger-Parker and Simpson's index is the dominance or evenness index. The Berger-Parker index expresses the proportional importance of the most abundant species. At the same time, the Simpson index represents the probability of 2 individuals being conspecifics and decreases with an increase in the dominance of predominant species. Shannon index is a synthetic index for judging the richness and evenness of a sample (Magurran, 2004). Finally, OTUs were annotated with the reference GreenGene database 13.8, collapsed at phylum, class, order, family, and genus levels, respectively, and visualized with a multi-layered pie chart produced Krona (Ondov et al., 2011).

Deducing of inferring functions of bacterial community

Inferring bacterial community functions were deduced with PICRUSt2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) (Douglas et al., 2019). Briefly, OTUs were placed into the reference phylogeny of GreenGene database 13.8 (Barbera et al., 2018; Czech et al., 2020). Then, the hidden-state prediction was run to get 16S copy numbers of OTUs to normalize the predicted KEGG Orthology (KO) functions abundances (Louca & Doebeli, 2017). After that, KEGG pathways abundances were inferred based on predicted KO acts abundances (Ye & Doak, 2009). The results were visualized with a multi-layered pie chart produced by Krona (Ondov et al., 2011).

The interaction, reaction, and relation network of KO functions involved in nitrogen metabolism was recolored in terms of their relative abundances with KEGG Mapper (Kanehisa & Sato, 2020), based on the reference map of 00910 obtained from a database of KEGG PATHWAY (https://www.kegg.jp/kegg/pathway.html).

In addition, Enzyme Commission (EC) functions abundances were also predicted and normalized based on 16S copy numbers obtained from the hidden-state prediction (Louca & Doebeli, 2017) to evaluate the composition of inferring digestive enzymes (EC 3.1, EC

3.2, and EC 3.4). The results were visualized with a multi-layered pie chart produced by Krona (Ondov et al., 2011).

Analysis of contributions of bacteria on inferring functions

Contributions (relative proportions) of bacteria to inferring functions at different levels were analyzed with QIIME 2 (Version 2019.10) (Bolyen et al., 2019), respectively.

Zootechnical measurement

Thirty PL were selected randomly and individually weighed to the nearest 0.1 mg with an electric balance (AUX220, Shimadzu, Japan) each week. The weekly increment rate of body weight (wiR) and specific growth rate (SGR) was calculated according to the following formulates.

$$wiR\ (g\ week^{-1}) = (fbw - ibw)/culture\ weeks$$
$$SGR\ (\%\ d^{-1}) = [(\ln fbw - \ln ibw)/culture\ days] \times 100\%$$

Wherein, fbw and ibw represented PL's final and initial mean body weight, respectively. At 28 d, when the experiment ended, all shrimp in each tank were harvested and counted individually to determine the survival rate (SR).

$$SR\ (\%) = (harvest\ counts\ of\ shrimp/\ stocking\ counts\ of\ shrimp) \times 100\%$$

Water quality monitoring

Water temperature, dissolved oxygen, and pH were detected each day by using electric analyzers (YSI-550A, Yellow Springs Instruments Inc., OH, USA). The water sample of each tank was passed through a 0.45-µm pore size microfilter (Xinya purification equipment Co., Ltd, Shanghai, China) once a week. Then, the filtrate was used to measure total ammonia nitrogen (TAN), nitrite, nitrate, and carbonate alkalinity, and a microfilter was used to measure total suspended solids (TSS), according to the standard methods (APHA, 1995). A water sample without filtration was used to determine the total nitrogen (TN) weekly (APHA, 1995). Biofloc volume represented with settleable solids (SS) was determined per week with an Imhoff cone by reading the sediment volume after a 15 min settlement of a 1-liter water sample (Avnimelech, 2015).

Statistical analysis

Growth and water quality data in the present study were expressed as mean ± standard deviation (SD) and statistically analyzed with the SPSS platform for windows (version 22.0, IBM Co., NY, USA). One-way ANOVA was executed as soon as the normality distribution of data was proved with Shapiro-Wilk's test. Tukey test or Dunnett's T3 test were adopted for post hoc multiple comparisons of data with equal or unequal variances certified using Levene's test, respectively, if a significant difference was found. Or else, a non-parametric Kruskal-Wallis test was conducted, such as TAN, nitrite, biofloc volume (settleable solids), and carbonate alkalinity. Percentage data were submitted to arcsine transformation before statistical analyses. Differences were considered significant at *P* < 0.05.

Results

Bacterial composition

The raw data produced from high-throughput sequencing has been deposited in the NCBI Sequence Read Archive database with the accession number SRR12281666. After quality control, a total of 68411 reads with high quality were obtained and clustered to 3712 OTUs, with a Coverage of 0.96, Chao1 index of 13321.2, Berger-Parker index of 0.07, Shannon index of 7.6, and Simpson index of 0.98. The taxonomy profile was shown in **Figure 1**, where twenty-five phyla were assigned and dominated by Proteobacteria

(45.8%), Bacteroidetes (21.1%), Planctomycetes (13.5%), Chlamydiae (10.3%) and Firmicutes (6.8%). At class level, Alphaproteobacteria (33%), Planctomycetia (11.8%), Saprospirae (11.4%), Chlamydiia (10.3%), Gammaproteobacteria (10.2%), Flavobacteriia (8%), Clostridia (3.6%) and Bacilli (3.2%), were the dominants (Figure 1). The first 10 predominant orders were Rhizobiales (14.1%), Rhodobacterales (13.0%), Saprospirales (11.4%), Chlamydiales (10.3%), Flavobacteriales (8.0%), Pirellulales (7.8%), Pseudomonadales (4.3%), Clostridiales (3.6%), Sphingomonadales (3.1%) and Lactobacillales (3.1%) (Figure 1). There were nineteen families and eleven genera at other levels with a proportion of more than 1.0% (**Figure 1**).
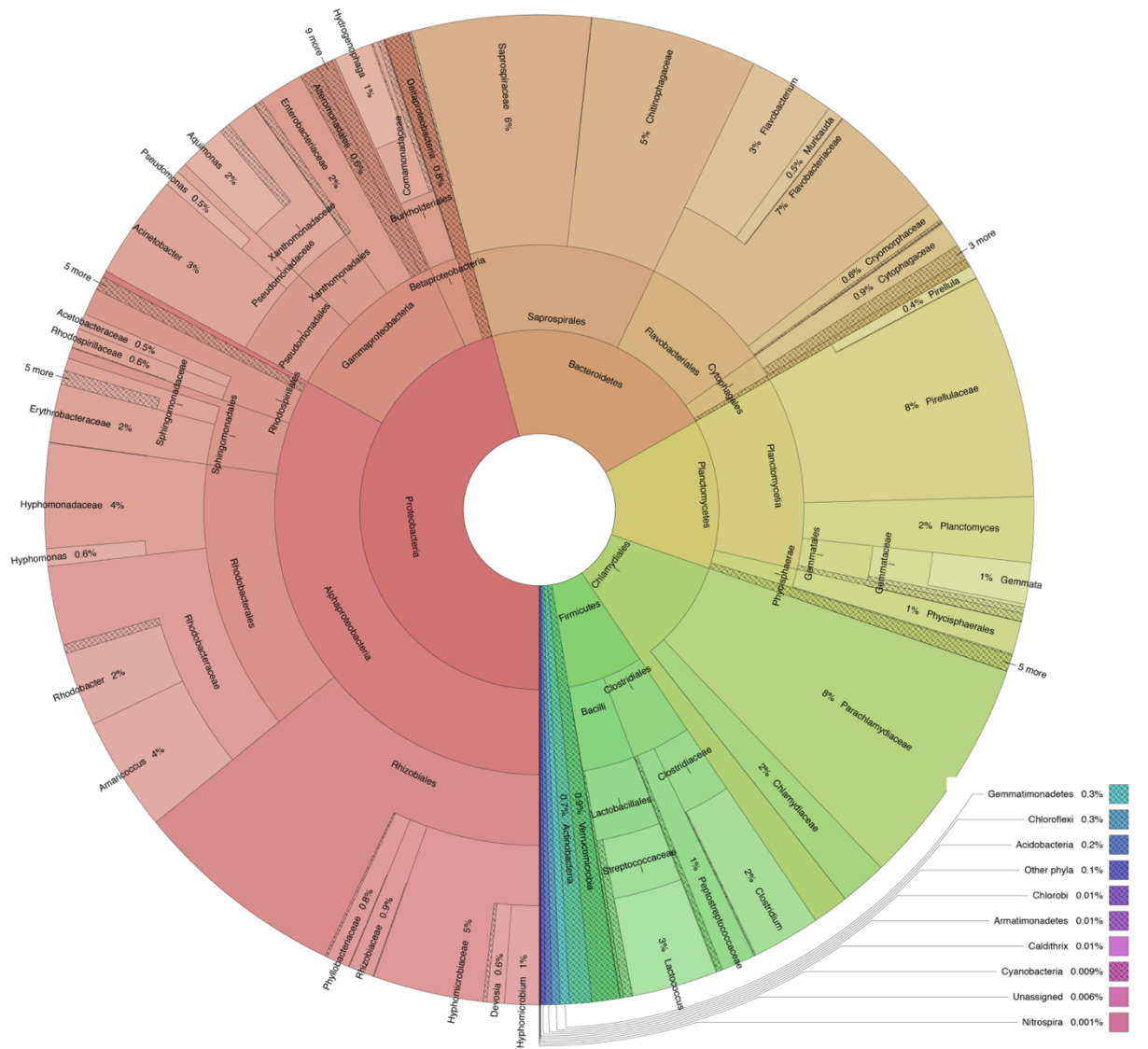

Figure 1 Five-layered pie chart for taxonomic compositions (relative abundance) of bacterial community at the end of the experiment (28 d) in the biofloc system rearing *Litopenaeus vannamei* postlarvae with a salinity of 5.0‰ at levels of phylum, class, order, family, and genera.

Inferring functions of bacterial community

Inferring functions (KEGG pathways) profile of bacterial community at three levels (levels 1-3) were analyzed. At level 1, Most inferring functions (81%) are related to metabolism (**Figure 2**). The proportions of other level-1 functions, such as genetic information processing, cellular processes, environmental information processing, and

organismal systems were 11%, 4%, 2%, and 0.4%, respectively (Figure 2). The most abundant level-2 functions were those relating to the metabolism of nutrients, secondary metabolites, and bioactive compounds, such as amino acids (21%, total of two categories, amino acid and other amino acids), carbohydrate (13%), lipid (8%), energy (5%), cofactors and vitamins (12%), terpenoids and polyketides (9%), xenobiotics (7%), and glycan (3%) (**Figure 2**). The most abundant Level-3 functions were Valine, leucine, and isoleucine biosynthesis (amino acid metabolism), C5-branched dibasic acid metabolism (carbohydrate metabolism), biosynthesis of ansamycins and biosynthesis of vancomysin group antibiotics (metabolism of terpenoids and polyketides), fatty acid biosynthesis and synthesis and degradation of ketone bodies (lipid metabolism), and nitrogen metabolism (energy metabolism) (**Figure 2**).
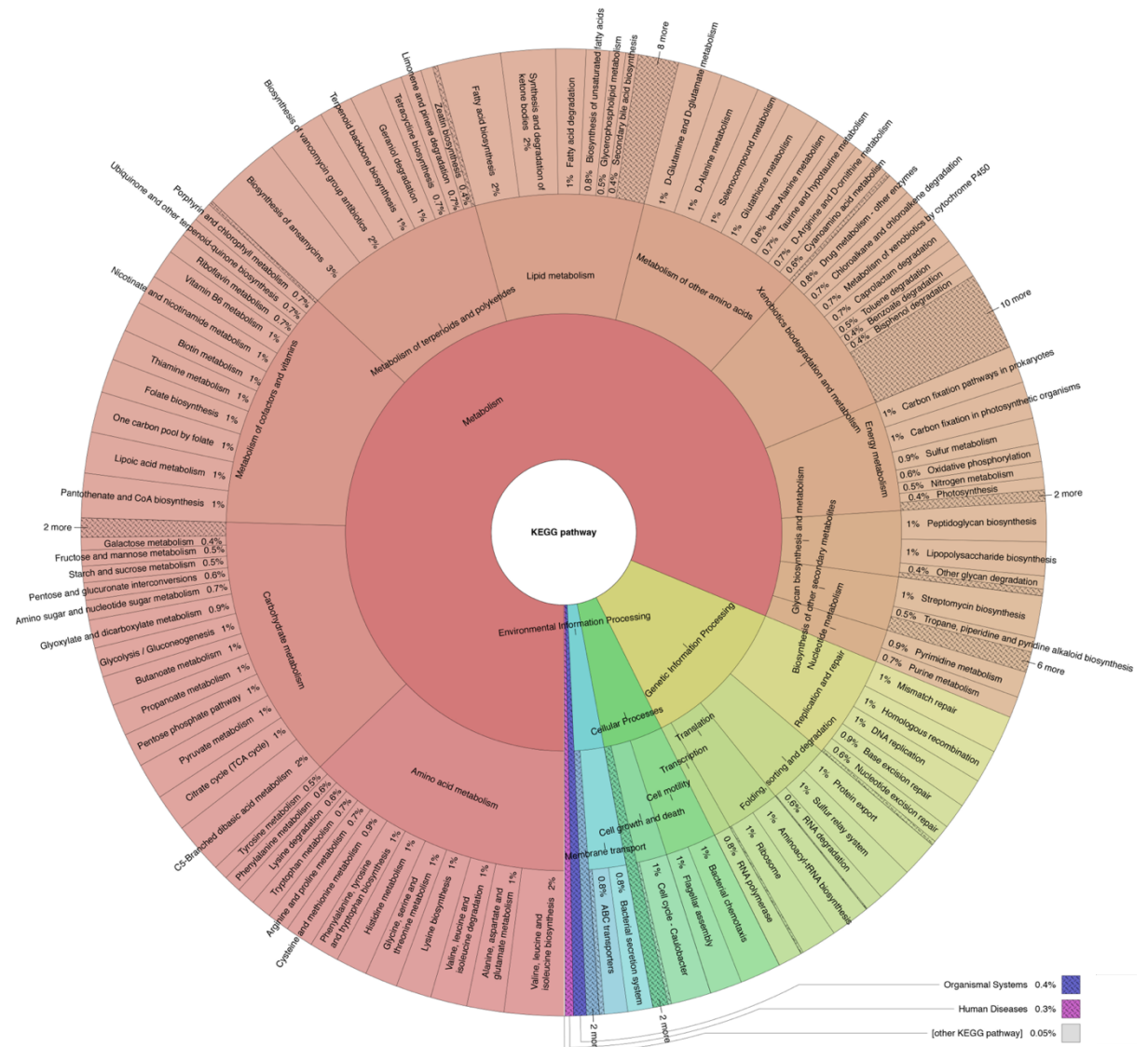

Figure 2 Three-layered pie chart for profiles (relative abundance) of level-1, level-2, and level-3 inferring bacterial community functions at the end of the experiment (28 d) in the biofloc system rearing *Litopenaeus vannamei* postlarvae with a salinity of 5.0‰. The inferring functions were represented with KEGG pathways.

Within the level-3 KEGG pathway of nitrogen metabolism, the most important KO functions were referred to as the conversion of ammonia to glutamate which accounted for 48.5% (**Figure 3**). The proportion of KO functions relating to transformations among inorganic nitrogen compounds, such as nitrification, denitrification, and dissimilatory and

assimilatory nitrate reduction, was 18.0% in total, including 1.2% for nitrite oxidization to nitrate, 7.0% for nitrate reduction to nitrite and 9.8% for nitrite reduction to ammonia (**Figure 3**).
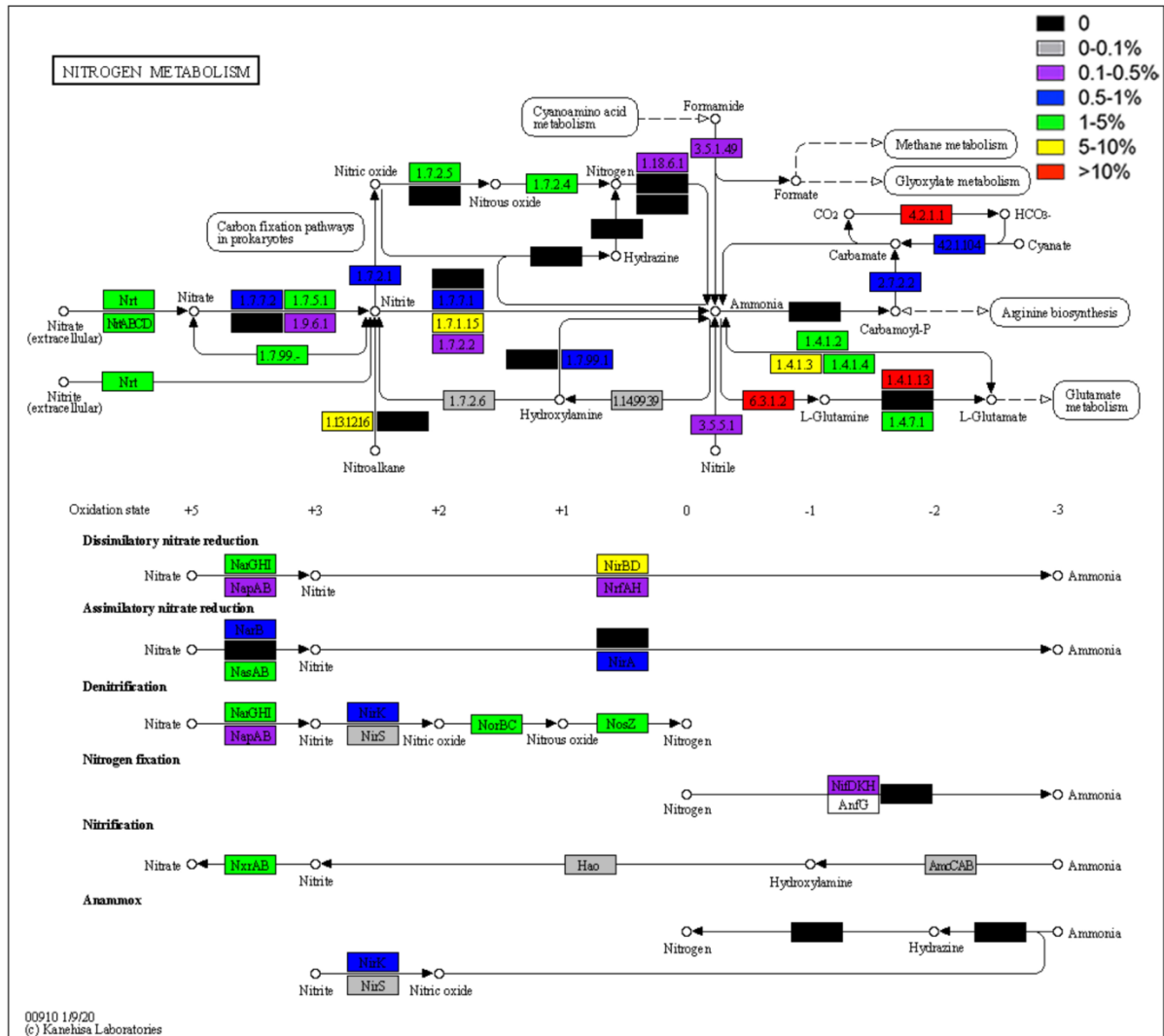

Figure 3 Profile of KEGG Orthology (KO) functions involved in the level-3 KEGG pathway of nitrogen metabolism of bacterial community at the end of the experiment (28 d) in the biofloc system w rearing *Litopenaeus vannamei* postlarvae with a salinity of 5.0‰ and recolored in terms of relative abundances of KO functions, based on the reference map of 00910 obtained from a database of KEGG PATHWAY (https://www.kegg.jp/pathway/map00910).

Analysis results based on predicted EC abundances showed that among enzymes with digestive activities, enzymes acting on ester bonds (EC 3.1), digesting carbohydrates (glycosylases, EC 3.2), and acting on peptide bonds (peptidases, EC 3.4) accounted for 53%, 15% and 32%, respectively (**Figure S1** – see supplementary material). And in those three categories, the usual digestive enzymes (Fänge & Grove, 1979; Terra & Ferreira, 2012), such as triacylglycerol lipase, esterases (carboxylesterases), phosphatases; α-amylase, cellulase, chitinase, lysozyme, glucosidases; trypsin and aminopeptidases, were observed (**Table 1**).

Table 1 Relative proportions of predicted digestive enzymes in each EC categories of bacterial community at the end of the experiment (28 d) in the biofloc system rearing Litopenaeus vannamei with a salinity of 5.0

EC Categories	Enzymes categories	Usual digestive enzymes	Proportions (%)
Enzymes acting on ester bonds (EC 3.1)	Lipases	Triacylglycerol lipases (EC 3.1.1.3)	0.5
		Esterases (carboxylesterases, EC 3.1.1.1)	0.3
	Phospholipases	Phospholipase A2 (EC 3.1.1.4)	0.2
		Phospholipase A1 (EC 3.1.1.32)	0.2
		Phospholipase B (EC 3.1.1.5)	2.0
		Phospholipase C (EC 3.1.4.3)	0.2
		Phospholipase D (EC 3.1.4.4)	0.1
	Phosphatases	Alkaline phosphatase (EC:3.1.3.1)	2.5
		Acid phosphatase (EC 3.1.3.2)	0.2
Glycosylases (EC 3.2)	Amylases	α-Amylases (EC 3.2.1.1)	4.9
	β-Glucanases	Cellulases (EC 3.2.1.4, EC 3.2.1.91)	4.0
	Xylanases	Xylanases (EC 3.2.1.8, EC 3.2.1.37)	1.2
	Pectinases	Pectinases (polygalacturonases, EC 3.2.1.15)	< 0.1
	Chitinases	Chitinase (EC 3.2.1.14)	1.0
	Lysozymes	Lysozyme (EC 3.2.1.17)	0.4
	Glucosidases	α-Glucosidases (EC 3.2.1.20)	6.0
		β-Glucosidase (EC 3.2.1.21)	7.8
	Trehalases	Trehalase (EC 3.2.1.28)	0.7
	Acetylhexosaminidases	β-N-acetyl-D-hexosaminidase (EC 3.2.1.52)	8.2
	β-Fructosidases	β-fructosidase (EC 3.2.1.26)	0.3
	α-Galactosidases	α-D-galactoside galactohydrolase (EC 3.2.1.22)	2.6
Peptidases (EC 3.4)	Serine proteinases	Trypsin (EC 3.4.21.4)	< 0.1
	Cysteine proteinase	Cathepsin L (EC 3.4.22.15)	< 0.1
	Aminopeptidases	Aminopeptidase N (EC 3.4.11.2)	2.8
		Aminopeptidase A (EC 3.4.11.7)	0.1

Contributions of bacteria to inferring functions

Four phyla Proteobacteria, Bacteroidetes, Planctomycetes, and Firmicutes were found to be very important for the inferring functions (KEGG pathways) and contributed to approximately 89.4% of total functions (**Figure 4**). For level-1 functions, Proteobacteria, Planctomycetes, and Firmicutes were the most important phylum to metabolism, organismal systems, and other functions, with contributions of 57.2%, 27.8%, and 46.1%, respectively (Figure 4). Proteobacteria played very important roles in almost all level-2 and level-3 pathways relating to metabolism (**Figures 5**). KO functions contained in the level-3 pathway of nitrogen metabolism were mainly contributed to phyla of Proteobacteria, Bacteroidetes, Planctomycetes, and Firmicutes (**Figure 6**).
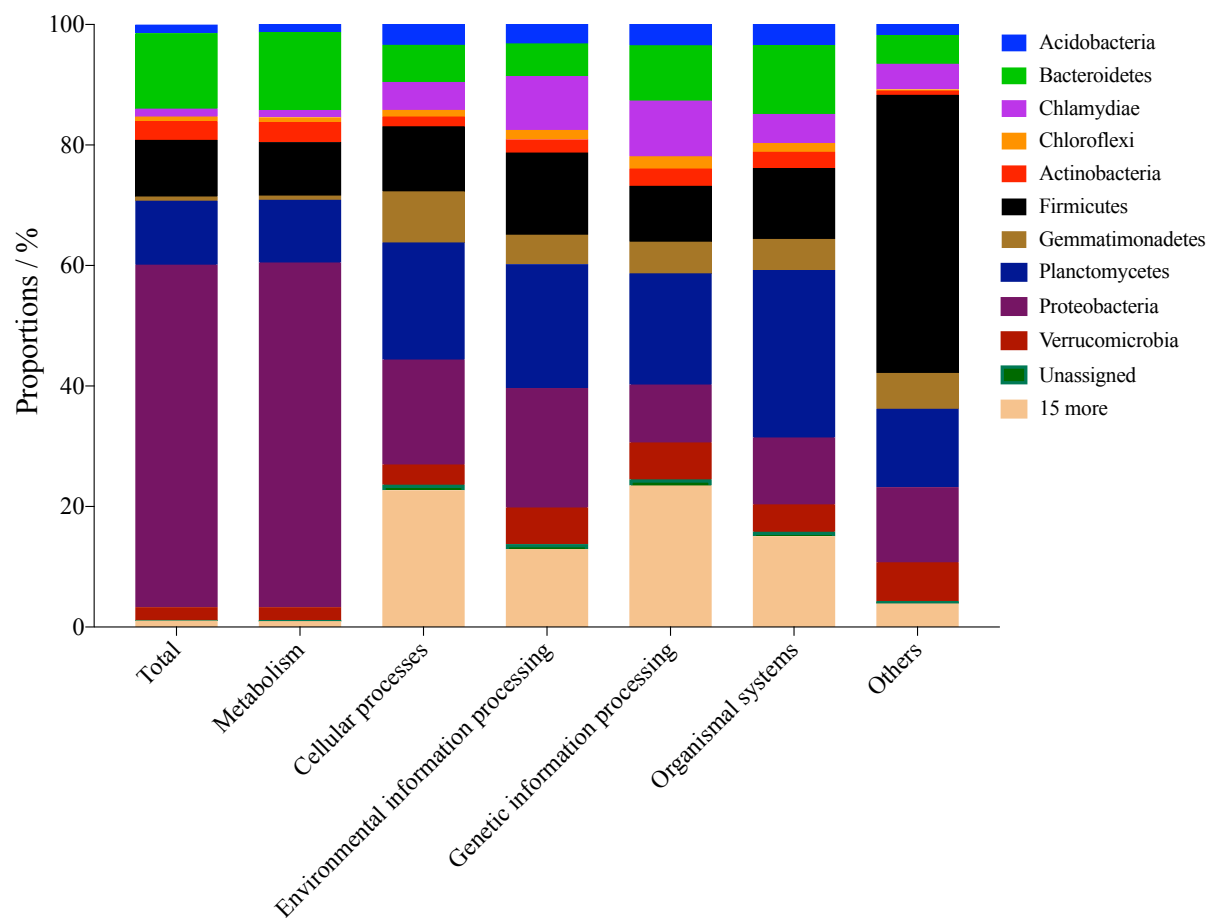
Figure 4 Stacked bar chart displaying contributions (relative proportions) of bacterial community at the end of the experiment (28 d) in the biofloc system rearing *Litopenaeus vannamei* postlarvae with a salinity of 5.0‰ at phylum level to level-1 inferring functions. The inferring functions were represented with KEGG pathways.
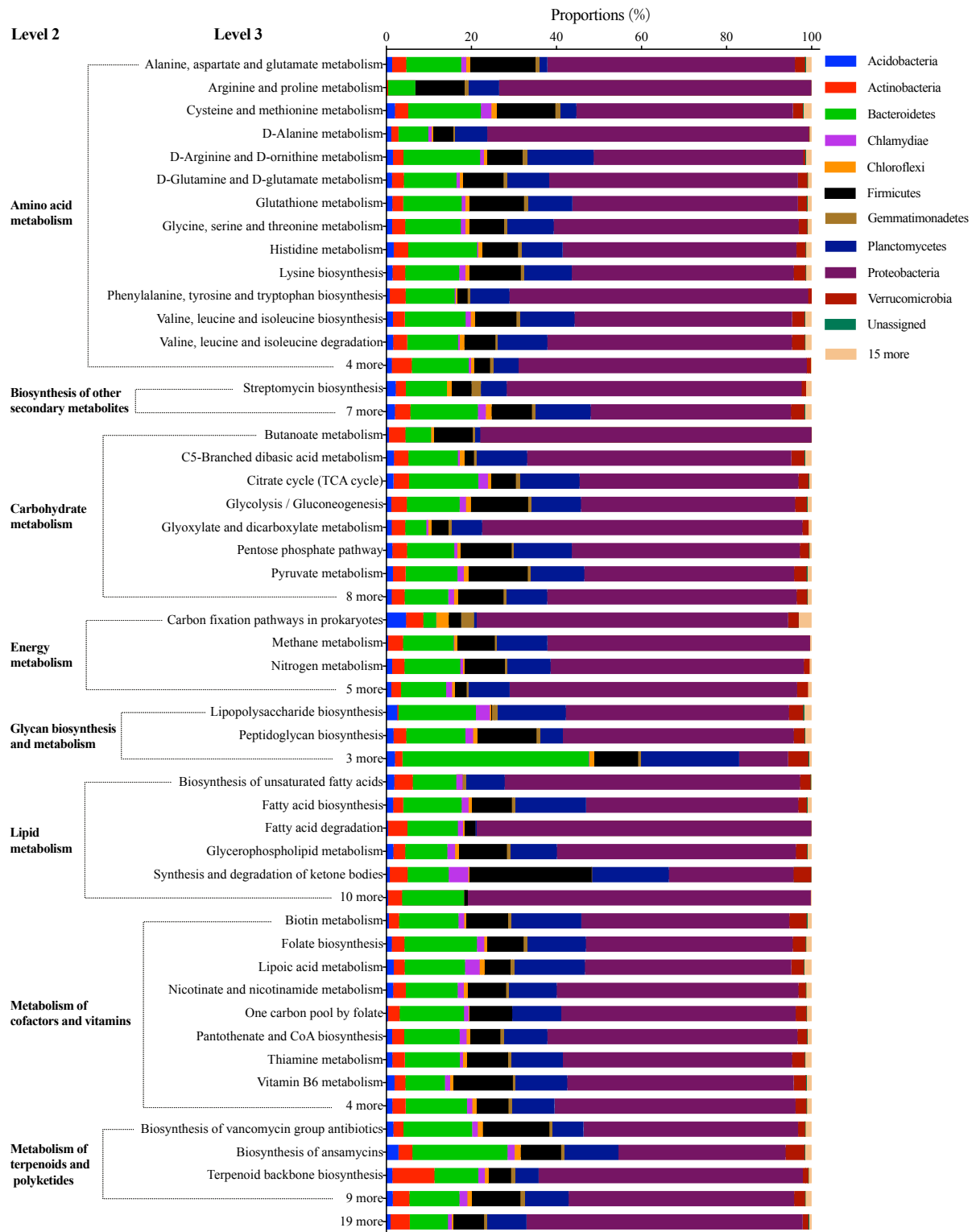
Figure 5 Stacked bar chart of contributions (relative proportions) of phyla of bacterial community at the end of the experiment (28 d) in the biofloc system rearing *Litopenaeus vannamei* postlarvae with a salinity of 5.0‰ to level-3 KEGG pathways relating to metabolism (level 1).
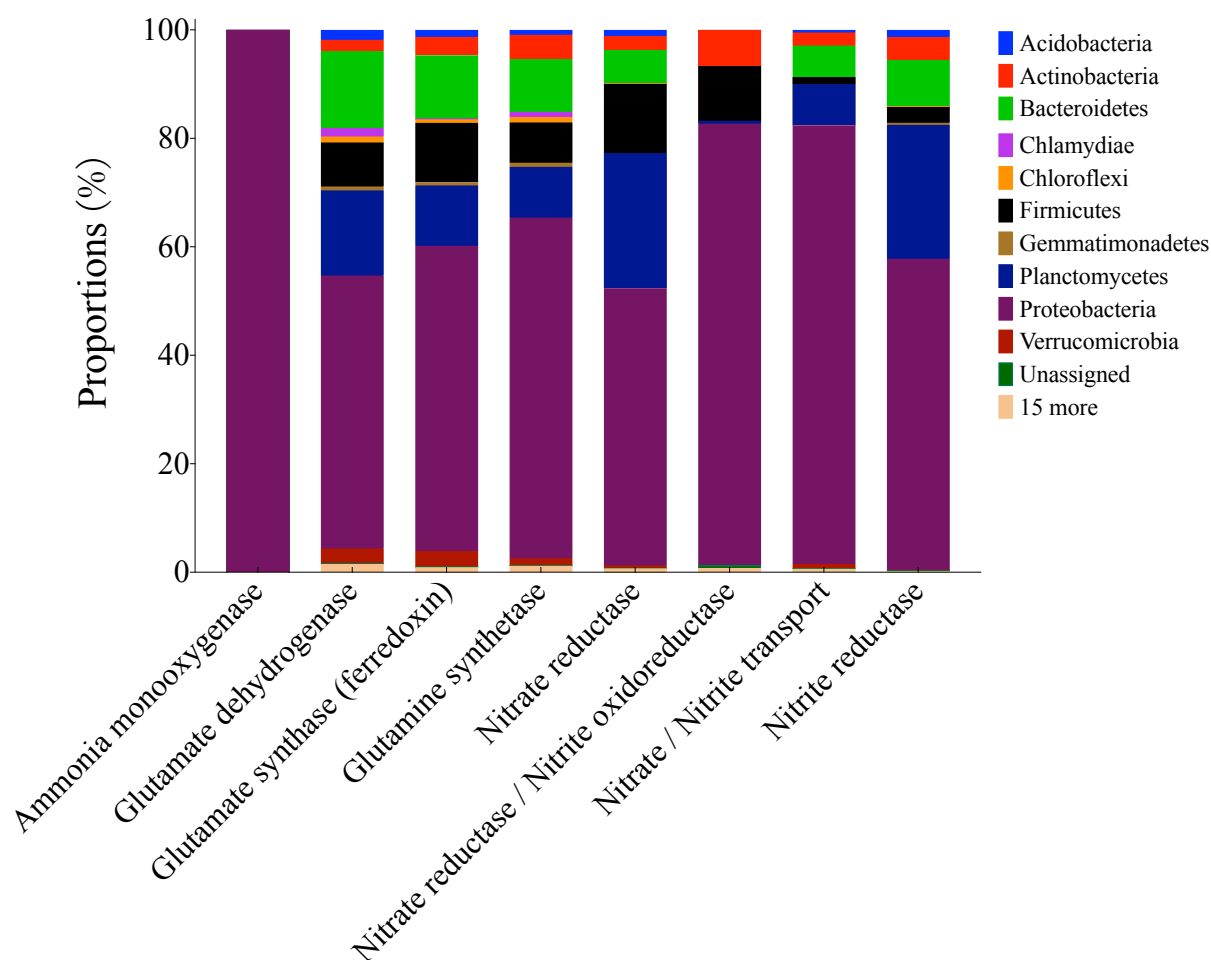
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Figure 6 Stacked chart for contributions (relative proportions) of bacterial community at the end of the experiment (28 d) in the biofloc system rearing *Litopenaeus vannamei* postlarvae with a salinity of 5.0‰ at phylum level on the main KEGG Orthology (KO) functions relating to transformations among organic and inorganic nitrogen compounds contained in KEGG pathway of nitrogen metabolism (level 3).

Growth performance of PL and water quality

At harvest, 91.6±11.8% of PL survived. The mean body weight of PL significantly increased to 120.9±16.7 mg at 28 d ($P < 0.05$, **Figure 7**). The concentrations of ammonia, nitrite, and nitrate non-significantly oscillated during the experiment ($P > 0.05$, **Figure 8a**), with values of 0.04±0.01, 0.2±0.1, and 12.9±2.5 mg L^{-1} at 28 d, respectively. Whereas, the total nitrogen significantly accumulated to 231.3±6.0 mg L^{-1} at 28 d ($P < 0.05$, **Figure 8a**). Similarly, biofloc and total suspended solids were also significantly accumulated to the levels of 127.0±63.0 mL L^{-1} and 240.0±62.0 mg L^{-1} at 28 d, respectively ($P < 0.05$, **Figure 8b**). The value of pH displayed a significant decreasing trend ($P < 0.05$, **Figure 8c**), with a final level of 7.4±0.1 at 28 d. The rest parameters, such as carbonate alkalinity, water temperature, and dissolved oxygen were all stable over the whole experimental period ($P > 0.05$, **Figure 8c** and **d**), with final values of 268.5±17.9 mg L^{-1} CaCO₃, 29.8±0.3 °C, and 6.8±0.1 mg L^{-1} at 28 d, respectively.
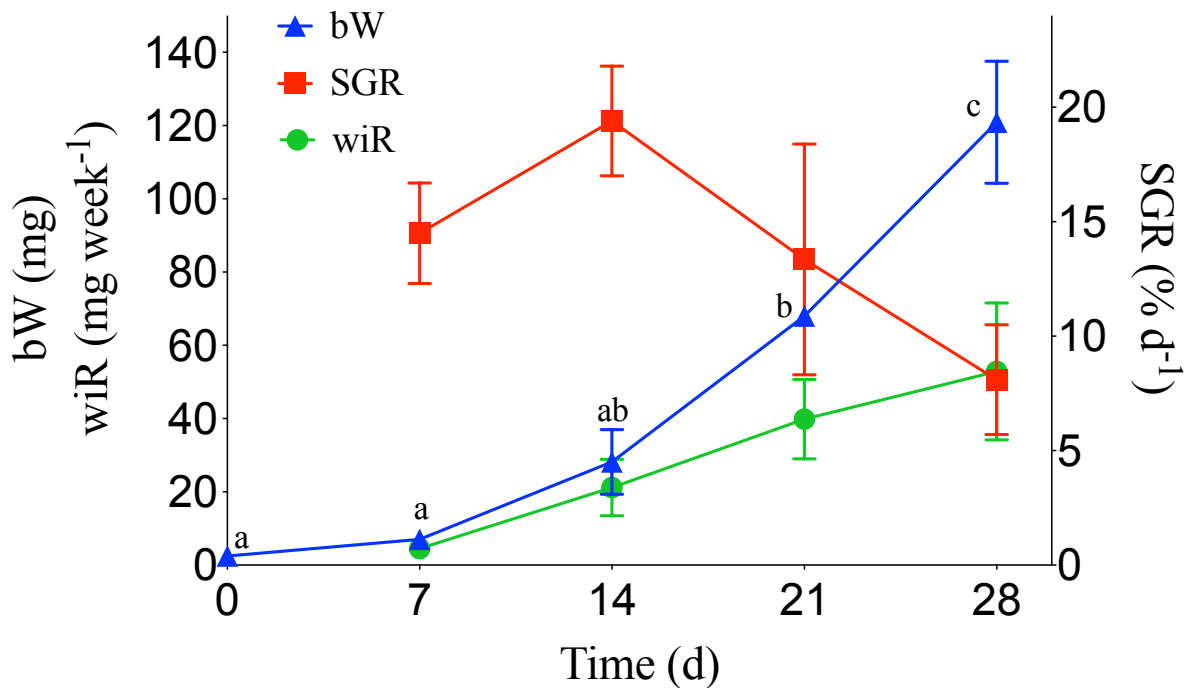
Figure 7 Growth performance of *Litopenaeus vannamei* postlarvae in the biofloc system with a salinity of 5.0‰ during the 28-days culture period. bW indicates mean body weight; SGR, specific growth rate; wiR, weekly increment rate of body weight. Error bar indicates ± standard deviation (SD). Symbols of a parameter with different low-case letters are significantly different ($P < 0.05$).
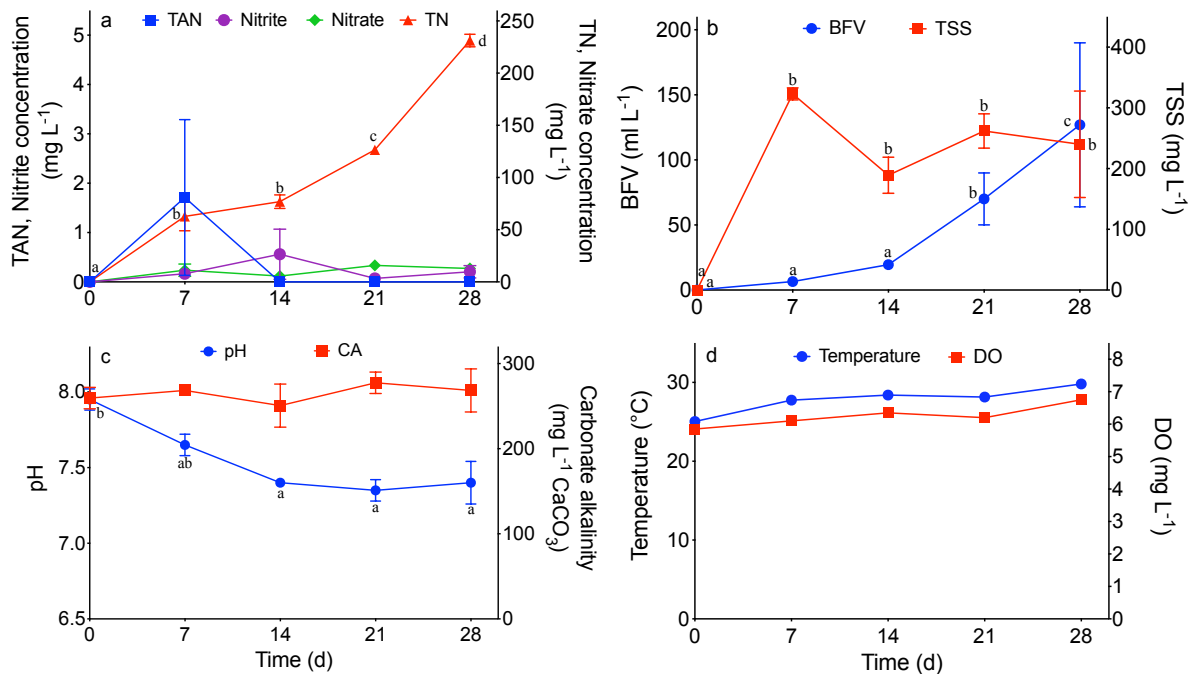

Figure 8 Performance of water quality parameters in the biofloc system rearing *Litopenaeus vannamei* postlarvae with a salinity of 5.0‰ during the 28-days culture period. (a) TAN represents total ammonia nitrogen; TN total nitrogen. (b) BFV indicates biofloc volume, TSS, and total suspended solids. (c) CA is an abbreviation of carbonate alkalinity. (d) DO means dissolved oxygen. Error bar indicates ± standard deviation (SD). Symbols of a parameter with different low-case letters are significantly different ($P < 0.05$).

Discussion

Bacterial community properties

There are two key aspects of diversity of bacterial community, richness and evenness (Magurran, 2004). Chao 1 index measures richness based on rare species, increasing value means increasing rare species, such as singletons. Several species dominance measurements, such as the Berger-Parker index and Simpson index, are used to determine evenness. Berger-Parker index expresses proportional importance of the most abundant species, increasing value means a reduction in the dominance of the most abundant species. Simpson index represents the probability of 2 individuals being conspecifics and increases with decreasing dominance of the abundant species. Comprehensively, the Shannon index indicates richness and evenness simultaneously. In the present study, high values of Chao1 index (13321.2), Shannon index (7.6), and Simpson index (0.98), but low values of Berger-Parker index (0.07) were observed, suggesting high diversity but low evenness of the bacterial community which was dominated by a few important species. In agreement with this, twenty-five phyla were observed in the present study, during which Proteobacteria, Bacteroidetes, Planctomycetes, Chlamydiae, and Firmicutes, together represented 97.5% of the bacteria community. Similarly, in the biofloc systems rearing *L. vannamei* with marine water, 19-22 phyla were found, and Proteobacteria (26-73.88%), Bacteroidetes (6.57-85%), Planctomycetes (5.03-42%) and Firmicutes (6.48%) were also the most predominant ones (Huerta-Rabago et al., 2019; Mariana et al., 2018; Martínez-Córdova et al., 2018; Vargas-Albores et al., 2019). Species belonging to those phyla, such as classes of Alphaproteobacteria, Planctomycetia, Saprospirae, Gammaproteobacteria and Flavobacteriia, and orders of Rhizobiales, Rhodobacterales, Saprospirales, Flavobacteriales, Pirellulales and Pseudomonadales, have strong adaptation capacity to different environments, especially conditions particularly rich in organic matter and suspended particles in the water column which are usually found in the BFT system, due to their abilities to use organic matter and nitrogen compounds for growth and particularity to attach to substrates for the requirement of support to grow on (Cardona et al., 2016; Kersters et al., 2006; Kirchman, 2002; Rank, 2009). Previous studies also revealed that in biofloc systems, although the proportions of the phyla mentioned above were low at initial, they should be predominant at last (Huerta-Rabago et al., 2019; Martínez-Córdova et al., 2018; Xu et al., 2019). Whereas, in the freshwater pond rearing fish with biofloc technology, Proteobacteria, Actinobacteria, Fusobateria, Chloroflexi and Saccharibacteria were the most dominant phyla (Liu et al., 2019), different from the results observed in the present study with a low salinity condition. Whereas, the dominant phyla in the present study were found to be similar to those in the marine biofloc systems rearing *L. vannamei* (Huerta-Rabago et al., 2019; Mariana et al., 2018; Martínez-Córdova et al., 2018; Vargas-Albores et al., 2019). It is hypothesized that the similar bacterial composition in the present study with those in seawater biofloc systems might be the result of successful adaptation of bacteria existing in marine-frying water to the low-salinity condition during the desalinating procedure and next operations. However, this adapting process is not clear and should be further studied for a deeper insight.

Properties of inferring functions of bacterial community

Vargas-Albores et al. (2019) documented that most of the predicted level-1 KEGG pathways of bacterial communities in shrimp-culture marine water biofloc systems with amaranth and wheat as biofloc promoters associated with metabolism (50-53%), genetic information processing (19-21%), environmental information processing (12-15%), cellular processes and signaling (4-6%) and organismal systems (1-2%), in agreement with the results in the present study. However, compared to this previous study, the proportion of functions relating to metabolism is higher in the current study (81%), but those of genetic information processing (11%) and environmental information processing (2%) are lower. Different salinities between the present study and the previous study by

Vargas-Albores et al. (2019) might be partially responsible for those differences in inferring functions of bacterial community. Different environmental osmotic pressure under different salinity conditions would make bacteria adjust their physio- and biochemical behaviors. Additionally, other aspects, such as carbon source, C:N and age of shrimp, would also affect functions of bacterial community.

At the level 2, the most abundant functions were found to be associated to metabolism, such as metabolism of amino acids, carbohydrates, lipids, nucleotides, vitamins and cofactors, xenobiotics and other metabolites (Vargas-Albores et al., 2019), also similar with the results in the present study. The high abundance of those metabolic functions suggests that bacteria might be able to take advantage of the suspended substrate and suitable for degradation of organic matter (Vargas-Albores et al., 2019), and in turn to improve water quality and formation of bacterial biomass which could be taken as supplementary food for shrimp to improve growth performance.

Contribution profile of bacteria to inferring functions

In the present study, contributions of phyla Proteobacteria, Bacteroidetes, Planctomycetes and Firmicutes to inferring functions were ratable to their proportions in the total bacterial community, respectively. For example, the most abundant phylum *Proteobacteria* (45.8%) attributed to 56.8% of total inferring functions, and played very important roles on almost all function categories. Species belonging to this phylum, such as classes of Alphaproteobacteria and Gammaproteobacteria, orders of Rhizobiales, Rhodobacterales and Enterobacteriales, are widely dispersed in the environment and play important roles in the nutrient cycling and the utilization of organic compounds (Berman, 2012; Cardona et al., 2016). However, the attribution of Chlamydiae to functions was only 1.3%, although it was the fourth predominant phylum (10.3%). Vargas-Albores et al. (2019) found that, compared to the taxonomic profile, the predicted functional profile shows a more effective pattern for the representation of the sample. This phenomenon should be deserved to note in future studies.

Inferring influence of bacterial community on growth performance of PL

Previous studies showed that the formation of biofloc was found to be favorable to the growth of PL (Kuhn et al., 2009; Xu & Pan, 2012). Generally, biofloc is considered to be a complementary food to improve the growth of shrimp, due to its rich in protein, lipid, amino acid, and other bioactive compounds (Ju et al., 2008; Kuhn et al., 2009; Xu & Pan, 2012). In the present study, inferring functions of the bacterial community relating to the metabolism of nutrients were also found, such as biosynthesis of essential and non-essential amino acids, unsaturated fatty acids, and cofactors and vitamins. Moreover, inferring digestive enzymes were also observed, which might be helpful for the growth of PL. In the biofloc system, bacteria could produce exogenous digestive enzymes, increasing the digestibility of foods and improving the growth of shrimp Fields (Panigrahi et al., 2019a; Wang et al., 2016; Xu et al., 2013). Additionally, inferring functions of the bacterial community relating to the biosynthesis of immunostimulants (LPS and peptidoglycan) and antibiotics (ansamycins, streptomycin, and vancomycins) were observed in the current study, which would be helpful to maintain the high survival rate of PL. Exposure to stimulation of immunostimulants could reinforce the immune system of shrimp (Li & Xiang, 2013; Panigrahi et al., 2019c), and functions of biosynthesis of antibiotics might partially explain why bacterial pathogens do not have the same virulence and are inhibited in biofloc systems (Ekasari et al., 2014; Panigrahi et al., 2019b). In the current study, the proportion of family *Vibrionaceae*, the most important opportunistic pathogenic bacteria groups for *L. vannamei* (Gomez-Gil et al., 1998; Kita-Tsukamoto et al., 1993), was only 0.003%.

Qualitative influence of bacterial community on water quality

The levels of ammonia, nitrite, and nitrate at 28 d were low in this study, indicating weak nitrification in the system. Actually, only two families belonging to autotrophic nitrifying bacteria, *Nitrospiraceae* and *Nitrosomonadaceae* (Sliekers et al., 2002), were found in the bacterial community at 28 d of the present study, with a total proportion of 0.004%. Besides, although some heterotrophic bacteria with nitrification capacity were also found in the bacterial community of the present study, such as genera *Acinetobacter*, *Bacillus*, *Paracoccus,* and *Pseudomonas* (Chen et al., 2019; Liu et al., 2019), their proportions were also very low. Correspondingly, the proportion of inferring functions of the bacterial community relating to inorganic transformation was low (18% of the level-2 KEGG pathway nitrogen metabolism) in the present study. Conversely, the conversion of ammonia to glutamate was the most important KO function relating to the level-3 KEGG pathway of nitrogen metabolism in this study. Coincidently, total nitrogen and biofloc significantly accumulated at the end of this study, indicating a massive formation of organic nitrogen or bacterial biomass. Heterotrophic bacteria could assimilate inorganic nitrogen to synthesize self-cellular protein, and as a result, massive bacterial biomass was produced and biofloc formed (Ebeling et al., 2006). Impressively, the level of biofloc volume increased to 127.0 ± 63.0 mL L^{-1} at the end of the experiment (28 d) in the present study, far higher than the acceptable value for shrimp (Avnimelech, 2015; Xu & Pan, 2012), meaning that removal treatment should be executed in subsequent studies, but the effect of this treatment on the bacterial community should be investigated as well.

It should be noted that in the present study, only the bacterial community at the end of the experiment (28 d) was determined, leading to that only the qualitative relationship between bacterial community and water parameters defined at the same time could be speculated and that no tighter associations or quantitative correlations could be obtained. Thereby, interestingly and meaningfully, more treatments would be built up to get a deeper insight into the effects of the bacterial community on growth performance and water quality in the following studies.

Acknowledgments

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