World J Microbiol Biotechnol (2013) 29:1645–1653
DOI 10.1007/s11274-013-1327-z

ORIGINAL PAPER

Bacillus subtilis SC02 supplementation causes alterations of the microbial diversity in grass carp water

Xiaoping Zhang · Luoqin Fu · Bin Deng · Quan Liang · Jiajia Zheng · Jiandong Sun · Haiyan Zhu · Lisha Peng · Yibing Wang · Shen Wenying · Weifen Li

Received: 30 November 2012 / Accepted: 18 March 2013 / Published online: 29 March 2013

Abstract This study was conducted to evaluate the effects of *Bacillus subtilis* SC02 supplement on water quality, microbial community diversity and structure in a grass carp (*Ctenopharyngodon idellus*) culture. Our selected strain, *B. subtilis* SC02, significantly reduced ammonia, nitrite and total nitrogen levels in water over an extended period compared with the control group. Pyrosequencing showed that the Shannon diversity index (Shannon) and species richness estimators (Chao) of the treatment group were higher, indicating that bacterial richness was significantly increased in the treatment group. The phyla *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* were dominant in the treatment groups, accounting for 45, 21.9 and 21.9 % of the sequence reads, respectively. However, in sharp contrast, the control fishes were predominantly occupied by *Proteobacteria* (82.1 %) and *Firmicutes* (8.4 %). At the genus level, the microbial communities were different between the control and treatment groups, although the two groups shared similar genera. Additionally, some genera (such as *Tepidimonas*, *Variovorax*, *Roseomonas*, *Rubritepida*, *Nitrobacter,* etc.) only appeared in the treatment group, and many other genera only existed in the control group. Therefore, we conclude that the addition of the SC02 strain in water improves water quality, which may ultimately be a result of changes in microbial community diversity in grass carp cultures.

Keywords *Bacillus subtilis* · Microbial community structure · Microbial community diversity · 454-Pyrosequencing

Introduction

Aquaculture is one of the fastest-growing food-producing sectors and provides approximately 40 % of the fish consumed throughout the world (Cole et al. 2009). Untreated wastewater from aquaculture, which contains high concentrations of nitrogen and phosphorus nutrients, can cause environmental deterioration (Nora'aini et al. 2005) and lead to a series of negative ecological impacts, such as a serious oxygen deficit, eutrophication or algal bloom, water deterioration and disease outbreaks (Cao et al. 2007). Nitrogen compounds, such as ammonium, nitrite and nitrate, may have adverse effects on aquatic animal health, environmental and economic development, threatening public health (Enelld and Lof 1983; Nora'aini et al. 2005). Therefore, improvements in wastewater management are essential for sustaining aquaculture practices.

Currently, water quality improvement has been especially achieved by using the biological approach of probiotics. Generally, probiotics are live microbial food supplements that improve the balance of the intestinal microbiota of the host animal (Fuller 1989). However, probiotics in aquaculture can be administered as either a food supplement or a water additive, with multiple benefits

Electronic supplementary material The online version of this article (doi:10.1007/s11274-013-1327-z) contains supplementary material, which is available to authorized users.

X. Zhang · L. Fu · B. Deng · Q. Liang · J. Zheng · J. Sun · H. Zhu · L. Peng · Y. Wang · W. Li (✉)
Key Laboratory of Molecular Animal Nutrition, Ministry of Education, Institute of Feed Sciences, College of Animal Science, Zhejiang University, Hangzhou 310058, People's Republic of China
e-mail: weifenli@zju.edu.cn; wfli@zju.edu.cn

S. Wenying
Department of Biology, Life Science College of Shaoxing University, Shaoxing 312000, People's Republic of China

(Moriarty 1998). Bacteria used successfully as probiotics primarily belong to the *Bacillus* family or lactic acid bacteria (LAB); however, other genera or species have been utilized in aquaculture (Sahu et al. 2008; Shen et al. 2010; Wang et al. 2008). Probiotics improve water quality via their ability to participate in the turnover of organic nutrients in ponds (Sahu et al. 2008). Microorganisms naturally present in pond cultures achieve better productivity, nutritional improvement, nutrient cycling and disease control and manage the environmental impacts of the effluent (Moriarty 1997). Therefore, a better understanding of microbial communities will improve the environment through the creation of a wastewater treatment system, which will also enrich microbial ecological theory (Oerther et al. 2001; DeAngelis et al. 2011).

Traditional cultures and independent molecular identification methods have limitations and do not provide sufficient information regarding microbial community structures (Hu et al. 2012). The recently developed massively parallel ('454') pyrosequencing (hereafter referred to as 454-pyrosequencing) method offers great promise in the high-throughput identification of hundreds of samples at reasonable cost and time consumption (Margulies et al. 2005; Sogin et al. 2006; Roesch et al. 2007) and can accurately elucidate the characteristics of microbial communities (Roesch et al. 2007). Thus, the utility of 454-pyrosequencing has been widely applied to study the composition of microbial communities, such as in wastewater treatment systems (Kim et al. 2011; Hu et al. 2012), the ocean (Sogin et al. 2006; Roberts et al. 2012) and soil (Lumini et al. 2010; Lin et al. 2012). However, the utilization of this technology to study microbial communities in aquaculture water has not been well documented. In this study, we used 454-pyrosequencing technology to evaluate the effects of *Bacillus subtilis* SC02 on water quality, microbial community structure and the diversity in a grass carp culture.

Materials and methods

Bacteria preparation

Bacillus subtilis SC02 with denitrification activity was isolated from a pond containing grass carp in the Zhejiang province of China. The isolation and identification of *B. subtilis* SC02 was performed according to the method described by Zheng et al. (2012). The bacteria were preserved at the Institute of Animal Nutrition and Feed Science, Laboratory of Molecular Feed Sciences, Zhejiang University, and bacterial purity was routinely checked during the investigation. The morphological, physiological and biochemical characteristics of *B. subtilis* SC02 were identified as described in the Bergey's Manual of Systematic Bacteriology (Holt et al. 1994). The phylogenetic tree is shown in Figure S1. *Bacillus subtilis* SC02 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 4,000 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 subtilis* SC02 preparations were stored at room temperature, and the purity was determined using plate spreading techniques, observing its growth and characteristic (Fig. S2).

Experimental design

Six buckets (500 L) were arranged in Dafan fishery cooperatives located in Shaoxing, Zhejiang Province, with an air compressor (ACO-318, Zhejiang Sunsun Industry Co. Limited). The buckets were filled with 340 L of fresh water from the Dafan river, which is located in Shaoxing, Zhejiang Province (30°03′38″N, 120°40′52″E). The culture water in the buckets was not changed during the experiment. Overall, 114 healthy fish with similar initial weight were fed a basal diet and acclimatized for 7 days before conducting the experiment. Next, the fish were divided into three replicates per group and 19 carps per replicate. The control group was fed a basal diet, and the treated group was fed a basal diet in addition to *Bacillus* preparations at a dose of 1×10^9 cfu/m^3 per 7 days in culture water. The viability of the bacterial mixture in rearing water was determined using the plate spreading technique. Fish were hand-fed 3 % of their body weight twice per day (09:00 and 17:00), and the ingredients and nutritional composition of a basal diet are shown in Table 1.

Sampling and water quality measurement

Water samples collected from each bucket (500 mL) were gathered from the same water level (15 cm below the surface). The rearing water in each bucket was mixed and collected, and the water was filtered through a 0.22-μm membrane and stainless steel filter holders (Sartorius, Germany) to isolate microbes, which were then stored at −80 °C for further study. The supernatant was used to measure water quality parameters.

Water quality parameters (e.g., water temperature) were measured daily, whereas ammonia nitrogen, nitrite nitrogen, nitrate nitrogen and total nitrogen were estimated every 3 days following standard methods (EPBC 2002).

Table 1 Ingredients and nutritional composition of the basal diet

Ingredients	Composition (%)	Nutritional index	Proximate composition (% wet weight)
Fish meal	1.0	Moisture	9.31
Soybean meal	5.0	Crude protein	28.40
Cottonseed meal	23.8	Crude fat	2.97
Rapeseed meal	27.2	Crude ash	12.08
Wheat flour	18.0	Calcium	0.80
Rice bran	8.0	Total phosphonium	1.29
Lee power	6.0		
Malt root	6.0		
Premix	5.0		

Premix provides following per kilogram of diet: Vitamin A 150,000 IU, vitamin D_3 30,000 IU, vitamin E 750 mg, vitamin K_3 150 mg, Fe 2.5 g, Cu 0.075 g, Zn 0.75 g, Mn 0.5 g, Mg 5 g, I 22.5 mg, Se 3.5 mg, Co 7.5 mg

DNA extraction

DNA extraction was performed according to the Lemarchand method (2005) with some modifications. Each tube was supplemented with 582 μL of sterilized double-distilled water, 160 μL of 0.5 M Na_2EDTA, 40 μL of 10 % SDS and 8 μL of Tris–HCl (pH 9.0), as well as with small fractions of a membrane that contained the desired bacteria. Eight microliters of lysozyme (20 mg/mL) and 1.6 μL of RNAase (20 mg/mL) were added to each aliquot, and the tubes were incubated at 37 °C for 1 h. The tubes were then spiked with 8 μL of proteinase K and incubated at 55 °C for 4 h. The samples were centrifuged at 13,000×*g* for 5 min at room temperature, and the supernatant fractions were collected.

An equal volume of Tris-phenol was added to the solution, and the solution was mixed for 10 min prior to centrifugation at 13,000×*g* for 5 min at 4 °C. The upper (aqueous) phase of each fraction was transferred into a separate tube that contained an equal volume of phenol–chloroform–isoamyl alcohol (25:24:1), homogenized, and centrifuged at 13,000×*g* for 5 min at 4 °C. The supernatant fractions were transferred into new tubes, and one volume of chloroform was added, mixed and centrifuged at 13,000×*g* for 5 min at 4 °C. The nucleic acids were precipitated by the addition of two volumes of ethanol at −20 °C for 20 min, and the solutions were centrifuged (13,000×*g*, 10 min, 4 °C). The nucleic acids were washed with two volumes of pre-cooled 70 % (v/v) ethanol dried at room temperature, dissolved in 30 μL of TE (pH 8.0) and pooled together.

454-Pyrosequencing

For each sample, the V3 region of the 16S rRNA gene (*E. coli* position 341 to 534) was amplified using a primer set (Casserly and Erijman 2003), and the 5′ terminus of each forward and reverse primer contained an 8-bp barcode sequence to tag specific samples (Table S1).

The PCRs were conducted in triplicate in 50 μL reactions with 1 μL of each forward and reverse primer (20 M), 1 μL of the template DNA, 5 μL of 10× PCR buffer, 4 μL of dNTPs (2.5 mM each), 0.5 μL of Taq DNA polymerase (5 U/μL) and 37.5 μL of sterilized double-distilled water. The amplification program consisted of an initial denaturation step at 94 °C for 5 min, followed by 35 cycles, in which each cycle consisted of 94 °C for 40 s (denaturation), 55 °C for 50 s (annealing), and 72 °C for 40 s (extension). A final extension of 10 min at 72 °C was added at the end of the program to ensure the complete amplification of the target region. During amplification, negative controls were also performed.

Next, the PCR products were visualized on agarose gels (1 % in TBE buffer) containing Goldview nucleic acid stain (Beijing Zoman Biotechnology Co. Ltd.) and cleaned using an Axyprep™ DNA gel extraction kit (Axygen Scientific Inc, USA). Prior to sequencing, the DNA concentration of each PCR product was quantified using a UV–Vis spectrophotometer (NanoDrop ND1000, USA). Equal amounts of three samples containing different sample-specific barcode sequences were pooled to provide a total quantity of 100 ng, and 454-pyrosequencing was performed at Tongji-SCBIT Biotechnology Co. Ltd. Shanghai, China.

454-Pyrosequencing data analysis

Sequences were processed and analyzed using the Mothur software package (Schloss et al. 2009) following the protocols described by Schloss et al. (2011) and Diaz et al. (2012). Sequences were assigned to samples by examining the 8-bp barcode. All poor-quality reads and primer-dimers were removed, and the good sequences were trimmed and compared against the sequences in the Silva database (Pruesse et al. 2007) via Mothur. The pre-cluster command in the Mothur platform was used to remove sequences that were likely caused by pyrosequencing errors (Huse et al. 2010; Roeselers et al. 2011). Mothur provided rarefaction analysis, constructed the distance matrices, assigned sequences to operational taxonomic units (OTUs, 97 % similarity) and calculated the Shannon diversity index (Shannon) and the species richness estimators (Chao). The formulas used to calculating these indices are shown in http://www.mothur.org/wiki/Calculators.

All of the sequences obtained from pyrosequencing in this study were compared with the RDP classifier with a set confidence threshold of 50 % (Wang et al. 2007). Then, the sequences were assigned to NCBI taxonomies with the MEGAN program (Huson et al. 2011) using the lowest common ancestor (LCA) algorithm and default parameters.

Statistical analysis

Data are presented as the mean ± standard deviation, and all mean values were compared using the Duncan's multiple range test (Statistical Package for the Social Sciences, SPSS, version 17.0). An independent sample t test was used for statistical comparison, and a value of $p < 0.05$ was considered statistically significant.

Results

Effects of *Bacillus subtilis* SC02 on water quality

Bacillus subtilis SC02 had an effect on the water quality of grass carp culture water (Fig. 1). Compared with the control, the levels of ammonia, nitrite and total nitrogen of the treated group significantly decreased. Specifically, ammonia decreased by 31.95 and 44.35 % ($p < 0.05$) on the 6th and 12th days (Fig. 1a), respectively. Nitrite decreased by 87.86 % ($p < 0.05$) on the 15th days (Fig. 1b), and total nitrogen decreased by 23.15, 17.95 and 41.68 % ($p < 0.05$) at the 6th, 9th and 15th days (Fig. 1d), respectively. In all trials, no significant differences were observed between groups regarding nitrate nitrogen content (Fig. 1c). The effect of *B. subtilis* SC02 on other water quality parameters is shown in Figure S3.

Effect of *Bacillus subtilis* SC02 on microbial diversity

To study the effect of *B. subtilis* SC02 on microbial community diversity and structure in a grass carp culture, the authors collected water microbes at the end of the trials, and 454-pyrosequencing was performed. After removing sequences of insufficient quality and sequences that could not be adequately classified, nearly 9,798 sequences (1,462 unique sequences) remained. To estimate the diversity of the control and treatment group samples, we identified OTUs corresponding with 3 % sequence divergence. Diversity indices at the 97 % sequence similarity level included observed richness (sobs), the Good's estimated coverage (coverage), the Shannon diversity index (Shannon) and species richness estimators (Chao), and these factors are shown in Table 2. The Good's coverage revealed the majority of bacterial 16S rRNA sequences present in each sample. The Chao values and rarefaction curves (Fig. 2) showed that the bacterial richness in the treated group was significantly higher than the control group. A hierarchically clustered heatmap analysis based on bacterial community profiles at the family level showed that the treated samples and control samples were clustered into two groups, indicating an effect of *B. subtilis* SC02 on microbial diversity (Fig. S4).

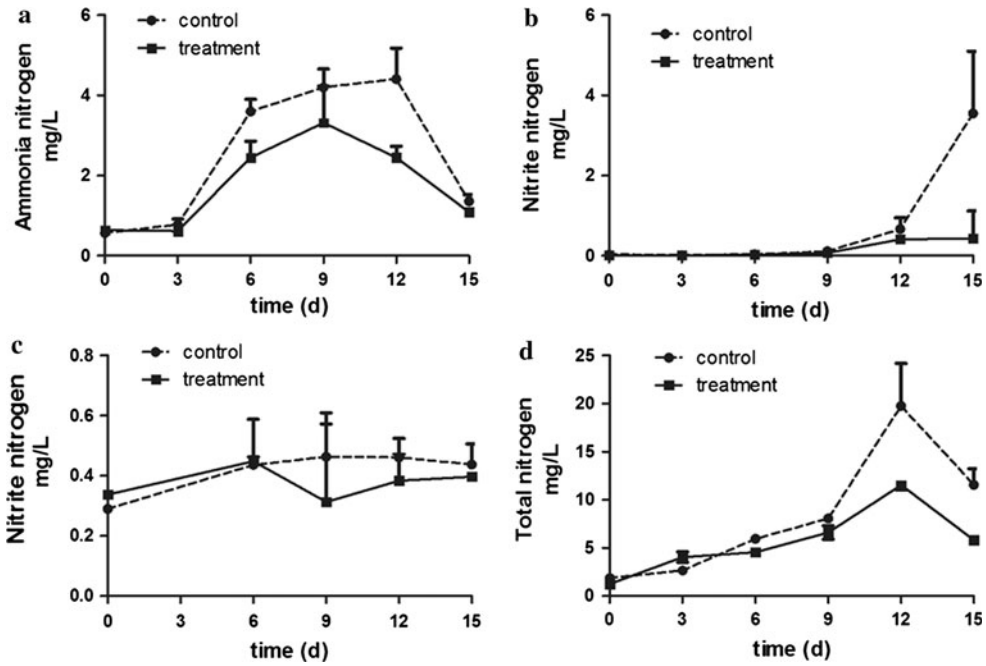

Fig. 1 Effect of *Bacillus subtilis* on the water quality in grass carp culture. **a** Ammonia nitrogen, **b** nitrite nitrogen, **c** nitrate nitrogen, and **d** total nitrogen

Table 2 Diversity estimates for the samples from control and treatment

	Control	Treatment group
Sobs	154 ± 26	187 ± 47
Coverage	94.44 ± 5.05 %	95.05 ± 0.40 %
Chao	231.68 ± 93.06	278.43 ± 54.54
Shannon	2.94 ± 0.44	3.93 ± 0.18

This table shows the observed richness (sobs), good's estimated coverage (coverage), Shannon diversity index (Shannon), and species rich ness estimators (Chao) (label = 0.03). However, a strong individual variation was observed between control and treatment (see text)

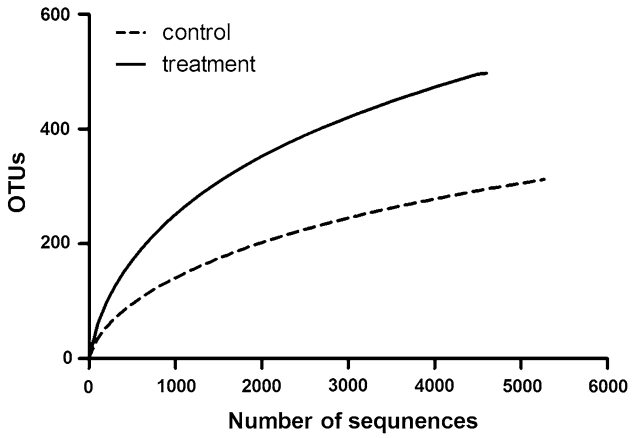

Fig. 2 Rarefaction results for the control and treatment groups

Effect of *Bacillus subtilis* SC02 on microbial community structure

All sequences were classified from phylum to genus according to the Mothur program using the default settings. The control and treatment groups had very dissimilar 16S rRNA profiles in phylum level distributions (Fig. 3). The Proteobacteria and Firmicutes phyla were dominant in the control group, comprising 82.1 and 8.4 % of the sequences, respectively; however, Proteobacteria (45 %), Bacteroidetes (21.9 %) and Actinobacteria (29 %) were dominant in the treatment group. In the treatment group, Proteobacteria, Alphaproteobacteria and Deltaproteobacteria were increased, while Betaproteobacteria and Gammaproteobacteria were decreased compared with the control group (Fig. 4). The microbial community structures of Alphaproteobacteria and Gammaproteobacteria at the family level are shown in Fig. S5, and marked divergences in composition of the microbial communities between the control and treatment groups were observed.

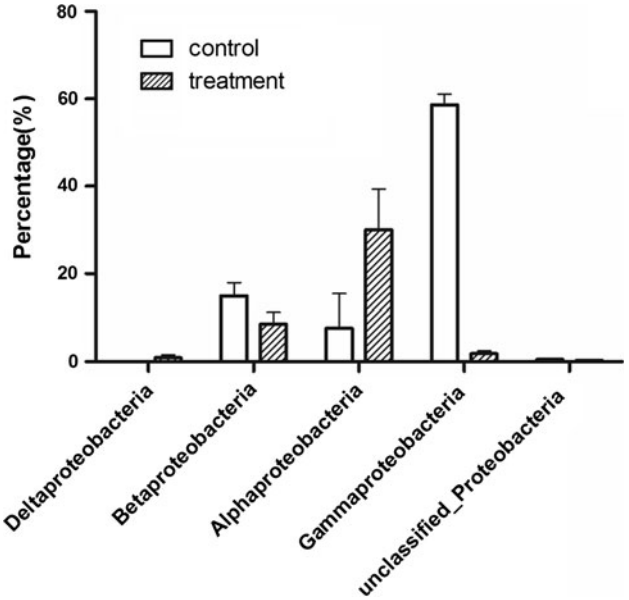

Fig. 4 Proteobacteria composition by class in samples from the control and treatment groups. The abundance is presented in terms of the percentage of the total number of sequences in a sample classified using the RDP Classifier at a confidence threshold of 50 %

To further compare the microbial communities of two samples from the control and treatment groups, an all-against-all comparison was conducted using MEGAN software. The pie charts and the leaves of the tree indicate the relative abundance of the genus in the two samples (Fig. 5). At the genus level, the microbial communities of

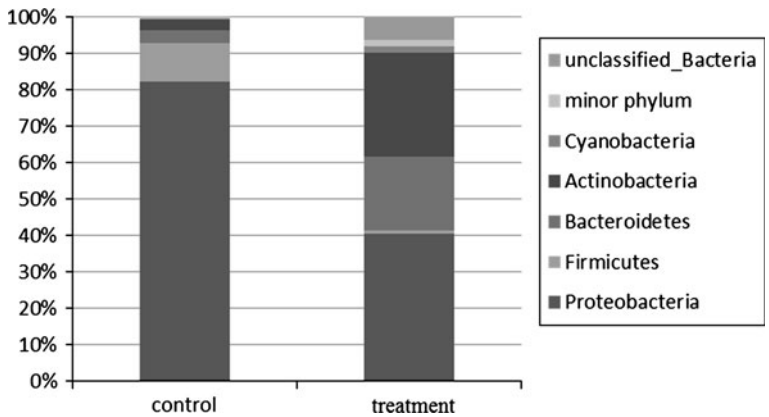

Fig. 3 Bacterial community structures in the control and treatment groups. The abundance is presented in terms of a percentage of the total effective bacterial sequences in the sample, which were classified using the RDP Classifier at a confidence threshold of 50 %. Minor phylum refers to the taxa with a maximum abundance of <1 % in any sample

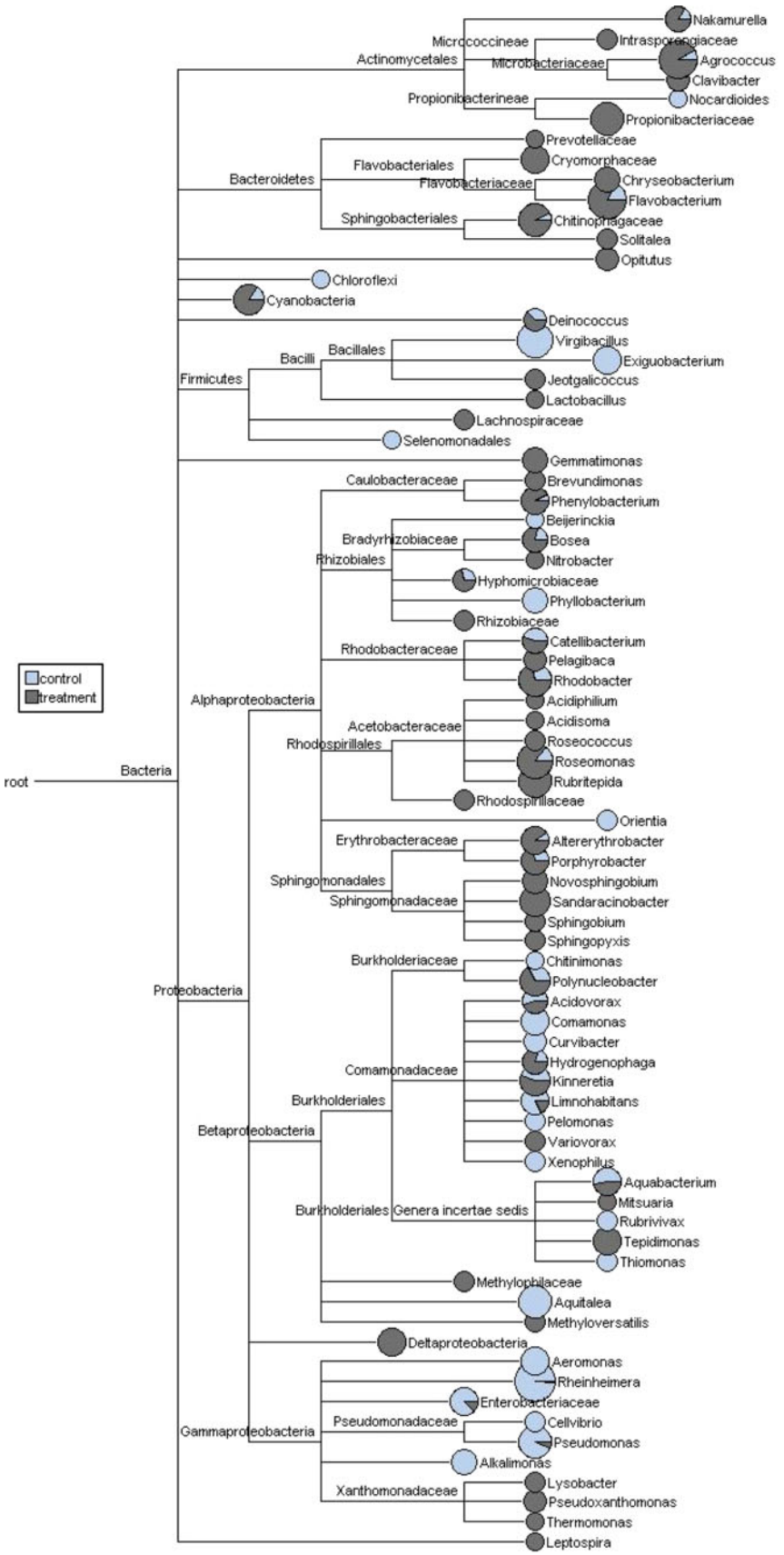

Fig. 5 Sequences from the control and treatment groups were compared using MEGAN 4. *Pie charts* indicate the relative abundance for each genus. The ratio of the *gray color* area to the *dark color* area in each *pie* represents the ratio of the relative abundance of the corresponding genus in the control group to the genus in the treatment group

the control and treatment samples were different, and 23 nodes were shared by the two groups. Some genera (such as *Tepidimonas*, *Variovorax*, *Roseomonas*, *Rubritepida*, *Nitrobacter*, etc.) only appeared in the treated group, while many other genera, which are marked in gray in Fig. 5, existed only in the control group.

Discussion

The present findings revealed that the levels of ammonia, nitrite and total nitrogen in the treatment group were significantly lower ($p < 0.05$) than in the control group in an extended range. It has been reported that the use of *Bacillus* sp. improved water quality and the survival, growth rates and health status of juvenile *Penaeus monodon* and reduced pathogenic vibrios (Dalmin et al. 2001). Wang et al. (2005) found that the use of commercial probiotics in *Penaeus vannamei* ponds improved the population density of beneficial bacterial flora, reduced nitrogen and phosphorus concentrations and increased shrimp yields. Additionally, denitrification activity promotes *B. subtilis* SC02 to improve water quality. Shi and Lee (2006) and Lu et al. (2011) reported that denitrifying bacteria can remove nitrogen, degrade naphthalene and accumulate phosphorus. The addition of immobilized denitrifying bacteria to a floating canna bed improved the removal efficiency of both TN and COD (Sun et al. 2009). However, the removal efficiency of NO_3^-–N by immobilized aerobic denitrifying bacteria HS-N62 pellets was enhanced, with no nitrite accumulation (Wang et al. 2012a, b).

In our study, the 454-pyrosequencing analysis revealed that the treatment group was dominated by Proteobacteria, Bacteroidetes and Actinobacteria. Proteobacteria comprise one of the largest divisions within prokaryotes and account for the vast majority of known Gram-negative bacteria (Stackebrandt 1992; Holt et al. 1994; Balows et al. 1992; Collier et al. 1998; Zinder 1998). It has been reported that α and β Proteobacteria were related to nitrogen removal (Labbé et al. 2007; Shapleigh 2011). Actinobacteria are well known for producing secondary metabolites, of which many are potent of antibiotics (Ventura et al. 2007), play a role in the breakdown and recycling of organic compounds (Goodfellow and Haynes 1984), and perform other undefined ecological roles (Jensen and Lauro 2008). Bacteroidetes comprise a large proportion of particle-associated bacteria (Lemarchand et al. 2006; Nold and Zwart 1998) and appear to play a particularly important role in the degradation of complex biopolymers (Kirchman 2002) and humic matter (Hutalle-Schmelzer et al. 2010). Our study also found that the levels of Bacteroidetes and Actinobacteria in the treated group were higher compared with the control group. Therefore, the increased number of Bacteroidetes and Actinobacteria observed in the treatment group may have improved the chemical quality of water compared with the control group. The added bacteria were not detected in the water, which may be due to the reduced numbers of *B. subtilis* SC02 and because the sequencing depth was not sufficient.

In this study, we found that adding *Bacillus* strain SC02 into the water increased microbial diversity. *B. subtilis* secretes antimicrobial substances, such as coagulin, amicoumacin and subtilisin, to suppress the growth of competing microbes and enteric pathogens (Cutting 2011). In addition, microorganisms participate in the turnover of organic nutrients and improve water quality (Moriarty 1997; Sahu et al. 2008), which creates a better living environment for the microorganism. Moreover, husbandry practices and environmental conditions alter the composition of microbial communities, which stimulates the proliferation of selected bacterial species (Balcazar et al. 2006).

In conclusion, rearing water with *Bacillus* strain SC02 supplementation alters the microbial community structure and may contribute to water quality improvement. However, further studies are needed to evaluate the impact of *Bacillus* strain SC02 on the fish population and fish intestinal flora.

Acknowledgments This study was supported by the National Basic Research Program, P. R. China (973 Program, 2009CB118705), and the Natural Science Foundation of Zhejiang Province (Grant No. Y3090171).

References

Balcazar JL, de Blas I, Ruiz-Zarzuela I, Cunningham D, Vendrell D, Muzquiz J (2006) The role of probiotics in aquaculture. Vet Microbiol 114:173–186

Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH (1992) The prokaryotes. Springer, New York

Cao L, Wang W, Yang Y, Yang C, Yuan Z, Xiong S, Diana J (2007) Environmental impact of aquaculture and countermeasures to aquaculture pollution in China. Environ Sci Pollut Res 14(7):452–462

Casserly C, Erijman L (2003) Molecular monitoring of microbial diversity in an UASB reactor. Int Biodeterior Biodegradation 52(1):7–12

Cole DW, Cole R, Gaydos SJ, Gray J, Hyland G, Jacques ML, Powell-Dunford N, Sawhney C, Au WW (2009) Aquaculture: environmental, toxicological, and health issues. Int J Hyg Environ Health 212(4):369–377

Collier L, Balows A, Sussman M (1998) Topley and Wilson's microbiology and microbial infections, vol 2, Systematic Bacteriology, vol 2. Arnold, London

Cutting SM (2011) *Bacillus* probiotics. Food Microbiol 28:214–220

Dalmin G, Kathiresan K, Purushothaman A (2001) Effect of probiotics on bacterial population and health status of shrimp in culture pond ecosystem. Indian J Exp Biol 39:939–942

DeAngelis KM, Wu CH, Beller HR, Brodie EL, Chakraborty R, DeSantis TZ, Fortney JL, Hazen TC, Osman SR, Singer ME, Tom LM, Andersen GL (2011) PCR amplification-independent methods for detection of microbial communities by the high-density microarray phylochip. Appl Environ Microbiol 77(18):6313–6322

Diaz PI, Dupuy AK, Abusleme L, Reese B, Obergfell C, Choquette L, Dongari-Bagtzoglou A, Peterson DE, Terzi E, Strausbaugh LD (2012) Using high throughput sequencing to explore the biodiversity in oral bacterial communities. Mol Oral Microbiol 27(3):182–201

Enell M, Lof J (1983) Environmental impact of aquaculture: sediment and nutrient loadings from fish cage culture farming. Vatten 39:364–375

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

Fuller R (1989) Probiotics in man and animal. J Appl Bacteriol 66:365–378

Goodfellow M, Haynes JA (1984) Actinomycetes in marine sediments. In: Oritz–Oritz L, Bojalil LF, Yakoleff V (eds) Biological, biochemical and biomed ical aspects of actinomycetes. Academic press, New York, pp 453–472

Holt JG, Krieg NR, Sneath PHA, Staley JT, Stanley TW (1994) Bergey's manual of determinative bacteriology. Williams and Wilkins, Baltimore, MD

Hu M, Wang XH, Wen XH, Xia Y (2012) Microbial community structures in different wastewater treatment plants as revealed by 454-pyrosequencing analysis. Bioresour Technol 117:72–79

Huse SM, Welch DM, Morrison HG, Sogin ML (2010) Ironing out the wrinkles in the rare biosphere through improved OTU clustering. Environ Microbiol 12:1889–1898

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

Hutalle-Schmelzer KML, Zwirnmann E, Krüger A, Grossart HP (2010) Enrichment and cultivation of pelagic bacteria from a humic lake using phenol and humic matter additions. FEMS Microbiol Ecol 72:58–73

Jensen PR, Lauro FM (2008) An assessment of actinobacterial diversity in the marine environment. Antonie Van Leeuwenhoek 94:51–62

Kim TS, Kim HS, Kwon S, Park HD (2011) Nitrifying bacterial community structure of a full-scale integrated fixed-film activated sludge process as investigated by pyrosequencing. J Microbiol Biotechnol 21(3):293–298

Kirchman DL (2002) The ecology of Cytophaga-Flavobacteria in aquatic environments. FEMS Microbiol Ecol 39:91–100

Labbé N, Laurin V, Juteau P, Parent S, Villemur R (2007) Microbiological community structure of the biofilm of a methanol-fed, marine denitrification system, and identification of the methanol-utilizing microorganisms. Microbial Ecol 53:621–630

Lemarchand K, Berthiaume F, Maynard C, Harel J, Payment P, Bayardelle P, Masson L, Brousseau R (2005) Optimization of microbial DNA extraction and purification from raw wastewater samples for downstream pathogen detection by microarrays. J Microbiol Methods 63(2):115–126

Lemarchand C, Jardillier L, Carrias JF, Richardot M, Debroas D, Ngando TS, Amblard C (2006) Community composition and activity of prokaryotes associated to detrital particles in two contrasting lake ecosystems. FEMS Microbiol Ecol 57:442–451

Lin X, Feng Y, Zhang H, Chen R, Wang J, Zhang J, Chu H (2012) Long-term balanced fertilization decreases arbuscular Mycorrhizal fungal diversity in an arable soil in North China revealed by 454 pyrosequencing. Environ Sci Technol 46(11):5764–5771

Lu XY, Zhang T, Fang HHP, Leung KMY, Zhang G (2011) Biodegradation of naphthalene by enriched marine denitrifying bacteria. Int Biodeterior Biodegradation 65:204–211

Lumini E, Orgiazzi A, Borriello R, Bonfante P, Bianciotto V (2010) Disclosing arbuscular mycorrhizal fungal biodiversity in soil through a land-use gradient using a pyrosequencing approach. Environ Microbiol 12(8):2165–2179

Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen ZT, Dewell SB, Du L, Fierro JM, Gomes XV, Godwin BC, He W, Helgesen S, Ho CH, Irzyk GP, Jando SC, Alenquer MLI, Jarvie TP, Jirage KB, Kim JB, Knight JR, Lanza JR, Leamon JH, Lefkowitz SM, Lei M, Li J, Lohman KL, Lu H, Makhijani VB, McDade KE, McKenna MP, Myers EW, Nickerson E, Nobile JR, Plant R, Puc BP, Ronan MT, Roth GT, Sarkis GJ, Simons JF, Simpson JW, Srinivasan M, Tartaro KR, Tomasz A, Vogt KA, Volkmer GA, Wang SH, Wang Y, Weiner MP, Yu PG, Begley RF, Rothberg JM (2005) Genome sequencing in microfabricated high-density picolitre reactors. Nature 437(7057):376–380

Moriarty DJW (1997) The role of microorganisms in aquaculture ponds. Aquaculture 15:333–349

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

Nold SC, Zwart G (1998) Patterns and governing forces in aquatic microbial communities. Aquat Ecol 32:17–35

Nora'aini A, Wahab MA, Jusoh A, Hasan MR, Ghazali N, Kamaruzaman K (2005) Treatment of aquaculture wastewater using ultra-low pressure asymmetric polyethersulfone (PES) membrane. Desalination 185:317–326

Oerther DB, De los Reyes FL, De los Reyes MF, Raskin L (2001) Quantifying filamentous microorganisms in activated sludge before, during, and after an incident of foaming by oligonucleotide probe hybridizations and antibody staining. Water Res 35(14):3325–3336

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glöckner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35:7188–7196

Roberts SB, Hauser L, Seeb LW, Seeb JE (2012) Development of genomic resources for Pacific Herring through targeted transcriptome pyrosequencing. PLoS ONE 7(2):e30908

Roesch LF, Fulthorpe RR, Riva A, Casella G, Hadwin AKM, Kent AD, Daroub SH, Camargo FAO, Farmerie WG, Triplett EW (2007) Pyrosequencing enumerates and contrasts soil microbial diversity. ISME J 1(4):283–290

Roeselers G, Mittge EK, Stephens WZ, Parichy DM, Cavanaugh CM, Guillemin K, Rawls JF (2011) Evidence for a core gut microbiota in the zebrafish. ISME J 5:1595–1608

Sahu MK, Swarnakumar NS, Sivakumar K, Thangaradjou T, Kannan L (2008) Probiotics in aquaculture: importance and future perspectives. Indian J Microbiol 48:299–308

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75:7537–7541

Schloss PD, Gevers D, Westcott SL (2011) Reducing the effects of PCR amplification and sequenc-ing artifacts on 16S rRNA-based studies. PLoS ONE 6:e27310

Shapleigh JP (2011) Oxygen control of nitrogen oxide respiration, focusing on α-proteobacteria. Biochem Soc Trans 39:179–183

Shen WY, Fu LL, Li WF, Zhu YR (2010) Effect of dietary supplementation with *Bacillus subtilis* on the growth, performance, immune response and antioxidant activities of the shrimp (*Litopenaeus vannamei*). Aquac Res 41:1691–1698

Shi HP, Lee CM (2006) Combining anoxic denitrifying ability with aerobic-anoxic phosphorus-removal examinations to screen denitrifying phosphorus-removing bacteria. Int Biodeterior Biodegradation 57:121–128

Sogin ML, Morrison HG, Huber JA, Welch DM, Huse SM, Neal PR, Arrieta JM, Herndl GJ (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". Proc Natl Acad Sci USA 103:12115–12120

Stackebrandt E (1992) Unifying phylogeny and phenotypic diversity. In: Balows A, Truper HG, Dworkin M, Harder W, Schleifer KH (eds) The prokaryotes. Springer, New York, pp 19–47

Sun LP, Liu Y, Jin H (2009) Nitrogen removal from polluted river by enhanced floating bed grown canna. Ecol Eng 35(1):135–140

Ventura M, Canchaya C, Tauch A, Chandra G, Fitzgerald GF, Chater KF, Sinderen D (2007) Genomics of Actinobacteria: tracing the evolutionary history of an ancient phylum. Microbiol Mol Biol Rev 71:495–548

Wang YB, Xu ZR, Xia MS (2005) The effectiveness of commercial probiotics in northern white shrimp (*Penaeus vannamei* L) ponds. Fish Sci 71:103–1039

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73(16):5261–5267

Wang YB, Li JR, Lin J (2008) Probiotics in aquaculture: challenges and outlook. Aquaculture 281:1–4

Wang P, Yuan Y, Li Q, Yang J, Zheng Y, He M, Geng H, Xiong L, Liu D (2012) Isolation and immobilization of new aerobic denitrifying bacteria. Int Biodeterior Biodegradation 10.1016/jibiod201206008

Wang X, Hu M, Xia Y, Wen X, Ding K (2012b) Pyrosequencing analysis of bacterial diversity in 14 wastewater treatment systems in China. Appl Environ Microbiol 78(19):7042–7047

Zheng JJ, Shen T, Fu LQ, Deng B, Li WF (2012) Identification and denitrification characteristics of a denitrifer *Pseudomonas Putida* F6. China Acta Hydrobiol Sinica 36:161–167

Zinder SH (1998) Bacterial diversity. In: Balows A, Duerden BI (eds) Topley and Wilson's microbiology and microbial infections, vol 2, systematic bacteriology. Arnold, London, pp 125–147