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Influence of *Bacillus subtilis* B068150 on cucumber rhizosphere microbial composition as a plant protective agent

Lihua Li · Jincai Ma · A. Mark Ibekwe (ID) · Qi Wang · Ching-Hong Yang

Abstract

Aim Our aim was to investigate factors that influenced bacterial and fungal community composition in the cucumber rhizosphere with the addition of *Bacillus subtilis* B068150 in soils.

Methods Using pyrosequencing, we tracked changes in total bacterial and fungal community composition and structures in cucumber rhizosphere planted in clay, loam and sandy soils and inoculated with *B. subtilis* B068150.

Results *B. subtilis* B068150 colonized the rhizosphere of cucumber without altering the indigenous microbial community composition. B068150 strain did not significantly affect microbial diversity in cucumber rhizosphere, but both soils texture and chemistry did, based on principal coordinates analysis (PCoA), hierarchical clustering in UniFrac, and canonical correspondence analysis (CCA). Shannon diversity values (H') suggest that rhizosphere diversity varied among the three soils with the significant highest diversity observed in loamy soil. Fungi were dominated by Ascomycota, Chytridiomycota, Basidiomycotina, with Ascomycota accounting for 29.1%. *Fusarium* was found only in cucumber rhizosphere grown in loamy soil; however, this did not affect the health of cucumber in three loamy soils during the three months study.

Conclusion *Bacillus subtilis* B068150 could be an environmental compatible plant protective agent in soils depending on the soil type.

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Introduction

Bacillus spp. is one of the most effective alternative biocontrol agents to synthetic chemical fungicides (Solankia et al. 2012). Some strains of *B. subtilis* are known biocontrol agents against *Fusarium oxysporum* and are marketed as biopesticides, biofertilizers, and soil amendments. In the rhizosphere, the complex interaction between an inoculant strain and the indigenous microbial rhizosphere community must be carefully examined (Kröber et al. 2014) due to the potential high levels of secondary metabolites being produced by *B. subtilis* (inoculant strains), and their possible impact on other microorganisms within the microbial community in the rhizosphere of host plants. Although the mode of action differs from strain to strain, the biocontrol agent must be able to colonize the root surface and soil for successful biological biocontrol to occur, and they must also be able to compete with the native microbial population for nutrients and for their survival (El-Hassan and Gowen 2006; Kokalis-Burelle et al. 2002). Therefore, the rhizosphere competence of bacterial inoculants is an important factor for successful biocontrol (Barret et al. 2011). The influence of inoculants on the indigenous rhizosphere microbial community has been hypothesized in many studies to be one of the controlling factors for successful biocontrol (Lottmann et al. 2000; Chowdhury et al. 2013). These authors evaluated the rhizosphere competence of the commercially available inoculant *Bacillus amyloliquefaciens* FZB42 on lettuce growth and health together with its impact on the indigenous rhizosphere bacterial community in field and pot experiments, and showed that the disease severity of bottom rot on lettuce was significantly reduced.

In agriculture, plants are cultivated under various ecological conditions. The complex relationships among inoculants, pathogens, plants, and ecological factors such as soil type can affect a biocontrol agent. So the present study was to investigate the influence of soil types on the rhizosphere competence and biocontrol activity of bacterial inoculants and their effects on the indigenous soil bacterial community. To determine the effect of *B. subtilis* B068150 as a plant protective agent, its impact on rhizosphere and soil bacterial and fungal communities was analyzed using pyrosequencing. During this study we assess the influence of soil types on the rhizosphere competence and biocontrol activity of strain B068150 and its effects on the indigenous soil bacteria and fungi. This study used the same DNA samples that were previously used for DGGE analysis (Li et al. 2016). The second study was done to gain deeper and more refined insights into microbial rhizosphere communities of cucumber after the inoculation with *B. subtilis strains* B068150, since DGGE can only differentiate only the major members of the community and in most cases about 1% of the community. Therefore, we used pyrosequencing approach to track changes in the taxonomic community profiles during the growth period of cucumber and to identify dominant rhizosphere microorganisms. Our overall objective was to determine the prevalence of different bacterial and fungal groups in cucumber rhizosphere and to assess the influence of strain B068150 on bacteria and fungi community composition and structure using deep sequencing.

Materials and methods

Strains and growth conditions

This study used *B. subtilis* strain B068150 that was stored on cryoprotect beads in MicroBank microbial storage tubes (Pro-Lab Diagnostics, Ontario, Canada) under −80 °C. This strain was tagged with nalidixic acid and rifampicin, and resistant to 50 μg ml^{-1} nalidixic acid and 150 μg ml^{-1} rifampicin in LB plates as previously described (Ma et al. 2011).

Inoculum preparation

A single bead was removed from storage at −80 °C under aseptic, and after a day of growth at 37 °C bacterial culture was streaked on a second LB plate with the antibiotics (rifampicin (150 μg ml^{-1}) and nalidixic acid (50 μg ml^{-1}) and single colonies from this growth were used for inoculums production. A loop of bacteria taken from a single colony was used to inoculate sterile LB broth (100 ml) in a 250 ml flask to obtain a batch culture. All experimental details were as previously described (Li et al. 2016). The concentration of *B. subtilis* B068150 was added to soils to obtain a final concentration of 1×10^6 colony forming units (CFU) per gram of dry soil.

Soils preparation and strain B068150 inoculation

Clay soil (Willows silty clay, saline-alkaline), sandy soil (Dello loamy sand), and loamy soil (Arlington sandy loam) were collected from Mystic Lake dry bed, the Santa Ana River bed, and fallow field at the University of California-Riverside, respectively (Ma et al. 2011). Details of soils texture and chemistry had previously been described (Ma et al. 2011; Li et al. 2016). All soil samples were passed through a 2-mm sieve and placed in black plastic cylinders (diameter, 16.5 cm; height, 25 cm). The soil moisture content was maintained at 50% water holding capacity (WHC) during the course of experiment. Based on water loss from each soil, after differences in soil weight after every 3 days, the amount of irrigation water was added automatically to each soil.

B. subtilis B068150 spore suspensions were mixed into the sand, loam, and clay soils to obtain a final concentration of 0.98×10^6 CFU/g, 0.85×10^6 CFU/g and 1.2×10^6 CFU/g dry weight soil, respectively, and control soil without strain B068150 was included. Plastic cylinders containing the three soils were kept in the greenhouse after introducing B068150 into soils and planting cucumber (Hybrid cucumber, Seminis vegetable seeds, Oxnard, CA). Surface-sterilized cucumber seeds were soaked in 3% NaOCl for 10 min, followed by repeated washing with sterile distilled water, and incubated at 30 °C for 24 h for germination (Li et al. 2012). All experiments were conducted under room temperature (22 ± 1 °C). Triplicate plastic cylinders containing the soils inoculated with *B. subtilis* B068150 were set up for each soil type.

Pot experimental design

Soil samples with no previous cucumber growth were used in this study as previously described (Li et al. 2016). Briefly, cucumber seeds were planted in the control soils and soils inoculated with B068150 in cylinders (16.5 cm in diameter, 25 cm in height), under the simulated natural conditions with the average daytime temperature at 30 °C, and 24 °C in the night. Moisture content of the soil sample was maintained constantly (50% of WHC) during the course of experiment by dripping water automatically to make up for evaporation. Based on evaporation, the amount of irrigation water applied to each soil was 27.33 ml, 38.67 ml and 18 ml for clay, loam, and sandy soil per day and per cylinder, respectively.

Non-rhizosphere soil samples from the pot experiments were collected at days 0, 3, 5, 10, 15, 20, 25, 28 and 31, and continued for days 39, 53, 74 and 91 for the rhizosphere soils as previously described (Li et al. 2016). After 31 days, the cylinders were filled with cucumber roots; hence the collection of non-rhizosphere soils was stopped. Cells were extracted from cucumber rhizosphere and non-rhizosphere soil samples inoculated with B068150 and cultured on the LB plates (25 μg ml^{-1} nalidixic acid, 50 μg ml^{-1} rifampicin) for the assessment of the colonization patterns of strain B068150 as previously reported (Li et al. 2016).

Both rhizosphere and none-rhizosphere soils were characterized (Ma et al. 2011) included clay, silt, and sand content, pH, electrical conductivity (EC), bulk density (BD), water-holding capacity (WHC), total organic carbon (OC), and total nitrogen (N) (Klute 1986). Soil microbial biomass carbon (MBC) was extracted by the chloroform-fumigation-extraction method (Vance et al. 1987). The texture and chemistry of the soils are summarized in Table 1. Water extractable organic carbon (WEOC) was measured by a total organic carbon analyzer (TOC-500, Shimadzu Corp., Kyoto, Japan) according to the method by Liang et al. 1998. The assimilable organic carbon (AOC) fraction in WEOC was determined using a luminous bacterium strain *Vibrio harveyi* (Ma et al. 2012).

DNA extraction, PCR amplification, and pyrosequencing

Microbial genomic DNA was extracted from 0.5 g samples of each soil sample collected during the sampling campaigns using a MO BIO Power Soil DNA isolation kit (MO BIO Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol. Extracted DNA (2 μL) was quantified using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington DE), and run on a 1.0% agarose gel before it was used for pyrosequencing.

DNA samples (days 0, 10 and 32) were submitted to Core for Applied Genomics and Ecology (SCAN) at the University of Nebraska Lincoln for PCR optimization and pyrosequencing analysis. The V1-V2 region of the 16S rRNA gene was amplified using bar-coded fusion primers with the Roche-454 A or B titanium sequencing adapters (in italics), followed by a unique 8-base barcode sequence (B) and finally the 5′ ends of primer A-8FM (5′-CCATCTCATCCCTGCGTGTCTCCGAC

Table 1 Total bacterial diversity on normalized data from cucumber rhizosphere soils

group	#seqs	OTUs	chao	invsimpson	npshannon	simpson	coverage
C.10	904	220	561.4516	19.37327	4.313165	0.051618	0.838496
C.32	904	220	490.0857	16.33473	4.315823	0.061219	0.847345
CB.0	904	140	279.3333	16.90367	3.798464	0.059159	0.914823
CB.10	904	196	526.24	18.89174	4.211155	0.052933	0.857301
CB.32	904	236	636.0968	25.60738	4.545923	0.039051	0.825221
L.10	904	375	1055.706	36.48485	5.43806	0.027409	0.707965
L.32	904	333	669.375	34.58363	5.248932	0.028915	0.769912
LB.0	904	325	862.5682	31.28591	5.118589	0.031963	0.75885
LB.10	904	285	655.2273	45.26517	5.037278	0.022092	0.799779
LB.32	904	363	1000.56	42.86002	5.394269	0.023332	0.720133
S.10	904	80	215	1.89317	1.744762	0.528215	0.939159
S.32	904	310	680.125	45.78306	5.203181	0.021842	0.790929
SB.0	904	172	352.5625	4.294164	3.325294	0.232874	0.880531
SB.10	904	259	550.383	12.38525	4.47948	0.080741	0.816372
SB.32	904	157	305.5313	3.294051	3.024085	0.303578	0.891593

(C) clay, (L) loam, (S) sand, (B) *B. subtillis* B068150 introduced

Treatments in this study (S0D, S10D, S32D: soils untreated and sampled in 0, 10 and 32 day. SB0D, SB10D, SB32D: *B subtilis* B068150-NR was introduced in to soils and sampled in 0, 10 and 32 day)

TCAGBBBBBBBBBAGAGTTTGATCMTGGCTCAG) and of primer B-357R (5′-CCTATCCCCTGTGT GCCTT-GGCAGTCTCA GBBBBBBBB CTGCTGCCTYCCGTA-3′ (Benson et al. 2010). All PCR reactions were quality- controlled for amplicon saturation by gel electrophoresis; band intensity was quantified against standards using GeneTools software, and the resulting products were quantified using PicoGreen (Invitrogen, Carlsbad, CA) and a Qubit fluorometer (Invitrogen) before sequencing using Roche-454 GS FLX titanium chemistry (Benson et al. 2010). Raw data were treated with the Pyrosequencing Pipeline Initial Process (Cole et al. 2009) of the Ribosomal Database Project (RDP) to match barcodes and to trim off the adapters, barcodes and primers using the default parameters, and to remove sequences containing ambiguous 'N' or shorter than 200 bps (Claesson et al. 2009). These raw reads were in addition denoised to remove sequences that are likely due to pyrosequencing errors (Huse et al. 2010; Roeselers et al. 2011), and chimeras were filtered out using Chimera Slayer (Haas et al. 2011). Bacterial pyrosequencing population data were further analyzed by performing multiple sequence alignment techniques using the dist.seqs function in MOTHUR, version 1.9.1 (Schloss et al. 2009). MOTHUR was also used to assign sequences to operational taxonomic units (OTUs, 97% similarity) and calculate both Shannon's diversity index values (H), and Chao estimates. The taxonomic classification of the bacterial sequences of each sample was carried out individually, using the RDP Classifier. A bootstrap cutoff of 80% suggested by the RDP was applied to assign the sequences to different taxonomy levels.

ITS1F (CTTGGTCATTTAGAGGAAGTAA) (Gardes and Bruns 1993) and ITS2R (GCTGCGTT CTTCATCGATGC) (White et al. 1990) containing Roche 454 pyrosequencing adaptor A and a sample-specific multiplex identifier (MID) were used for the analysis of fungi. The PCR reactions were performed as described previously (Wubet et al. 2012). For quality filtering and analysis of the 454 ITS sequences MOTHUR (Schloss et al. 2009) was used. Sequences with ambiguous bases, homo-polymers and primer differences of more than eight bases were removed as well as all primer and barcode sequences. All sequence reads with a quality score lower than 20 and a read length of less than 200 bp were removed. We used the keep first 200 bp commands in MOTHUR to eliminate sequencing noise resulting in a sequence read fragment covering the ITS2 region for further analysis. Data were checked for chimeric sequences using the UCHIME algorithm (Edgar et al. 2011). Next, we used CD-HIT-EST (Li and

Godzik 2006) to assemble the reads into Operational Taxonomic Units (97% similarity) and were assigned to the OTUs using the UNITE database's 454 pipeline (Tedersoo et al. 2010). OTUs that were identified to the family level were subjected to a BLASTn search (e.g., Johnson et al. 2008) against the NCBI GenBank database (Benson et al. 2015).

Statistics and analysis of pyrosequencing data

General linear model ANOVA (repeated measures) procedure in randomized complete block design was used with soil type and inoculation as the main plot and sampling time as the subplot to analyze the data on colonization of strain B068150 in rhizosphere and non-rhizosphere soil. Both ANOVA and general linear model ANOVA used SPSS (SPSS statistics 17.0, 2008, Chicago, IL), and the means were separated by Duncan's tests at $p < 0.05$. The rhizosphere and non-rhizosphere colonization data were converted to $log10$ values before the statistical analysis.

Principal coordinates analysis (PCoA) and hierarchical clustering in UniFrac were carried out using MOTHUR. The hierarchical clustering was conducted to group the bacterial communities of different samples into different taxonomic groups using the RDP Classifier at 80%, and OTUs generated using RDP Complete Linkage Clustering from all the samples, and Greengenes database (McDonald et al. 2012). PCoA was conducted based on RDP Classifier results, OTUs, and weighted UniFrac (Hamady et al. 2010). Phylogenetic trees were constructed using the relaxed neighbor-joining algorithm in Clearcut (version 1.0.9) (Sheneman et al. 2006), and between-site comparisons of phylogenetic structure were conducted using the parsimony test in Treeclimber (Schloss and Handelsman 2006). Parsimony test scores with P-values <0.05 were considered to represent significant differences. The microbial diversity indices were analyzed using R software version v3.1.0. (The R foundation for Statistical Computing: http://www.r-project.org/). Canonical correspondence analysis (CCA) was performed to determine the most significant variables shaping microbial community composition and structure. The Mantel test ($p < 0.1$) was used to select the most significant variables and to examine the correlation between community structure and these variables (Zhou et al. 2012).

Results

Bacterial diversity, taxonomic structure, and estimated richness

A total of 34,344 16S rRNA sequence tags with 6279 OTUs were generated through 454 pyrosequencing (Table S1), with an average read length of about 200 bp. Thus, moderate diversity pyrosequencing analysis ($\geq$ 3,000 reads per sample) was performed with read depth used for normalization of 418.6. Tags which did not have 100% homology to the original sample tag designation were not considered, as they might be suspect in quality. All data were normalized and the data reanalyzed to show normal distribution of variances (Table 1). Pyrosequencing results showed that rhizosphere soil bacteria were affiliated with 18 phylum-level groups (Table S2). Shannon diversity values (H') suggest that diversity varied among the different treatments with the significant highest diversity observed in loamy soil collected from the University of California plot ($p = 0.014$). Chao also confirmed that the highest estimate was captured in loamy soil compare to both clay and sand ($p = 0.019$). Pearson correlation coefficients showed that there were no significant correlation between bacteria diversity and colonization of *B. subtilis* B068150 in cucumber rhizosphere in clay, loamy and sandy soil. This was different from results obtained from DGGE analysis (Li et al. 2016).

The dominant bacteria in the three cucumber rhizosphere soil samples were Proteobacteria, Actinobacteria, and Firmicutes. Across all soils, Proteobacteria was the most abundant phylum accounting for 58.0% of the bacterial population in the rhizosphere. The second most abundance phylum was Firmicutes, and the highest percentages were found in clay and loamy soils, and *Bacillus* belongs to Firmicutes phylum. Phyla that were less abundant but still were found in most of the soils examined included the Acidobacteria, Bacteroidetes, BRC1, Chloroflexi, Cyanobacteria, Deinococcus-Thermus, Gemmatimonadetes, Nitrospira, OD1, OP10, Planctomycetes, Tenericutes, Thermomicrobia, TM7 and Verrucomicrobia (Table S2). Furthermore, additional analysis at the genus level showed the relative abundances (> 1%) of some bacterial genera including *Agrobacterium*, *Agromyces*, *Bacillus*, *Bosea*, *Bradyrhizobium*, *Herbaspirillum*, *Kaistobacter*,

Massilia, Rhodobacter, Skermanella, and unclassified bacteria (Fig. 1). Based on this analysis, *Bradyrhizobium* was the most dominant genus in sandy soil.

Addition of *Bacillus* B068150 resulted in a decrease in *Bacillus* in day 10 and a subsequent increased in day 32 in the cucumber rhizosphere in both clay and loam in comparison to sand, with no changes (Fig. 1). It was generally observed that the introduction of *Bacillus* B068150 into any of the soils resulted in the initial drop in percent of *Bradyrhizobium* during the first 10 days with a subsequent increase in population after 32 days. The addition of B068150 also resulted in the decrease of abundance of *Massilia, Skermanella,* and *Herbaspirillum* (Fig. 1). However, the differences in bacterial community between the treated samples and control became significantly smaller in clay and loam in day 32 (Fig. 1, Clay and Loam). Examination of the top most abundant bacterial OTUs showed *Bradyrhizobium* to produce the highest sequence tags in this study (Table S5). This is quite different from our previous study as we did not observe *Bradyrhizobium* from any of the major 25 DGGE bands (Li et al. 2016). *Streptomyces* was another genus that showed very high sequence tags in all the samples, and again was not observed in our previous study. Further examination showed that some uncultured bacteria and *Bacillus* were the only bacterial groups that were detected in both studies.

Fungal diversity, taxonomic structure, and estimated richness

After removing singleton OTUs and filtering reads by basal quality control, the pyrosequencing analysis of the ITS recovered 85,581 high-quality sequences across all samples with a range of 406–27,582 high-quality sequences (Table S3). Shannon diversity index showed a significant increase from day 0 to 32 after the inoculation of *Bacillus* to soil one (clay soil). However, there was no such continuous effect on soil one without *Bacillus*. No significant differences in diversity were observed in loamy and sandy soil after the inoculation of *Bacillus*. Based on 97% species similarity, 656 fungal OTUs were observed (Table S3). All data were normalized and the data reanalyzed to show normal distribution of variances (Table 2).

Fungi in all three soils were dominated by Ascomycota, Chytridiomycota, Basidiomycotina. The predominant phylum was Ascomycota, accounting for more than 29.1% (Table S4). After adding B068150 fungi abundance and Shannon diversity in clay soil increased. However, DGGE analysis results from our previous study showed that the Shannon diversity was highest in sandy soil (Li et al. 2016). Both analysis of DGGE and 454-pyroseqencing showed that there were no significant correlation between fungi diversity and colonization of *B subtilis* B068150 in cucumber rhizosphere in clay, loamy and sandy soil.
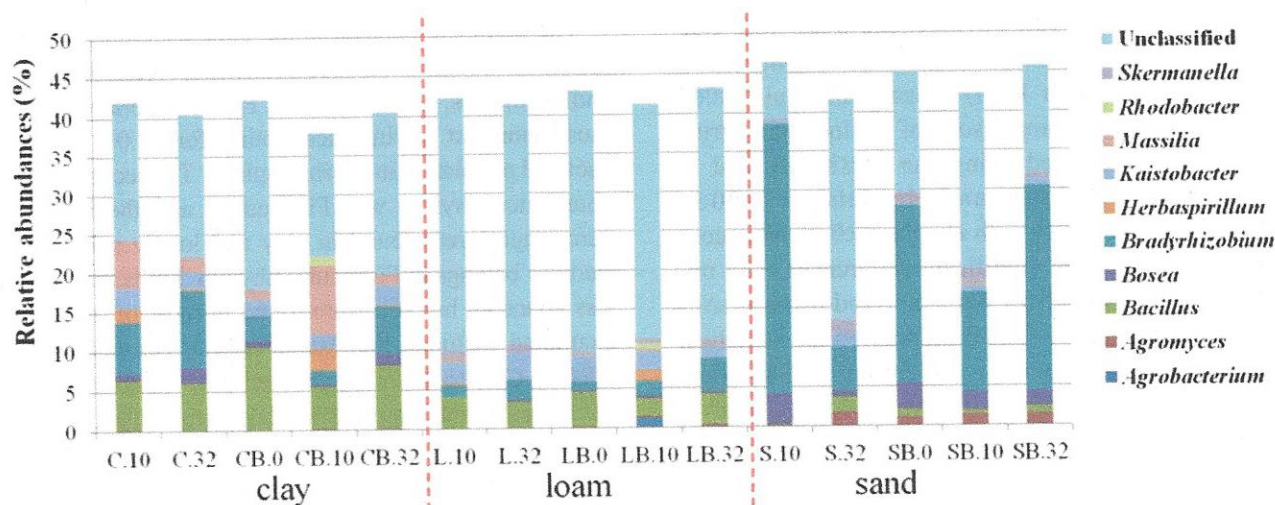

Fig. 1 Community composition (%) of bacterial genera in cucumber rhizosphere soil samples of clay, loam and sand. Major taxa detected are displayed with relative sequence abundances >1%. (C) clay, (L) loam, (S) sand, (B) *B. subtillis* B068150 introduced. Treatments in this study (S0D, S10D, S32D: soils untreated and sampled in 0, 10 and 32 day. SB0D, SB10D, SB32D: *B subtilis* B068150-NR was introduced into soils and sampled in 0, 10 and 32 day)

Table 2 Total fungal diversity on normalized data from cucumber rhizosphere soils

group	#seqs	OTUs	chao	invsimpson	npshannon	simpson	coverage
C.10	406	4	4	1.094128	0.225597	0.913969	1
C.32	406	15	17.5	2.815679	1.504879	0.355154	0.987685
CB.0	406	19	24	4.492377	2.001469	0.222599	0.985222
CB.10	406	15	15	5.245645	1.999569	0.190634	0.997537
CB.32	406	12	27	4.857894	1.754974	0.205851	0.985222
L.10	406	47	64.5	9.267839	2.956468	0.1079	0.963054
L.32	406	40	51	6.345709	2.630724	0.157587	0.970443
LB.0	406	50	56	8.840323	3.031216	0.113118	0.970443
LB.10	406	53	72	9.065498	3.03288	0.110308	0.950739
LB.32	406	43	54.25	7.602645	2.85278	0.131533	0.975369
S.10	406	9	9	2.155272	1.167317	0.463979	0.997537
S.32	406	13	14.5	3.066465	1.483822	0.326108	0.992611
SB.0	406	33	38.5	2.54575	1.807262	0.392812	0.972906
SB.10	406	6	6	1.925816	0.99707	0.51926	1
SB.32	406	27	28.5	10.23211	2.665665	0.097732	0.990148

(C) clay, (L) loam, (S) sand, (B) B. subtillis B068150 introduced. Treatments in this study (S0D, S10D, S32D: soils untreated and sampled in 0, 10 and 32 day. SB0D, SB10D, SB32D: *B subtilis* B068150-NR was introduced into soils and sampled in 0, 10 and 32 day)

Furthermore, the abundances (>1%) of fungal genera including *Alternaria, Aspergillus, Candida, Chaetomium, Fusarium, Hohenbuehelia, Monacrosporium, Nectria, Penicillium, Thielavia* in cucumber rhizosphere in three soils are presented in Fig. 2. As shown in the bacteria community, the differences between the treated samples and control became significantly smaller in clay and loamy soils in day 32. With the addition of *Bacillus* B068150, the abundance of *Aspergillus, Monacrosporium* and *Thielavia* increased in rhizosphere in both clay and loamy soils while decreased in sandy soil. *Alternaria, Chaetomium* and *Hohenbuehelia* also decreased in rhizosphere of loamy in day 32. Interesting, *Fusarium* was found in rhizosphere of cucumber grown in loamy as compared to none detectable levels in clay and sandy soils. There was an increasing abundance of *Fusarium* after the addition of *Bacillus* B068150 (Fig. 2, Loam). However, this did not affect the health of cucumber in three soils during the three months that the study was conducted. It was also interesting to find *Fusarium oxysporun* in loamy soil that was not treated with *Bacillus* (Table S6). It is interesting to note that *F. oxysporum* is one of the main plant pathogens that cause disease in cucumber. This was not observed in our previous study based on DGGE.

Factors affecting bacterial and fungi composition

In order to identify which factors influenced bacterial and fungal community composition in the cucumber rhizosphere in soils, CCA was performed. CCA results showed that soil textural contents (sand, silt, clay), were major parameters affecting soil bacterial and fungal community structures (Fig. 3). CCA showed that soils clustered together irrespective of the addition of *Bacillus*. In addition, sand and silt were the most important textural factors affecting bacterial (Fig. 3a) and fungal separations (Fig. 3b). However, the influence was stronger with bacteria (F = 4.348, $P < 0.001$) than with fungal communities (F = 2.100, $P < 0.01$). For bacteria, the model explained 41.7% of the variance with 28.9% explained by CCA1 and 12.8% by CCA2 (Fig. 3a), and for fungi, CCA1 explaining 14.8% and CCA explaining 11.1% (Fig. 3b).

CCA results also showed the effects of soil chemistry on bacterial and fungal community structures with or without the addition of *Bacillus* (Fig. 4). CCA showed the effects of soil chemical properties significantly affected ($P < 0.001$) bacterial community structures, and the model explained that CCA1 was affected by 28.9% of the variance and CCA2 by 12.8% (Fig. 4a). Soil chemical properties also
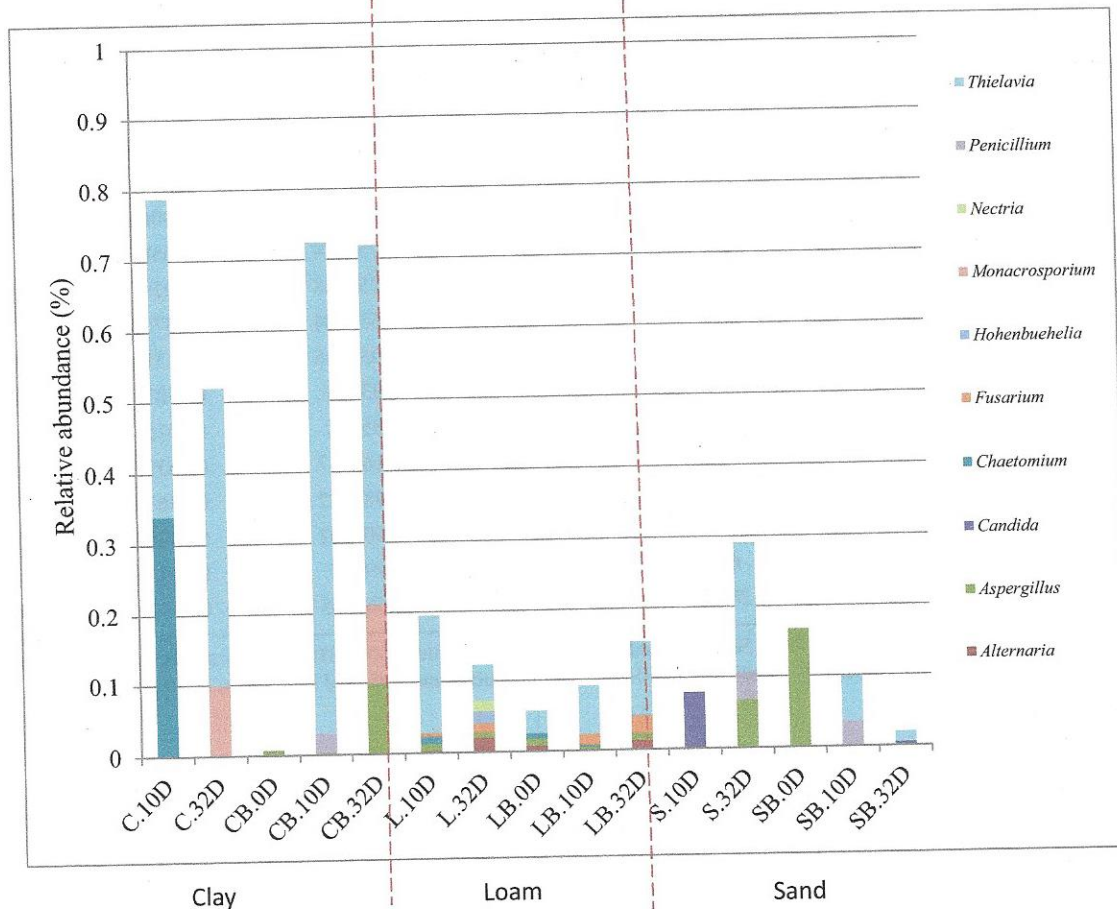
Fig. 2 Community composition (%) of fungal genera in cucumber rhizosphere soil samples of clay, loam and sand. Major taxa are displayed detected with relative sequence abundances >1%. (C) clay, (L) loam, (S) sand, (B) *B. subtillis* B068150 introduced.

Treatments in this study (S0D, S10D, S32D: soils untreated and sampled in 0, 10 and 32 day. SB0D, SB10D, SB32D: *B subtilis* B068150-NR was introduced into soils and sampled in 0, 10 and 32 day)

significantly affected fungal community structure ($P < 0.001$), with CCA1 explaining 14.8%, and CCA2 explaining 11.1% (Fig. 4b).

Hierarchical clustering analysis based on the Bray-Curtis distance with normalized data revealed that the bacterial communities in the three soils were separated based on soil but not the addition of B068150 (Fig. S1). Furthermore, samples collected from the same time-series clustered together within that soil type except in sandy soil that showed soils sampled in different days clustering together. UniFrac-weighted principal coordinate analysis (PCoA) based on the OTU composition of normalized data (Fig. 5) also clearly demonstrated variations among these different soil samples, with the first two axes explaining 15.51 and 12.41% of the total variation for the bacterial data. In addition, bacterial communities from the three soils samples were clearly

separated from each other with clay and sandy soils separated on PCoA 1 while loamy soil was separated on PCoA2. For fungal community analysis, separations were similar to what were observed based on the CCA analysis; therefore, these data were not presented.

Discussion

Rhizosphere colonization of *B. subtilis* B068150 in three contrasting soils

In our recent study (Li et al. 2016) we showed that rhizosphere colonization was a function of soil types. The effects were different in different soils, as the abundance of *B. subtilis* B068150 in rhizosphere was significantly higher than in non-rhizosphere in the three soils
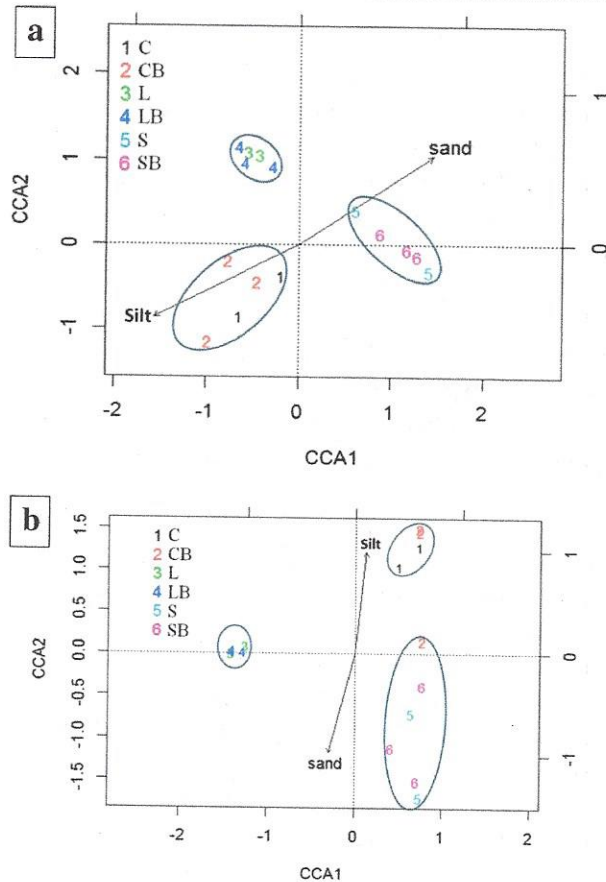
Fig. 3 Canonical correspondence analysis (CCA) results of soil textural contents (sand, silt, clay) on bacterial and fungi communities (C, clay. L, loam. S, sand. B, *B. subtillis* B068150 introduced). **a** For bacterial communities, the first two components explained 41.77% of the variance with CCA1 = 28.95%, CCA1 = 12.82%. F = 4.348, P = 0.001. **b** For fungi communities, the model explained 25.93% of the first two components with CCA1 = 14.86%, CCA1 = 11.07%. F = 2.100, $P = 0.001$
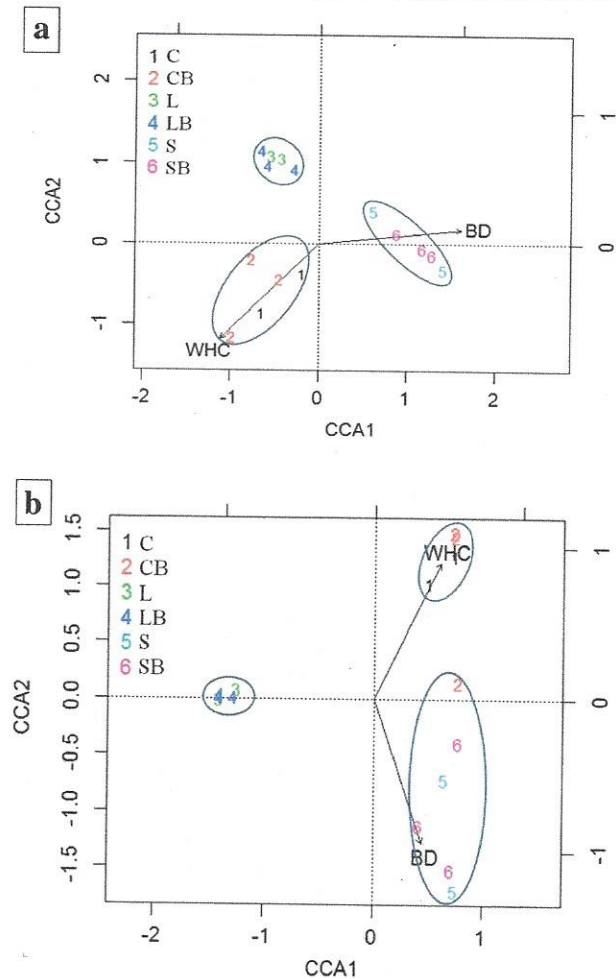

Fig. 4 Canonical correspondence analysis (CCA) was used to evaluate the effects of soils chemistry on bacterial and fungal community structure (C, clay. L, loam. S, sand. B, *B. subtillis* B068150 introduced). **a** For bacterial communities, the model explained 41.77% of the variance: CCA1 = 28.95%, CCA1 = 12.82%. F = 4.305, $P = 0.001$. **b** For fungi communities, the model explained 25.93% of the two components: CCA1 = 28.95%, CCA1 = 12.82%. F = 2.100, P = 0.001

(clay, $P < 0.021$; loam, $P < 0.04$; sand, $P < 0.002$). Many studies have shown that colonization in roots and soil by a biocontrol agent is a prerequisite for successful biological control (Duffy and Weller 1996; Cavaglieri et al. 2005). The population of *B. subtilis* B068150 initially decreased in the three rhizosphere soils (Li et al. 2016), and then stabilized over the remaining study period. This phenomenon has been observed in other studies (Pedersen et al. 1995; Akiba 1986; Liu et al. 2012), and the stabilization in the number of viable cells may be related to spore formation (Backman et al. 1994; van Elsas et al. 1986), and cucumber root exudates (Gamalero et al. 2004; Liu et al. 2006). The colonization results showed that the survival of strain B068150 in cucumber rhizosphere was successful, and based on this assumption, should provide a positive effect on

biocontrol of Fusarium wilt as previously reported (Compant et al. 2005). These authors suggested that rhizosphere competence of biocontrol agents comprises effective root colonization combined with the ability to survive and proliferate along growing plant roots over a considerable time period, in the presence of the indigenous microflora. Our previous study showed that strain B068150 can colonize the rhizosphere of cucumber plant during three months of cultivation (Li et al. 2016). High and effective rhizosphere colonization suggests greater ability to compete with native rhizosphere microbiota for survival and niche developments. As previously reported, the important factor in the
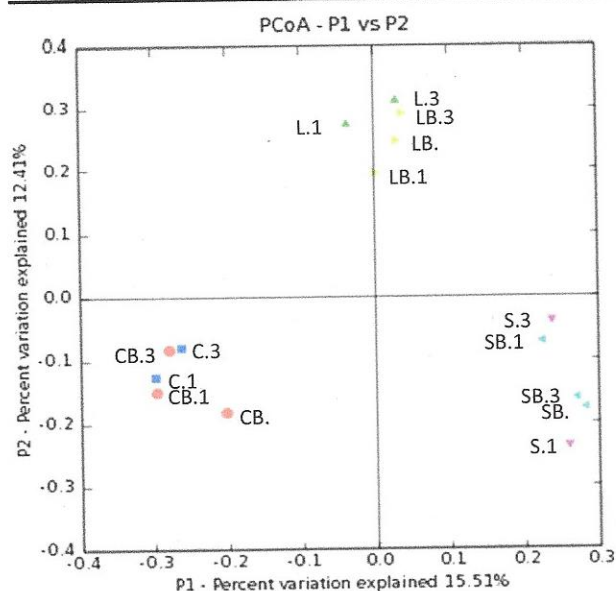
Fig. 5 UniFrac-weighted principal coordinate analysis (PCoA) based on the OTU composition of normalized data. (C) clay, (L) loam, (S) sand, (B) *B. subtillis* B068150 added

protection of cucumber Fusarium wilt was the ability for the biocontrol agent to successfully compete for the same niches and resources with the pathogens on the plant roots (Arguelles-Arias et al. 2009; Bulgarelli et al. 2013).

Rhizosphere microbial community affected
by biocontrol agents

Fusarium was found in rhizosphere of loamy soil and increased after the addition of *Bacillus* B068150 (Fig. 2). Cucumber plants were healthy without any diseases during the three months. However, this *Fusarium* may not be the pathogenic species since some members of *F. oxysporum* are commercially marketed as biopesticides, biofertilizers and soil amendments (Hervás et al. 1998). The use of microorganisms to control plant diseases has been practiced during the last century. Several products based on the application of plant-associated bacteria are commercially available, and these include some *Bacillus* spp. (Choudhary and Johri 2009) that are used as biological control agents (BCA) (Winding et al. 2004). Microbial biocontrol agent applied into soils directly to control plant diseases should have no impact on other organisms or on biogeochemical cycles, but only on the target population (Winding et al. 2004; Cavaglieri et al. 2005; Príncipe et al. 2007). The relative abundances of bacterial and fungal phyla reported in this

study generally agreed with previous studies of soil microbial communities associated with soils inoculated with *Bacillus* with antagonistic activity to *F. oxysporum* f. sp. *Cucumerinum* on cucumber rhizosphere (Zhou and Wu 2012). We observed similar results for bacteria as previously reported (Liu et al. 2014), in which Acidobacteria and Proteobacteria were the top two abundant bacterial phyla in succession cropping soils. The general trends observed in this study that Proteobacteria and Actinobacteria are often abundant in all rhizosphere soils agreed with other reports (Janssen 2006). For fungi, Ascomycota and Basidiomycota were the two main phyla and this was consistent with previous studies in which Ascomycota and Basidiomycota were the top two abundant phyla in continuous cropping peanut and soybean soils (Li et al. 2010, 2014a, b). Our study also showed that Ascomycota was the most dominant phyla (Table S4, S6), and it has been suggested that the succession of fungal communities might be related to changes of residue quality during decomposition (Ma et al. 2013). During this study sequences assigned to Ascomycota members were dominant during the different stages of cucumber growth, revealing that they were key drivers in many activities in cucumber rhizosphere as *Fusarium* is a genus in the Ascomycota phyla.

Rhizosphere microbial community associated
with strain B068150 changed with time

This study was conducted in a greenhouse with cucumber inoculation with B068150 that resulted in microbial diversity increase of the indigenous microbial population in the cucumber rhizosphere in clay and loam after 32 days. The relative abundance of some major bacterial phyla in the rhizosphere with strain B068150 increased significantly greater than the control initially, and then stabilized at about day 32, especially with the major microbial population of Ascomycota, Proteobacteria and Actinobacteria in clay and loamy soils. That meant the effects of B068150 was temporary, and it could be accepted as the previous report (Domsch et al. 1983). For example, application of *B. amyloliquefaciens* FZB42 may have a negative impact on the indigenous microbial rhizosphere community; however, it was observed to be a temporary shift (Kröber et al. 2014). In another study, *B. amyloliquefaciens* SQR9 was shown to suppress the growth of *F. oxysporum* in cucumber rhizosphere and protect the host plant from pathogen

invasion through efficient root colonization (Xu et al. 2014; Cao et al. 2011). In this study, *F. oxysporum* was detected only in loamy soil on day 32 after planting of cucumber (Table S6). This pathogen was not detected in samples treated with BO68150. Assessing the effects of other BCAs on microbial abundance generally involves measuring changes in the number of culturable bacteria or fungi in soil or rhizosphere in various test systems, and such methods have been used for *Pseudomonas* BCAs and the *B. cereus* BCA UW85 (Winding et al. 2004). In this study, we used 454 pyrosequencing that allows for deep sequencing of bacterial and fungi composition in cucumber rhizosphere, and we found substantial differences in bacterial and fungal abundance with the introduction of B068150.

Comparison between the 454- pyrosequencing and previous PCR-DGGE study

Pyrosequencing is a method capable of identifying both major and minor bacterial components (Li et al. 2014a, b). Pyrosequencing results showed that rhizosphere soil bacteria were affiliated with 18 phylum-level groups (Table S2) and fungi were 5 phyla (Table S4). It was higher than the DGGE results with 4 phyla for bacteria and 3 phyla for fungi (Li et al. 2016). Therefore, we got OTUs from pyrosequencing enough to identify that soil chemical properties significantly affected bacterial and fungal community composition in the cucumber rhizosphere soils. The introduction of B068150 did not influence the microbial communities significantly in clay, loamy and sandy soil. It has been reported that next-generation sequencing approaches such as 454 pyrosequencing showed much greater detection capability than the traditional sequencing methods for diversity studies in natural habitats (Kautz et al. 2013). Detail data analyses of bacterial and fungal reads of normalized data from the different treatments are presented in Tables S5 & 6. Comparisons of this data to our DGGE data from Li et al. 2016 show that very few bacterial and fungal sequence data were similar. This may be attributed to the primers used for both studies as primers for DGGE were from the V3 region of 16S rRNA whereas primers for pyrosequencing were from the V1-V2 section. Also, fungal primers for DGGE were ITSEF4F and ITSEF3R whereas primers for pyrosequencing were ITS1F and ITS2R. These primer sets are slightly different and may not produce the exact sequences for different species for direct comparison.

In this study, the Shannon diversity of microbial community was significant higher in rhizosphere of loamy soil than clay and sandy soils. However, the denaturing gradient gel electrophoresis (DGGE) analysis results showed that the Shannon diversity was highest in sandy and the introduction of B068150 had a significant negative correlation on bacterial diversity in cucumber rhizosphere in clay (Li et al. 2016). Moreover, analysis of 454- pyrosequencing showed that there were no significant correlation between microbial diversity and colonization of *B subtilis* B068150 in cucumber rhizosphere in clay, loamy, and sandy soil. The finding of *F. oxysporum* in the current study shows that the technique was powerful enough to detect this species that is associated with cucumber Fusarium wilt.

Conclusion

Our study showed that strain B068150 is able to colonize the rhizosphere of cucumber plant during three months of cultivation in clay, loam, and sandy soils. The difference between the treated samples with *B. subtilis* B068150 and control became small in clay and loam in day 32. Compare to clay and sand, both Shannon diversity and Chao estimates of microbial community were significant highest in rhizosphere of loamy soil. *Fusarium* was found in the rhizosphere of loamy soil and increased after adding of *Bacillus* B068150, but no negative effect on plant growth. Sand, silt and soil chemistry influenced bacterial and fungal community structures in the cucumber rhizosphere in soils, not the addition of *Bacillus* B068150.

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