CrossMark

ORIGINAL PAPER

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

Quan Liang[1] · Xiaoping Zhang[1,2] · Khui Hung Lee[3] · Yibing Wang[1] ·
Kan Yu[3] · Wenying Shen[4] · Luoqin Fu[1] · Miaoan Shu[1] · Weifen Li[1]

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, Prosthecobacter, 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

Quan Liang and Xiaoping Zhang have contributed equally to this work.

✉ Miaoan Shu
shuma@zju.edu.cn

✉ Weifen Li
weifenli@zju.edu.cn; wfli@zju.edu.cn

1 College of Animal Science, Zhejiang University, 310058 Hangzhou, People's Republic of China

2 College of Biosystems Engineering and Food Science, Zhejiang University, 310058 Hangzhou, People's Republic of China

3 Faculty of Medicine, Dentistry and Health Science, University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia

4 Department of Biology, Life Science College of Shaoxing University, 312000 Shaoxing, People's Republic of China

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

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 (Verschuere et al. 2000). Because *Bacillus* bacteria secrete many exoenzymes (Moriarty 1996, 1998), these bacteria have been used widely as putative probiotics. It has been reported that Bacillus bacteria has nitrification and denitrification functions (Yang et al. 2011; Zhang et al. 2012, 2013; Yao et al. 2014). Briefly, this research aims to study the effects of isolated bacteria *Bacillus licheniformis* BSK-4 on nitrogen removal and microbial community structure using the 454-pyrosequencing technology in grass carp culture.

Materials and methods

Bacteria preparation

Bacillus licheniformis BSK-4 with nitrogen removal activity was isolated from a grass carp culture pond in the Zhejiang province of China. The isolation and identification of *B. licheniformis* BSK-4 were performed according to the method described by Zhang et al. (2013). *Bacillus licheniformis* BSK-4 broth was inoculated into a conical flask with Luria–Bertani media and incubated at 30 °C for 24 h in a shaking incubator (180 rpm). Pure bacterial cells were harvested by centrifugation (at 4000 rpm for 10 min at 4 °C), and the collected cells were washed three to four times with sterilized 0.85 % sodium chloride solution. The pellet was collected, mixed with corn starch and then dried at 50–60 °C in an oven. *Bacillus licheniformis* BSK-4 preparations were stored at room temperature, and the purity was determined using plate spreading techniques to observe its growth and characteristic (Fig. S1). The final density was approximately 3×10^9 CFU/g of dry powder.

Experimental design

The experiments used six tanks with three replicate tanks per treatment and 120 fish of uniform size (mean ± standard error: 14.46 ± 0.9 g) were randomly divided into the tanks (20 fish per tank). Each replicate was maintained in an aquarium of 1400 L capacity and fed a diet at 3 % of body weight twice daily. The control group was fed a basal diet, and the treated group was fed a basal diet in addition to *B. licheniformis* BSK-4 at a dose of 1×10^8 cfu/m^3 per

7 days in culture water. The ingredients and nutritional composition of a basal diet are shown in the previous study (Zhang et al. 2013). The culture water in the buckets was not changed during the 18-day experimental period. Nitrogen compounds, such as ammonia nitrogen, nitrite nitrogen, nitrate nitrogen and total nitrogen, were estimated every 3 days following standard methods (EPBC 2002).

DNA extraction and pyrosequencing

The protocol of sampling, DNA extraction, PCR amplification and 454-pyrosequencing performed in this study was followed our previous study (Zhang et al. 2014). All analyses described in the current study were performed with version 1.6 of the QIIME (Caporaso et al. 2010), which was the same as in our previous study (Zhang et al. 2014). Briefly, Sequences were demultiplexed and quality filtered with split_libraries.py by using default parameters. Similar sequences were clustered into operational taxonomic units (OTUs) using the threshold of 97 % identity. Representatives of the most abundant reads were selected from each OTU for subsequent analysis. Representative OTUs were aligned using PyNAST (Caporaso et al. 2010) with the default database as a reference. ChimeraSlayer was used to identify and discard chimeric of the successfully aligned reads (Haas et al. 2011). A representative sequence from each OTU was classified directly with the RDP Classifier with a 50 % confidence threshold (Cole et al. 2005).

Statistical analysis

Data is presented as mean ± standard deviation. All means were compared using *t test* (Statistical Package Social Science, SPSS, version17.0). A value of $P < 0.05$ was considered to be statistically significant.

Results

Nitrogen removal

Bacillus licheniformis BSK-4 had an effect on the nitrogen content of grass carp culture water (Fig. 1). Compared with the control group, ammonia was significantly increased ($P < 0.05$) from 12 to 18th day (Fig. 1a). From 9 to 18th day (Fig. 1b), nitrite was decreased by 88.64 % ($P < 0.05$), 96.70 % ($P < 0.05$), 95.85 % ($P < 0.05$) and 88.80 % ($P < 0.05$), respectively. Nitrate was decreased by 54.97 % ($P < 0.05$), 48.56 % ($P < 0.05$) and 72.37 % ($P < 0.05$) on the 3rd, 15th and 18th, respectively (Fig. 1c). Total nitrogen was decreased by 35.95 % ($P < 0.05$) on the 18th day (Fig. 1d).

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
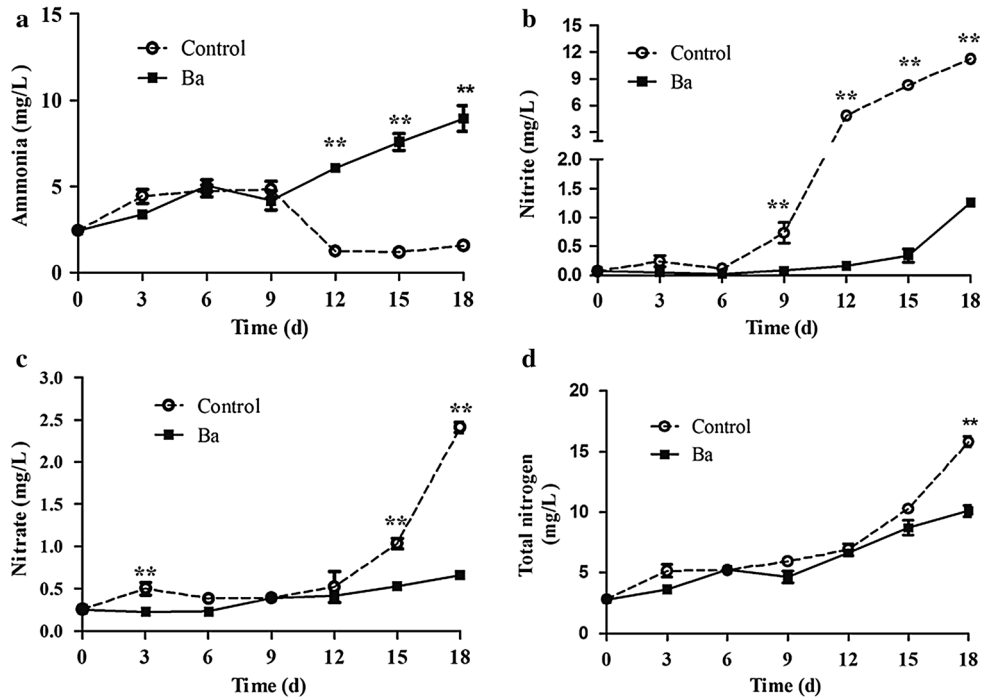

Fig. 2 Bacterial community structures in control and treatment group. **a** Bacterial community structures at phylum level. **b** Proteobacteria composition by class in samples from control and treatment
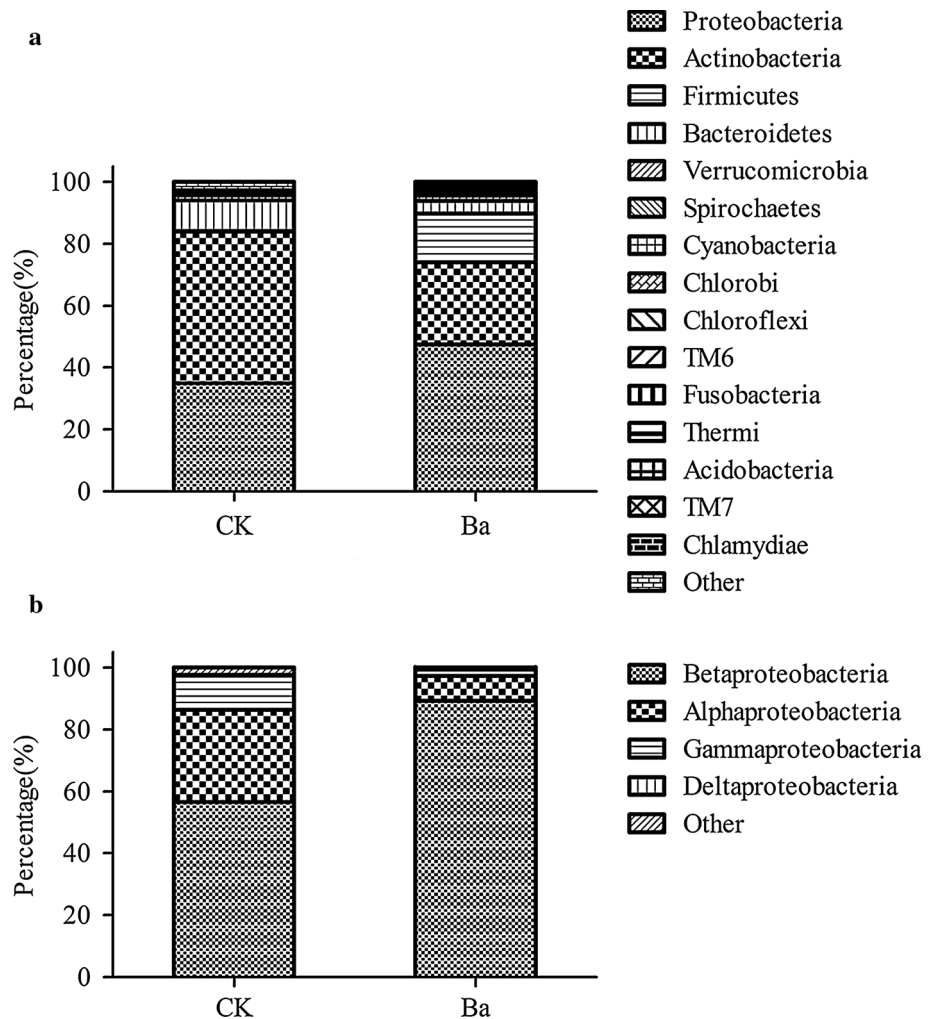
Fig. 3 Comparison of the taxonomic analyses of control and treatment groups computed by MEGAN4
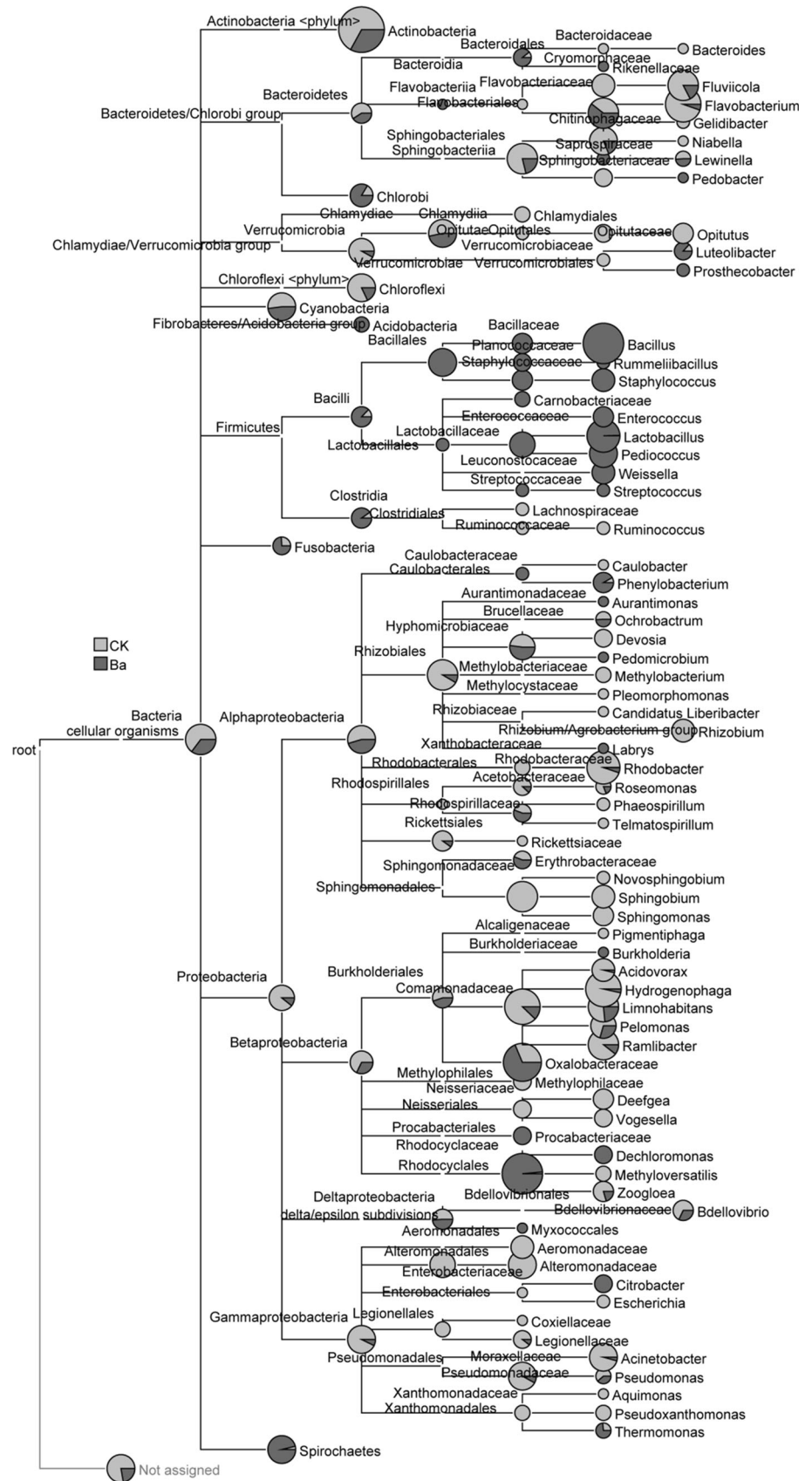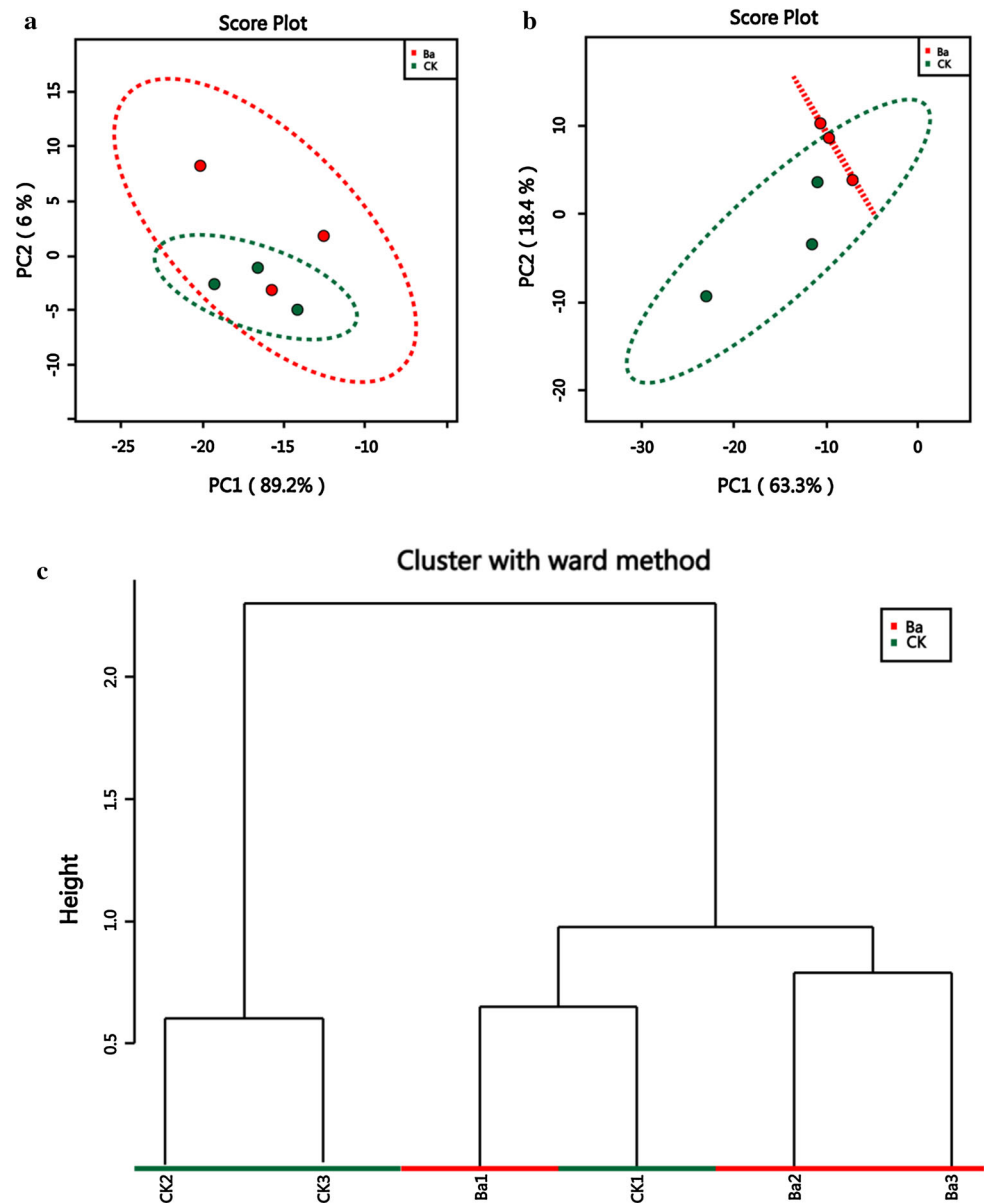
Fig. 4 Similarity analysis between control and treatment groups. Water microbiome sequencing data of treatments and controls analyzed by principal component analyses at phyla levels (**a**) and genus levels (**b**). Cluster analysis of the water microbiome sequencing data of controls and treatments at 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, Prosthecobacter, 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, Phenylobacterium*, 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.
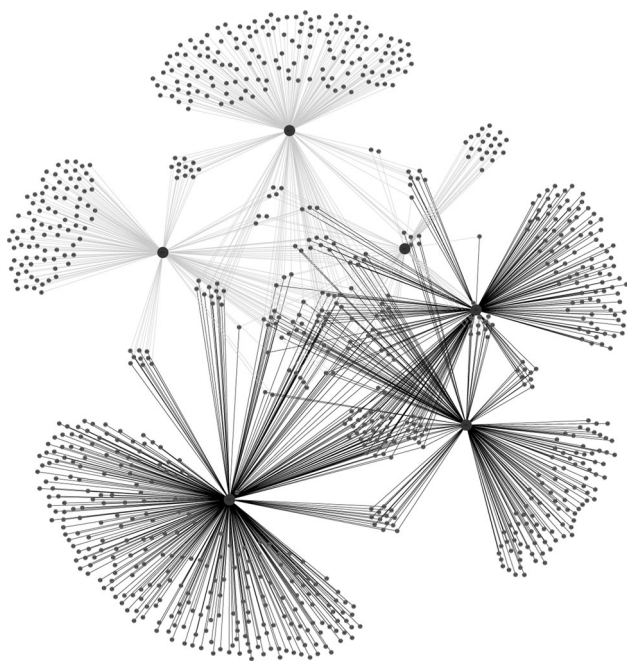
Fig. 5 Network based analysis of the water bacterial communities. *Dark circles* are representative sequences from each OTU clustered based on the relatedness of the sequences (97 % similarity). Each sample is connected with the OTUs through edges color-coded (*dark* control; *gray* treatment)

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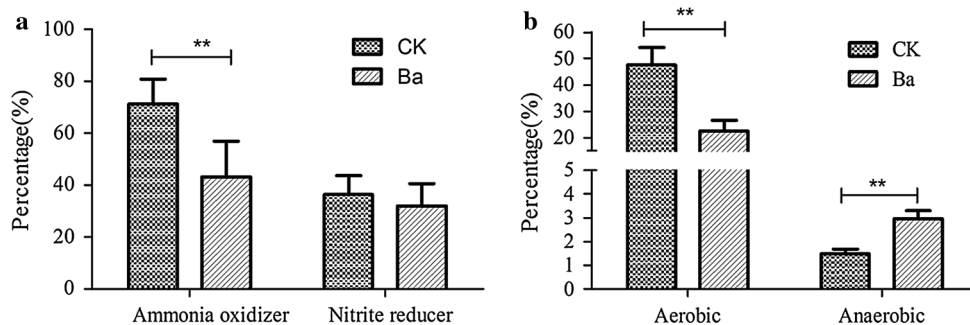
significant higher than that in control group from the 12th day, which was associated to the reduce number of ammonia oxidizer. Fu et al. (2012) suggested that there were a lot of heterotrophic denitrifying bacteria involved in various nitrogen metabolic pathways in grass carp culture water, and the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

References

Arndt D, Xia J, Liu Y, Zhou Y, Guo AC, Cruz JA, Sinelnikov I, Budwill K, Nesbø CL, Wishart DS (2012) METAGENassist: a comprehensive web server for comparative metagenomics. Nucleic Acids Res 40(W1):W88–W95

Badri DV, Chaparro JM, Zhang R, Shen Q, Vivanco JM (2013) Application of natural blends of phytochemicals derived from the root exudates of *Arabidopsis* to the soil reveal that phenolic-related compounds predominantly modulate the soil microbiome. J Biol Chem 288(7):4502–4512

Boopathy R, Lyles C (2008) Shrimp production and biological treatment of shrimp wastewater in the United States. Pandey A (ed) New horizons in biotechnology. Asia Tech Publishers, New Delhi, pp 235–252

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7(5):335–336

Chen X, Wu Z, Luo Y, Gao H, Chen X (1999) Effects of furazolidone on bacterial flora in water and the body surface muxus of grass carp (*Ctenopharyngogon idellus*). J Huazhong Agric Uni 1:68–71 **(article in Chinese with English abstract)**

Cole JR, Chai B, Farris RJ, Wang Q, Kulamm SA, CcGarrell DM, Garrity GM, Tiedje JM (2005) The ribosomal database project (RDP-II): sequences and tools for high-throughput rRNA analysis. Nucleic Acids Res 33(Database issue):D294-6

de Paiva-Maia E, Alves-Modesto G, Otavio-Brito L, Olivera A, Vasconcelos-Gesteira TC (2013) Effect of a commercial probiotic on bacterial and phytoplankton concentration in intensive shrimp farming (*Litopenaeus vannamei*) recirculation systems. Lat Am J Aquat Res 41(1):126–137

Deng B, Fu LQ, Zhang XP, Zheng JJ, Peng LS, Sun JD, Zhu HY, Wang YB, Li WF, Wu XX, Wu D (2014) The denitrification characteristics of *Pseudomonas stutzeri* SC221-M and its

application to water quality control in grass carp aquaculture. PLoS ONE 9(12):e114886

EPBC (Environmental Protection Bureau of China) (2002) Methods for monitor and analysis of water and wastewater (4rd ed). China Environmental Science Press, Beijing

Fu LQ, Sun JD, Deng B, Liang Q, Zhang XP, Zheng JJ, Hu CH, Li WF (2012) Analysis of cultivable heterotrophic bacteria involved in nitrogen cycle in grass carp (*Ctenopharyngodon idellus*) pond. J Fish China 36(10):1572–1578 **(article in Chinese with English abstract)**

Huson DH, Mitra S, Ruscheweyh HJ, Weber N, Schuster SC (2011) Integrative analysis of environmental sequences using MEGAN4. Genome Res 21(9):1552–1560

Haas BJ, Gevers D, Earl AM, Feldgarden M, Ward DV, Giannoukos G, Ciulla D, Tabbaa D, Highlander SK, Sodergren E, Methé B, DeSantis TZ, Human Microbiome Consortium, Petrosino JF, Knight R, Birren BW (2011) Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Res 21(3):494–504

Knowles R (1982) Denitrification. Microbiol Rev 46(1):43–70

Lee H, Shoda M (2008) Removal of COD and color from livestock wastewater by the Fenton method. J Hazard Mater 153(3):1314–1319

Li M, Wu YJ, Yu ZL, Sheng GP, Yu HQ (2007) Nitrogen removal from eutrophic water by floating-bed-grown water spinach (*Ipomoea aquatica* Forsk.) with ion implantation. Water Res 41:3152–3158

Moriarty DJW (1997) The role of microorganisms in aquaculture ponds. Aquaculture 151(1):333–349

Moriarty DJW (1996) Microbial biotechnology: a key ingredient for sustainable aquaculture. INFO Fish Int 4:29–33

Moriarty DJW (1998) Control of luminous Vibrio species in penaeid aquaculture ponds. Aquaculture 164:351–358

Othman I, Anuar AN, Ujang Z, Rosman NH, Harun H, Chelliapan S (2013) Livestock wastewater treatment using aerobic granular sludge. Bioresour Technol 133(2013):630–634

Othman R, Hatta FAM, Ayuni F, Ramya R, Hanifah NA, Yaman M, Baharuddin ZM (2015) Phytoremediation model system for aquaculture wastewater using *glossostigma elatinoides* and *hemianthus callitrichoides*. Int J Sust Energ Environ Res 4(1):1–7

Russo RC, Thurston RV, Emerson K (1981) Acute toxicity of nitrite to rainbow trout (*Salmo gairdneri*): effects of pH, nitrite species, and anion species. Can J Fish Aquat Sci 38(4):387–393

Smoot ME, Ono K, Ruscheinski J, Wang PL, Ideker T (2011) Cytoscape 2.8: new features for data integration and network visualization. Bioinformatics 27(3):431–432

Thurston RV, Russo RC, Vinogradov GA (1981) Ammonia toxicity to fishes. Effect of pH on the toxicity of the unionized ammonia species. Environ Sci Technol 15(7):837–840

Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol R 64(4):655–671

Wu DX, Zhao S M, Peng N, Xu CP, Wang J, Liang YX (2014) Effects of a probiotic (*Bacillus subtilis* FY99-01) on the bacterial community structure and composition of shrimp (*Litopenaeus vannamei*, Boone) culture water assessed by denaturing gradient gel electrophoresis and high-throughput sequencing. Aquac Res. doi:10.1111/are.12545

Yang XP, Wang SM, Zhang DW, Zhou LX (2011) Isolation and nitrogen removal characteristics of an aerobic heterotrophic nitrifying–denitrifying bacterium, *Bacillus subtilis* A1. Bioresour Technol 102(2):854–862

Yao RL, Qiu LN, Zhang WW, Gong AJ, Wang ZY, Cai H (2014) Isolation and characteristics of heterotrophic nitrification-aerobic denitrification bacterium, *Bacillus cereus* X7 at high salinity. Adv Mater Res 864:111–114

Zhang QL, Liu Y, Ai GM, Miao LL, Zheng HY, Liu ZP (2012) The characteristics of a novel heterotrophic nitrification–aerobic denitrification bacterium, *Bacillus methylotrophicus* strain L7. Bioresour Technol 108:35–44

Zhang XP, Fu LQ, Deng B, Liang Q, Zheng JJ, Sun JD, Zhu HY, Peng LS, Wang YB, Shen WY, Li WF (2013) *Bacillus subtilis* SC02 supplementation causes alterations of the microbial diversity in grass carp water. World J Microbiol Biotechnol 29(9):1645–1653

Zhang XP, Shu MA, Wang YB, Fu LQ, Li WF, Deng B, Liang Q, Shen W (2014) Effect of photosynthetic bacteria on water quality and microbiota in grass carp culture. World J Microbiol Biotechnol 30(9):2523–2531

Zhang Y, Bleeker A, Liu J (2015) Nutrient discharge from China's aquaculture industry and associated environmental impacts. Environ Res Lett 10(4):045002

Zhou T, Wang Y, Tang J, Dai Y (2013) Bacterial communities in Chinese grass carp (*Ctenopharyngodon idellus*) farming ponds. Aquac Res 45(1):138–149