



Nitrogen removal and water microbiota in grass carp culture following supplementation with *Bacillus licheniformis* BSK-4

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Abstract This experiment was designed to study the effects of *Bacillus licheniformis* BSK-4 on nitrogen removal and microbial community structure in a grass carp (*Ctenopharyngodon idellus*) culture. The selected strain *Bacillus licheniformis* BSK-4 significantly decreased nitrite, nitrate and total nitrogen levels in water over an extended, whereas increased ammonia level. Pyrosequencing showed that Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes were dominant in grass carp culture water. Compared with the control group, the number of Proteobacteria and Firmicutes were increased, while Actinobacteria and Bacteroidetes decreased in treatment group. At the genus level, some genera, such as *Bacillus*, *Prostheco bacter*, *Enterococcus*, etc., appear only

in the treatment, while many other genera exist only in the control group; *Lactobacillus*, *Luteolibacter*, *Phenylobacterium*, etc. were increased in treatment group compared to those in control group. As above, the results suggested that supplementation with *B. licheniformis* BSK-4 could remove some nitrogen and cause alterations of the microbial composition in grass carp water.

Keywords *Bacillus licheniformis* BSK-4 · Nitrogen removal · Microbial community structure · Grass carp · 454-Pyrosequencing

Introduction

China's aquaculture industry accounts for the largest share of the world's fishery production, and provides a principal source of protein for the nation's booming population (Zhang et al. 2015). However, the industry has its own problems. Typically aquaculture wastewater is characterized by increased nitrogen species (ammonia, nitrites, and nitrates), organic carbon, phosphates, suspended solids, and high biological oxygen demand (BOD) and chemical oxygen demand (COD) (Boopathy and Lyles 2008), which is recognized as a serious global problem because of its severe influence to surrounded lakes and slow moving rivers (Lee and Shoda 2008; Othman et al. 2013). For instance, aquaculture waste may decrease dissolved oxygen level and load high nutrient and inorganic contaminants which subsequently may cause water deterioration (Othman et al. 2015). Nitrogen and phosphorus at enough concentrations may become toxic to plants and alter their membrane permeability, protein synthesis, oxidation stress response, enzyme activities, photosynthesis and respiratory processes (Li et al. 2007). Additionally, ammonia and

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nitrite are toxic to aquatic life in aquatic systems (Russo et al. 1981; Thurston et al. 1981). Therefore, appropriate administration should be introduced in order to minimize the negative impacts of aquaculture practices.

The use of probiotics is prevalent in the aquaculture industry as a mean of improving water quality by balancing bacterial load and replacing the use of antibiotics/biocides, producing supplemental digestive enzymes, lowering incidence of diseases, improving immune response and survival (Verschul.

Fig. 1 Effect of *Bacillus licheniformis* BSK-4 on the nitrogen removal in grass carp culture. **a** Ammonia nitrogen, **b** nitrite nitrogen, **c** nitrate nitrogen, and **d** total nitrogen. ** indicated significant differences ($p < 0.05$) compared with treatment group at each time point

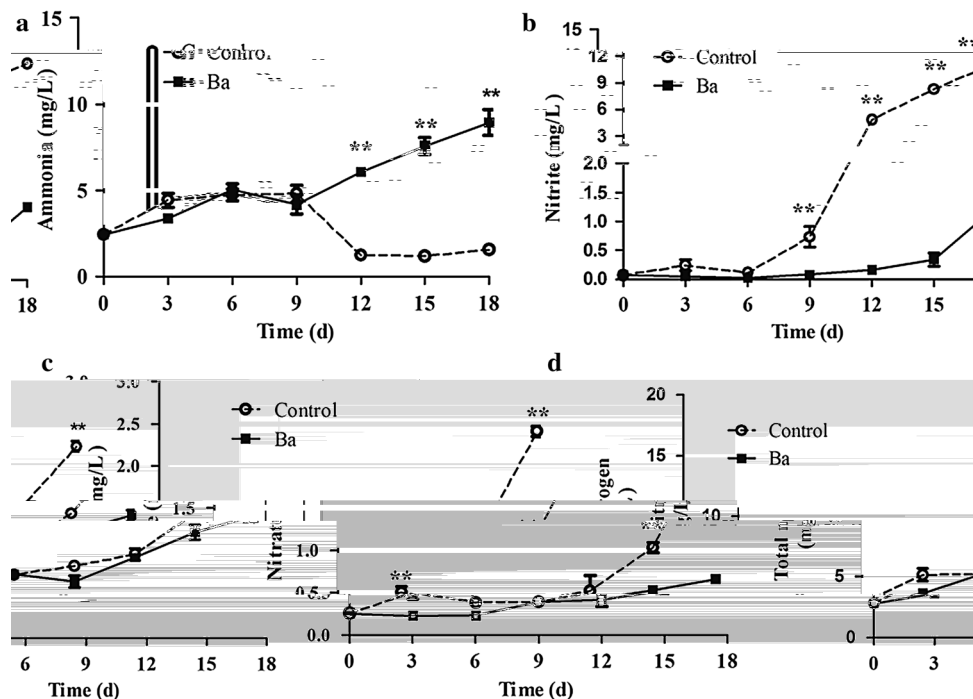


Fig. 2 Bacterial community structures in control and treatment group. **a** Bacterial community structures at phylum level. **b** Proteobacteria composition by class in samples from control and treatment

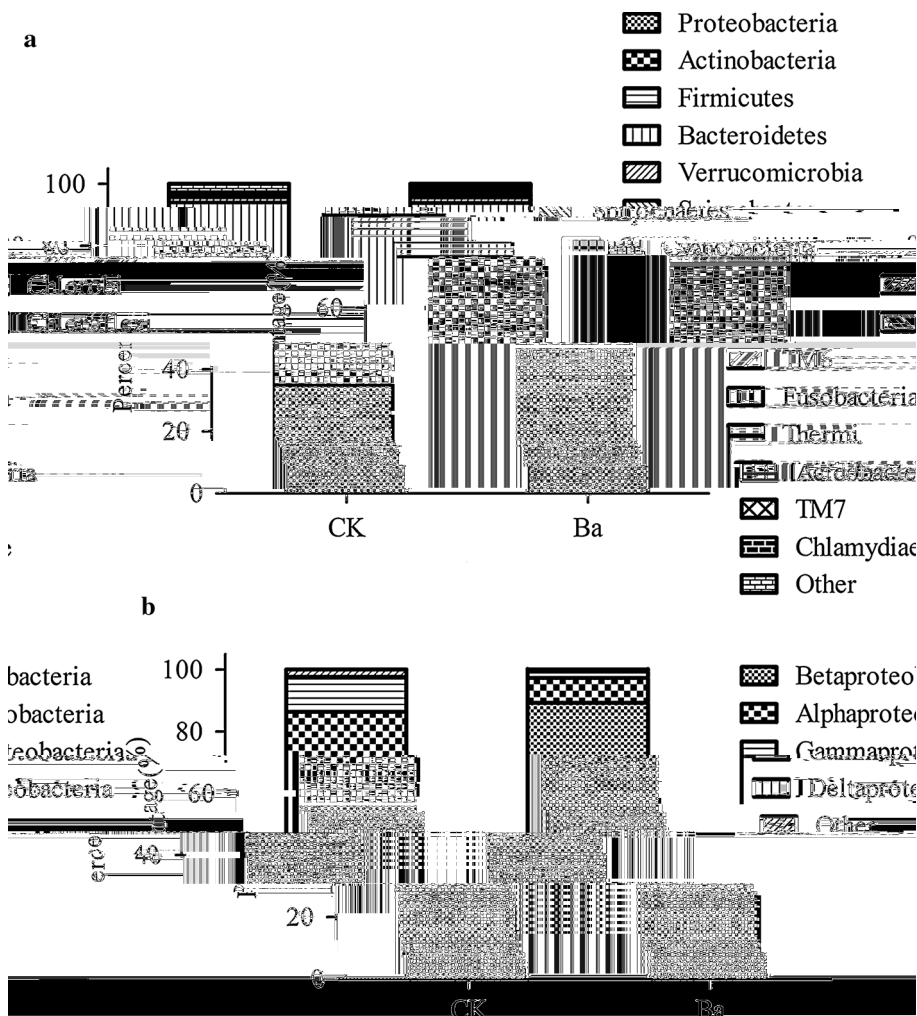


Fig. 3 Comparison of the taxonomic analyses of control and treatment groups computed by MEGAN4

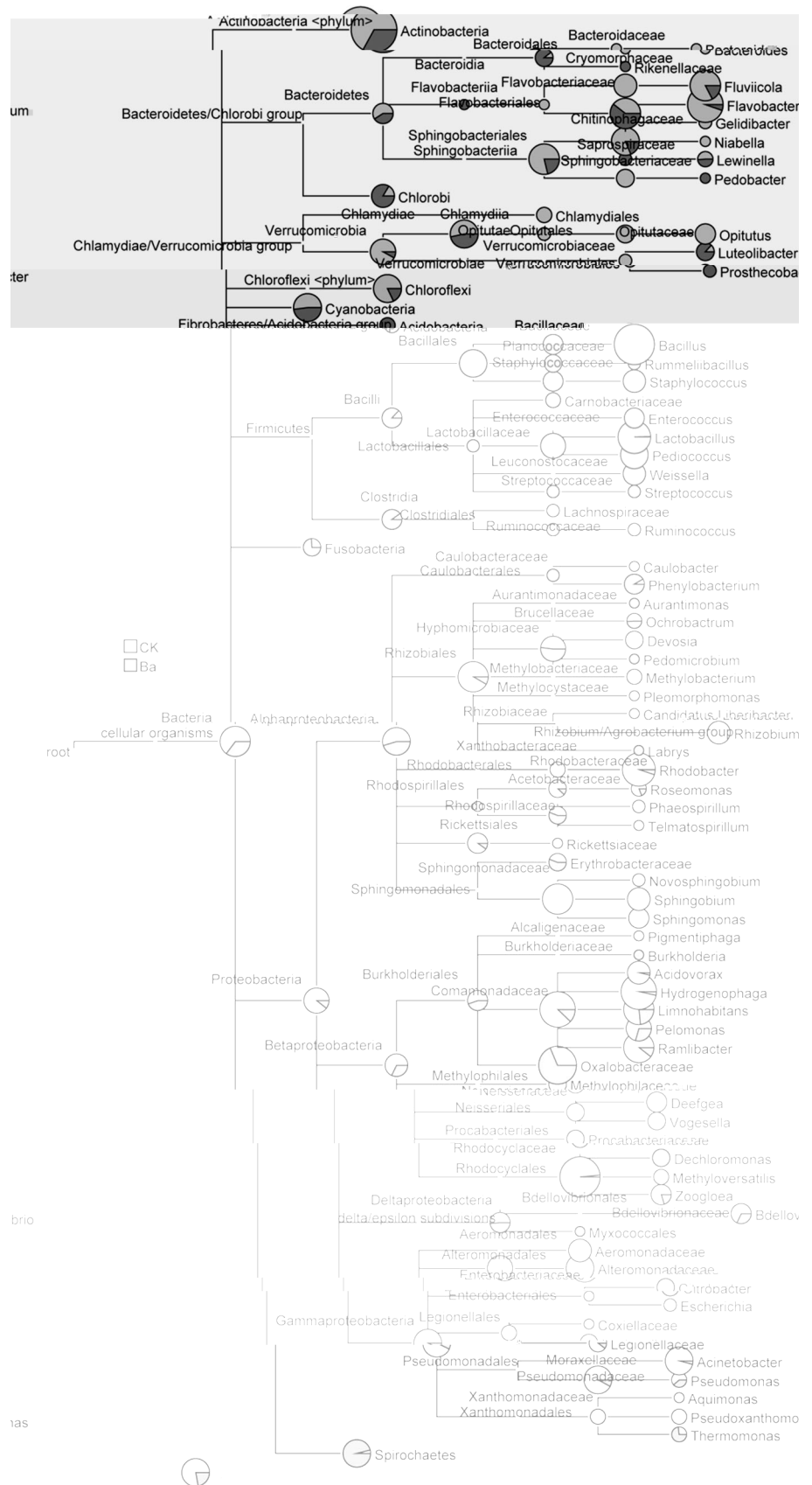
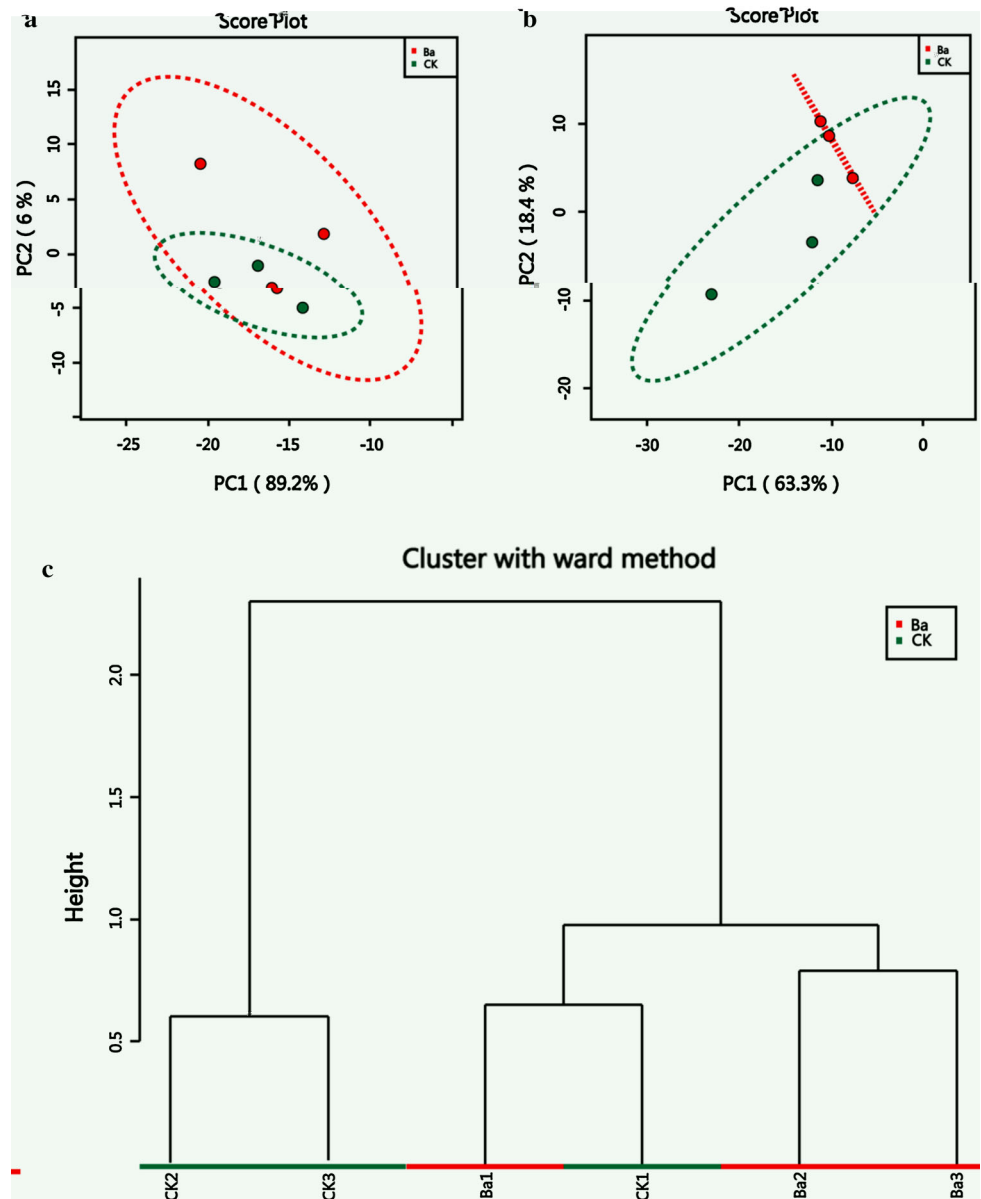


Fig. 4 Similarity analysis between control and treatment groups. Water microbiome sequencing data of treatments and controls analyzed by principal component analyses at phyla levels (a) and genus levels (b). Cluster analysis of the water microbiome sequencing data of controls and treatments at genus level by Ward method (c)



Microbial community structure

Sequences from pyrosequencing were assigned taxonomic affiliations at the phylum level using RDP's classifier tool. At the phylum level (Fig. 2a), Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes were dominant for two groups, accounting for more than 93 % of the total. Compared with the control group, the number of Proteobacteria and Firmicutes was increased, while Actinobacteria and Bacteroidetes decreased in treatment group. Within Proteobacteria phyla (Fig. 2b), where Betaproteobacteria and Deltaproteobacteria were higher, Alphaproteobacteria and Gammaproteobacteria were lower in treatment group compared to those in control group.

MEGAN (Huson et al. 2011) was used to further compare the microbial communities of the samples from the control and treatment groups, the tree created by shown in Fig. 3. The pie charts beside the leaves of the tree indicated the relative abundance of the microbes in the control and treatment samples. The microbial communities of the two groups were quite different from phylum to genus level. Some genera, such as *Bacillus*, *Prostheco bacter*, *Enterococcus*, etc., appear only in the treatment, while many other genera exist only in the control group (Fig. 3). At the genus level, *Lactobacillus*, *Luteolibacter*, *Phenyllobacterium*, etc. were increased in treatment group compared to those in control group (Fig. 3). As above, the results suggested that supplementation with *Bacillus* bacteria could cause alterations of the microbial composition in grass carp water.

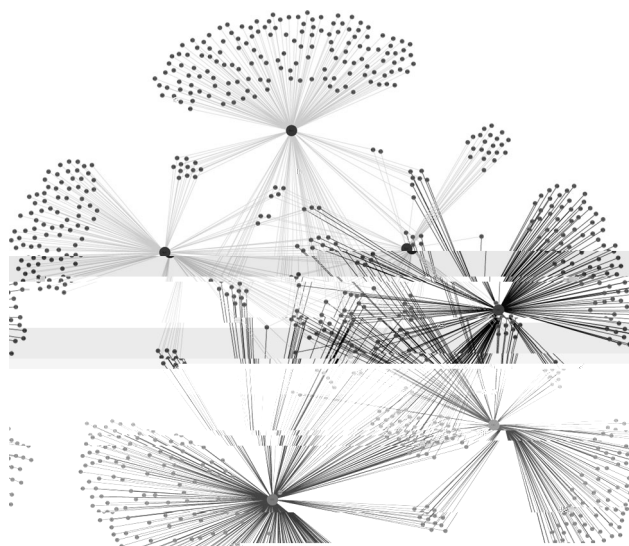


Fig. 5 Network based analysis of the water bacterial communities. Dark circles are representative sequences from each OTU clustered based on the relatedness of the sequences (97 % similarity). Each sample is connected with the OTUs through edges color-coded (dark control; gray treatment)

Similarity between communities

METAGENassist (Arndt et al. 2012) was applied to perform a multivariate data analysis following the protocol described in the previous study (Badri et al. 2013). Principal component analyses on pair-wise and normalized OTUs between all treatments were performed to identify the main factors driving community composition differences. No significant differences were observed between the treatments and control group at phylum level (Fig. 4a). However, we observed that the control and treatment group formed two different clusters at the genus level (Fig. 4b). The second principal component (18.4 %) revealed that the controls separated from their respective treatments. This pattern was recapitulated by hierarchical clustering using

Ward method where controls clustered separately from the treatments (Fig. 4c). The authors used the Cytoscape program to visualize the OTU network between samples (Smoot et al. 2011), which indicated a high number of shared OTUs between samples highlighting a co-occurring community (Fig. 5). The results indicated that these control and treatment group did not only have different OTU, but also share some OTUs.

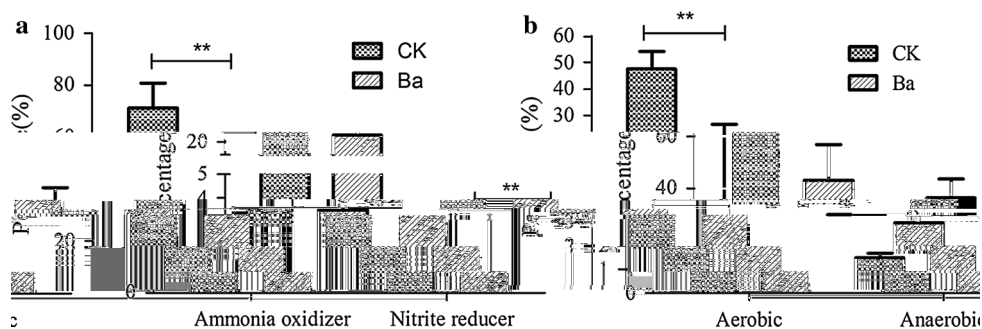
Taxonomic to phenotype mapping

The authors assigned the OTUs from taxonomic to phenotype mapping by METAGENassist webserver tool (Arndt et al. 2012) based on oxygen requirement and metabolism. Based on our analyses, we observed that the water supplementation with *B. licheniformis* BSK-4 significantly reduced the number of sequence reads assigned to ammonia oxidizer compared with the control group, while there was no difference in nitrite reducer between the two groups (Fig. 6a); The strain BSK-4 significantly enriched the number of sequence reads assigned to anaerobic and significantly reduced the aerobic bacteria compared with the control group (Fig. 6b).

Discussion

The results showed that *B. licheniformis* BSK-4 could decrease the level of nitrite nitrogen, nitrate nitrogen and total nitrogen to some extent. Denitrification refers to the dissimilator reduction, by essentially aerobic bacteria, of one or both of the ionic nitrogen oxides (nitrate, and nitrite) to the gaseous oxides (nitric oxide, and nitrous oxide), which may be further reduced to dinitrogen (Knowles 1982). It was reported that other denitrification bacteria *Bacillus subtilis* SC02 (Zhang et al. 2013) and *Pseudomonas stutzeri* F1M (Zhang et al. 2013) could also decrease the nitrogen level in grass carp culture water. As above, the decreased nitrogen level by BSK-4 might be related to its denitrification capabilities. However, in the study, the ammonia concentration in the treatment was

Fig. 6 Taxonomic to phenotypic mapping based on the metabolism (a) and oxygen requirement (b)



significant higher than that in control group from the 12th day, which was associated to the reduce number of ammonia oxidizer. Fu et al. (2012) suggested that there were a lot of heterotrophic denitrifying bacteria involved in various nitrogen metabolic pathways in grass carp culture water, and the proportion of heterotrophic ammonia production bacteria were much higher than that of ammonia degrading bacteria, which might result in high ammonia content.

Microorganisms have major roles in productivity, nutrient cycling, the nutrition of the cultured animals, water quality, disease control and environmental impact of the effluent in pond culture (Moriarty 1997). Therefore, a better understanding of the bacterial communities in grass carp farming ponds may be useful to optimize the aquaculture system and enhance stability and health of intensive grass carp culture. Some studies have been carried out to identify the bacterial communities in water column of grass carp farming ponds. Chen et al. (1999) used culture-dependent methods to isolate 11 genera of bacteria that belong to four phyla (Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes) from the water column and the mucus on body surface of grass carp. Zhou et al. (2013) applied PCR-denaturing gradient gel electrophoresis (PCR-DGGE) to detect the water column and sediment of 12 commercial grass carp farming ponds. Total 32 bacterial species, which belonged to seven phyla (Proteobacteria, Bacteroidetes, Actinobacteria, Cyanobacteria, Acidobacteria, Fibrobacteres and Fusobacteria), were identified. In the present study, a 454-pyrosequencing analysis revealed that Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes were the major phylum in the grass carp culture water. These results are consistent with those of Zhang et al. (2013, 2014) and Deng et al. (2014). As above, Proteobacteria was the most common phylum in grass carp farming ponds.

The results also indicated that *B. licheniformis* BSK-4 supplementation caused alterations of the microbial community structure in grass carp water. de Paiva-Maia et al. (2013) applied a commercial probiotic in intensive shrimp farming (*Litopenaeus vannamei*) with a recirculation system, they found the probiotic causes changes in the total heterotrophic bacteria in the sediment and percentage values of Pyrrophyta concentration, improving the environmental quality of the sediment and water in ponds with closed recirculation systems. Wu et al. (2014) showed that *Bacillus subtilis* FY99-01 could increase the abundance of Flavobacteria, whereas decrease the abundance of α -Proteobacteria in the late phase of shrimp culture. They also found that using probiotics could increase the abundance of beneficial microalgae (Bacillariophyta and Chlorophyta), and decrease the abundance of *Vibrionaceae*. Zhang et al. (2014) showed that rearing water with photosynthetic

bacteria could decrease the number of Proteobacteria, and Bacteroidetes, and increase the number of Actinobacteria. The authors also indicated that the treatment group enjoyed a higher microbial diversity than that of the control group (Zhang et al. 2014). As above, adding microbial preparations to aquaculture water could alter the microbial community structure, which is associated with the nitrogen removal (Zhang et al. 2013, 2014; Deng et al. 2014).

In conclusion, supplementation with *B. licheniformis* BSK-4 could remove some nitrogenous waste to improve water quality, and cause alterations of the microbial composition in grass carp water, which has some connection with the nitrogen removal.

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