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ENVIRONMENTAL BIOTECHNOLOGY

Contrasting beneficial and pathogenic microbial communities across consecutive cropping fields of greenhouse strawberry

Ying Huang[1,2] · Xu Xiao[2] · Hongying Huang[1] · Jinquan Jing[2] · Hejuan Zhao[2] · Lin Wang[2] · Xi-En Long[3]

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Abstract

Soil weakness across consecutive cropping fields can be partially explained by the changes in microbial community diversity and structure. Succession patterns and co-occurrence mechanisms of bacteria and fungi, especially beneficial or pathogenic memberships in continuous cropping strawberry fields and their response to edaphic factors remained unclear. In this study, Illumina sequencing of bacterial 16S ribosomal RNA and fungal internal transcribed spacer genes was applied in three time-course (1, 5, and 10 years) fields across spring and winter. Results showed that the richness and diversity of bacterial and fungal communities increased significantly ($p < 0.05$) in 1-year field and decreased afterwards across two seasons. Network analysis revealed beneficial bacterial and fungal genus (*Bacillus* and *Trichoderma*) dominated under 1-year field whereas *Fusarium* accumulated under 10-year field at either season. Moreover, *Trichoderma harzianum* and *Bacillus subtilis* that have been reported to effectively control *Fusarium* wilt in strawberries accumulated significantly under 1-year field. Canonical correspondence analysis showed that beneficial bacterial *Rhodospirillales* and *Rhizobiales* and fungal *Glomerales* accumulated in 1-year field and their distributions were significantly affected by soil pH, microbial biomass C (MBC), and moisture. On the contrary, fungal pathogenic species *Fusarium oxysporum* strongly increased under 10-year field at the winter sample and the abundance was positively ($p < 0.01$) correlated with soil moisture. Our study suggested that the potential of microcosm under 1-year field stimulates the whole microbial diversity and favors different beneficial taxa across two seasons. Soil pH, moisture, and MBC were the most important edaphic factors leading to contrasting beneficial and pathogenic memberships across consecutive strawberry cropping fields.

Keywords Microbial communities · Continuous cropping · Strawberry soils · Edaphic factors · Illumina sequencing

Introduction

Strawberries *(Fragaria* × *ananassa* Duch) are recognized as a highly valuable soft fruit in the world. The acreage of strawberry in China has risen from 3.3×10^3 hm^2 in 1985 to 9.0×10^4 hm^2 in 2012, accounting for 35.7% of the global planting area (Zhang et al. 2016). The growing season of strawberry has been extended from the beginning of August to May of the next year by successive planting under polytunnels. However, declined crop yield, soil degradation, and plant disease frequently occur during greenhouse strawberry cultivation after planting in the same site for a long time which is called continuous cropping obstacle. The factors contributing to this phenomenon are occurrence of soil-borne pathogens, enrichment of autotoxic substances, and deterioration of soil biological and physicochemical properties (Zhou and Wu 2012). Notably, shift in soil biological property is a critical factor contributing to continuous cropping obstacle (Kong et al. 2011; Zhao et al. 2014).

Ying Huang and Xu Xiao contributed equally to this work.

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✉ Hongying Huang
hhymicrolab@163.com

✉ Xi-En Long
xienlong@gmail.com

[1] Laboratory for Agricultural Wastes Treatment and Recycling, Circular Agriculture Research Center, Jiangsu Academy of Agricultural Sciences, No. 50 Zhongling Street, Xuanwu District, Nanjing 210014, Jiangsu Province, China

[2] Nanjing Institute of Agricultural Sciences in Jiangsu Hilly Area, No.6 Xianyin South Road, Qixia District, Nanjing 210046, Jiangsu Province, China

[3] College of Environment Science and Engineering, Central South University of Forestry and Technology, Changsha 410004, China

Soil microorganisms are keystone in maintaining soil health. Especially, the diversity and structure have been regarded as important indicators of soil function and quality. Recently, a number of studies have focused on the disruption of soil microbial communities under greenhouse continuous cropping regime in cucumber (Zhou and Wu 2012), tomato (Mo et al. 2016), and vanilla (Xiong et al. 2015). For example, in a greenhouse potting experiment of cucumber, continuous cropping significantly increased fungal pathogenic communities, especially *Fusarium* (Zhou and Wu 2012). Besides, there are also some studies linking plant disease with variations in the amount of pathogenic or beneficial microbes (Xiong et al. 2015; Liu et al. 2015). Xiong et al. (2015) found that soil sickness after long-term successive cropping of vanilla is related to the reduction of beneficial microbes and accumulation of the pathogens.

Anthracnose, *Fusarium* wilt, and gray mold are the main fungal diseases of strawberry (Baroncelli et al. 2015; Henry et al. 2017), particularly *Fusarium* wilt caused by *Fusarium oxysporum* is the most serious disease influencing strawberry plant and yield (Maas and Galleta 1996; Koike and Gordon 2015). Meanwhile, common bacterial angular leafspot, caused by *Ralstonia solanacearum* and *Xanthomonas fragariae*, respectively, was demonstrated to be widely distributed in strawberry cultivation areas (Maas and Galleta 1996). On the other hand, the relative abundances of beneficial bacterial *Arthrobacter* and *Bacillus* and fungal *Glomus* and *Trichoderma* were regarded as worldwide antagonists against pathogen *Colletotrichum acutatum* and *Fusarium* wilt disease, respectively (Freeman et al. 2004; Nam et al. 2009; Wang et al. 2015). However, the exact influence of consecutive cropping on both bacterial and fungal communities as well as the link between specific microorganisms (beneficial or pathogenic) and edaphic properties in strawberry fields has been rarely reported. We expect that shift in bacterial and fungal community under continuous cropping fields is directly attributed to the effect of successive cropping on soil properties. Previous studies suggested that soil microbial populations were influenced by environmental factors such as soil moisture (Romanowiczet al. 2016), pH (Lauber et al. 2008), and N availability (Frey et al. 2004), but which of these factors dominantly affected community structure of microbial communities remained unclear.

In this study, we used Illumina sequencing to analyze soil bacterial and fungal community composition under successive cropping regime in three time-course (1, 5, and 10 years) greenhouse strawberry fields across two seasons. We aimed to reveal the most important edaphic factors that significantly affect the distribution and compositions of beneficial and pathogenic microbes. We hypothesized that both continuous cropping and season controlled the distribution of pathogenic and beneficial microbes, which resulted to soil weakness in the long-term cropping field.

Materials and methods

Study site and soil sampling

Soil samples were collected from four adjacent fields at Fujiabian village, suburban Nanjing (31° 57′ N, 119° 01′ E), Jiangsu province, China, where strawberry soils represent a wide range of cropping years. Strawberries were grown as perennial crop and cultivated in greenhouses from August to next May annually. No plantation of greenhouse strawberry was performed ranging from June to July. To alleviate continuous cropping obstacle, soil disinfection with methyl bromide fumigation was carried out by closing a greenhouse at high temperature in July and weeds were removed before strawberry seedlings transplanting on August. All the greenhouses were located in the fields had the same strawberry variety (*Benihoppe*). Combined fertilizers of organic manure (colzacake) with chemical fertilizer (N-P-K compound fertilizer) were applied into the fields via drip irrigation system. Soil is classified as yellow-brown soil according to the Universal Soil Classification System. Three continuous cropping fields 1 year (Y1), 5 years (Y5), and 10 years (Y10) at winter (the first fruiting stage of strawberry, December 8) and spring (the last fruiting stage of strawberry, April 10) were used to compare effect of soil age on the microbial community structure at two seasons. A general phenomenon was observed when soil samples were collected. Owing to vegetative period almost finished in this stage, the taste, quality, or yield of strawberry declined and therefore, irrigation or fertilization was rarely carried out by farmers during the last fruiting stage in strawberry daily management. Few flower or fruit was found in strawberry plant in the second soil sampling on April. The adjacent bare soil (BS) that has received no fertilization was also sampled and regard as control.

Soil samples from the fields were collected inside the greenhouse: the area of each greenhouse was 10 × 60 m. Each greenhouse contained five sampling plots. Eighteen random soil cores (5-cm diameter×20-cm length) were obtained from the 0–20-cm layer in an S-pattern in each plot, approximately 4 cm from the nearest plant. The freshly collected soils were mixed to form one composite sample and then sieved through a 2-mm mesh to remove plant debris and possible small animals before used. Subsamples of each replicate were stored at − 80 °C prior to DNA extraction.

Soil chemical analysis

Total soil C and N were measured using a PerkinElmer 2400 CHN analyzer (PerkinElmer, USA). Microbial biomass C was calculated with the extraction coefficient 0.38 following the chloroform fumigation extraction. Soil pH and electrical conductivity (EC) were measured using a glass electrode and a

conductivity meter (soil/water, 1:2.5). Total organic C was determined by dichromate oxidation. Inorganic N (NH_4^+-N and NO_3^--N) was extracted with 2 M KCl by shaking (1 h, 200 rpm) and filtering through a 0.45-m-pore-size polysulfone membrane, before colorimetric determination using a continuous flow analyzer. Available P and K of the soil samples were determined using molybdenum blue method and flame photometry method, respectively. Soil moisture was determined after soils were oven-dried for 48 h at 105 °C. Microbial biomass C (MBC) was calculated with the extraction coefficient 0.38 following chloroform fumigation extraction. Soil physicochemical properties are presented in Table 1.

DNA extraction and Illumina sequencing

DNA was extracted from 0.5-g soil using the FastDNA SPIN kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instruction. The DNA was dissolved in 50 μL of sterilized deionized and free of nucleases water and stored at − 20 °C before used in Illumina sequencing.

The primer sets of 16S-515F/16S-907R (Turner et al. 1999) and ITS1F/ITS2R (White et al. 1990) were used to amplify the V4-V5 region of the bacterial 16S ribosomal RNA (rRNA) gene and the internal transcribed spacer (ITS) region of fungal rRNA fragments, respectively (SI Table S1). To generate amplicons, two pairs of primers were modified by adding Illumina adapter consensus sequences A and B to the 5′-ends of the forward and reverse primers, respectively. The 50-μL amplification contained 5-μL template DNA (10 ng), 5 μL of each primer (10 μM), and 25-μL 2 × KAPA HiFi Hotstart Ready Mix (KAPA BIOSYSTEMS, Boston, MA, USA). The PCRs, performed on a GeneAmp® PCR System9700 (Applied Biosystems, USA), consisted of the following program: 3-min initial denaturation at 95 °C, 25 cycles of 30-s denaturation at 95 °C, 30-s annealing at 55 °C, and 30-s extension at 72 °C, followed by a 10-min final extension at 72 °C. Then, the amplicons were purified with AMPure XP beads (Beckman Coulter Genomics, Danvers, MA, USA). Multiplexing was performed using the Illumina Nextera XT Index kit (Illumina Inc., San Diego, CA, USA) with dual 8-base indices. Incorporating the amplicons with the indices, the PCRs consisted of 3-min initial denaturation at 95 °C, 8 cycles of 30-s denaturation at 95 °C, 30-s annealing at 55 °C, and 30-s extension at 72 °C, followed by a 5-min final extension at 72 °C. Following by library pooling, the barcoded libraries were purified with AMPure XP beads (Beckman Coulter Genomics, Danvers, MA, USA) and then were quantified using the Qubit dsDNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA). By using MiSeq bacterial v3 Reagent Kit, the libraries were sequenced by 2 × 300 bp paired-end sequencing on a MiSeq platform (Illumina, San Diego, CA, USA).

Table 1 Soil physicochemical properties (means and standard deviations, $n = 3$)

Season	Soil source	pH	EC	TC (g kg^{-1})	TN (g kg^{-1})	TOC (g kg^{-1})	NH_4^+-N (mg kg^{-1})	NO_3^-N (mg kg^{-1})	MBC (mg kg^{-1})	Available P (mg kg^{-1})	Available K (mg kg^{-1})	Soil moisture (%)
Winter, 2015	BS	5.85 ± 0.11a	0.17 ± 0.02 g	5.78 ± 0.29f	0.61 ± 0.01f	5.12 ± 0.10g	3.95 ± 0.23f	3.76 ± 0.13e	7.58 ± 0.53e	1.51 ± 0.13g	35.65 ± 3.78f	22.49 ± 1.51c
	Y1	5.46 ± 0.05b	0.26 ± 0.01f	8.52 ± 0.09e	1.02 ± 0.02e	7.95 ± 0.17f	4.59 ± 0.21f	23.74 ± 2.18d	43.57 ± 1.12a	21.36 ± 0.76e	49.98 ± 1.00e	25.53 ± 0.17b
	Y5	5.05 ± 0.11d	0.35 ± 0.02d	14.32 ± 0.18b	1.46 ± 0.01c	12.87 ± 0.17c	11.55 ± 0.82e	25.18 ± 0.47d	22.48 ± 0.36 c	56.54 ± 0.48d	76.31 ± 1.54d	26.59 ± 3.08a
	Y10	5.20 ± 0.02c	0.31 ± 0.02e	17.45 ± 0.24a	2.85 ± 0.01a	16.65 ± 0.08a	17.07 ± 0.45c	31.87 ± 0.31c	10.54 ± 0.79e	94.84 ± 3.23a	76.65 ± 2.11d	22.28 ± 0.18c
Spring, 2016	Y1	5.07 ± 0.02e	0.37 ± 0.01c	9.2 ± 0.26e	1.32 ± 0.02d	8.6 ± 0.1e	14.44 ± 1.73d	49.61 ± 2.71b	39.83 ± 1.12b	12.64 ± 0.87f	117.56 ± 1.50 c	11.25 ± 0.49d
	Y5	4.52 ± 0.03g	0.57 ± 0.04a	11.5 ± 0.30d	1.36 ± 0.03d	11.3 ± 0.2d	39.8 ± 1.81b	88.16 ± 5.77a	12.9 ± 0.32d	79.68 ± 0.71c	206.28 ± 1.10a	7.05 ± 0.34e
	Y10	4.70 ± 0.02f	0.45 ± 0.02b	13.3 ± 0.62c	2.49 ± 0.01b	14.5 ± 0.1b	43.25 ± 1.48a	88.06 ± 4.57a	13.3 ± 0.71d	83.49 ± 1.51b	173.27 ± 1.56b	6.31 ± 0.02f

BS, bare soil; *Y1*, *Y5*, and *Y10*, year 1, 5, and 10 of continuous strawberry cropping; *EC*, electrical conductivity; *TC*, total carbon; *TN*, total nitrogen; *TOC*, total organic carbon; NH_4^+-*N*, ammonia; NO_3^--*N*, nitrate; *MBC*, microbial biomass carbon

Quantitative PCR

Quantification of fungal ITS rRNA and bacterial 16S rRNA gene was performed on an ABI StepOne Plus real-time PCR system (Applied Biosystems, Germany). For all the genes, the 20-μL reaction mixtures consisted of 10 μL SYBR Premix Ex Taq (TaKaRa, Japan), 1 μL of each primer (10 μM), 0.4 μL ROX Reference Dye, and 2 μL template DNA (1–10 ng). The primers used in the gene amplification are listed in Table S1. Standard plasmid carrying the target gene was generated by cloning the gene as described previously (Huang et al. 2014). Plasmid DNA containing the correct gene was extracted using a Qiagen Plasmid Mini Kit (Qiagen Nordic). The concentration of plasmid DNA was determined using a NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE) for the calculation of gene copy numbers. A standard curve was obtained using 10-fold serial dilutions of standard plasmid DNA. Product specificity was confirmed by melt curve analysis (67–95 °C) and visualization through agarose gel electrophoresis. For all assays, amplification efficiencies were 91–98%, and r^2 values were 0.99–1.00.

Processing of high-throughput sequencing data

Data obtained from Illumina Miseq sequencing were analyzed using the Mothur 1.32.1 toolkit (Schloss et al. 2009). The process contained (i) removed any sequences with ambiguous bases and anything longer than 275 bp; (ii) classified the sequences into operational taxonomic units (OTU) by setting a 0.03 distance limit; (iii) calculated α-diversity indices (Chao1, ACE, and Shannon diversity index) to evaluate richness of microbial composition between treatments (Chase et al. 2011); and (iv) calculated β-diversity using the unweighted UniFrac metric (Lozupone et al. 2006).

Data analysis

A two-way analysis of variance (ANOVA) followed by a Newman-Keuls multiple comparison test was performed to evaluate statistical differences ($p < 0.05$) in the soil properties, microbial community abundance, and structure among treatments, on the basis of cropping years and seasons. Univariate statistical and Pearson's correlation analyses were performed using SPSS 18.0 (SPSS Inc., Chicago, IL, USA) and Origin Pro8G (OriginLab corporation, Northampton, USA). To explore the relationships between the community structure and the main environmental factors, canonical correspondence analysis (CCA) was performed using the community ecology package "vegan" (Oksanen et al. 2010). Five important edaphic factors (pH, NH_4^+, TOC, MBC, and moisture) were selected for the CCA ordination diagrams by 1000 times permutation test. P values < 0.05 were considered significant. ADONIS analysis was used to detect the significance of microbial community composition between treatments.

To understand the co-occurrence interactions of bacteria and fungi, especially beneficial or pathogenic memberships in continuous cropping strawberry fields, network analysis was performed on the abundance of genus taxa that were significant between treatments. Abundance data at the genus level were merged and used to generate taxonomic matrices. Spearman's correlation coefficient was then calculated and the correlations between taxa were obtained according to abundances dataset using Cytoscape version 3.2.0 (Shannon et al. 2003). To highlight the significant interactions, strong positive ($r > 0.7$) and strong negative ($r < -0.7$) were shown in the network diagrams. A statistics software package STAMP (Statistical Analysis of Metagenomic Profiles, http://kiwi.cs.dal.ca/Software/STAMP, v2.1.3) was used to analyze taxonomic profiles (Parks et al. 2014). The STAMP ANOVAs analysis was carried out using multiple groups, post hoc tests (Tukey-Kramer at 0.95), an effect size (Eta-squared), and multiple test correction using Benjamini-Hochberg FDR (false discovery rate) procedure (Benjamini and Hochberg 1995). A FDR threshold of 0.05 was used to represent the statistical significance.

Sequence accession numbers

Sequence data have been deposited in the NCBI database with the accession number SRR5273074-SRR5273094 for bacterial 16S rRNA gene and SRR5273053-SRR5273073 for fungal ITS rRNA gene.

Results

Soil physicochemical properties

Changes in soil physicochemical parameters across three time-course fields at two seasons are displayed in Table 1. A two-way ANOVA followed by Newman-Keuls multiple comparison test showed that all the soil parameters were influenced significantly ($p < 0.05$) by both soil age and seasons (Table S2). Soil TC, TOC, TN, EC, NH_4^+-N, and NO_3^--N increased significantly ($p < 0.05$) with soil age, being highest in plot under 10-year field, and lowest in plot under bare soil at winter or spring soil samples. Soil pH decreased significantly with soil age, ranging from 4.52 ± 0.03 to 5.75 ± 0.11, with the lowest values under 5-year field at spring. Compared with bare soil, MBC increased significantly ($p < 0.05$) under 1-year field across two seasons and then decreased with soil age. Available P and K accumulated significantly ($p < 0.05$) with increasing soil age and the contents of available P and K under a 10-year field at spring samples were 56.44 and 2.15 times higher than those under bare soil, respectively. A

significant ($p < 0.05$) increase was observed in EC, NH_4^+-N, NO_3^--N, and available P and K across different treatments at spring soil samples compared with winter soil samples.

Abundance of bacterial 16S rRNA and fungal ITS rRNA gene

Gene copy numbers of bacterial 16S rRNA and fungal ITS rRNA gene showed significant responses to soil age-related changes (SI Fig. S1). Bacterial 16S rRNA gene abundance (4.29×10^8 to 4.82×10^9 copies g^{-1} dry soil weight) was about 10–100 times higher than fungal abundance (4.72×10^6 to 1.69×10^8 copies g^{-1} dry soil weight) under different conditions. Two seasons showed a similar change in bacterial abundance. Bacterial abundance was first increased significantly ($p < 0.05$) at 1-year field and then decreased with increasing cropping years. The lowest bacterial abundance was observed at 5-year field at winter samples. A two-way ANOVA followed by multiple comparison test showed that the fungal abundance was influenced by both soil age and seasons. Similar to bacteria, ITS rRNA gene abundance increased significantly at 1-year field and then decreased with age at winter samples. Furthermore, higher fungal abundances were observed in spring soil samples between treatments and the highest fungal abundance was detected under 5-year field. Results from Pearson's correlation analysis suggested that soil MBC had a significantly ($p < 0.01$) positive correlation with bacterial 16S rRNA gene abundance (SI Table S5). For fungi, ITS rRNA gene abundance was negatively related to soil pH and moisture but positively associated with soil EC, NH_4^+-N, and NO_3^--N.

Richness and diversity of bacteria and fungi

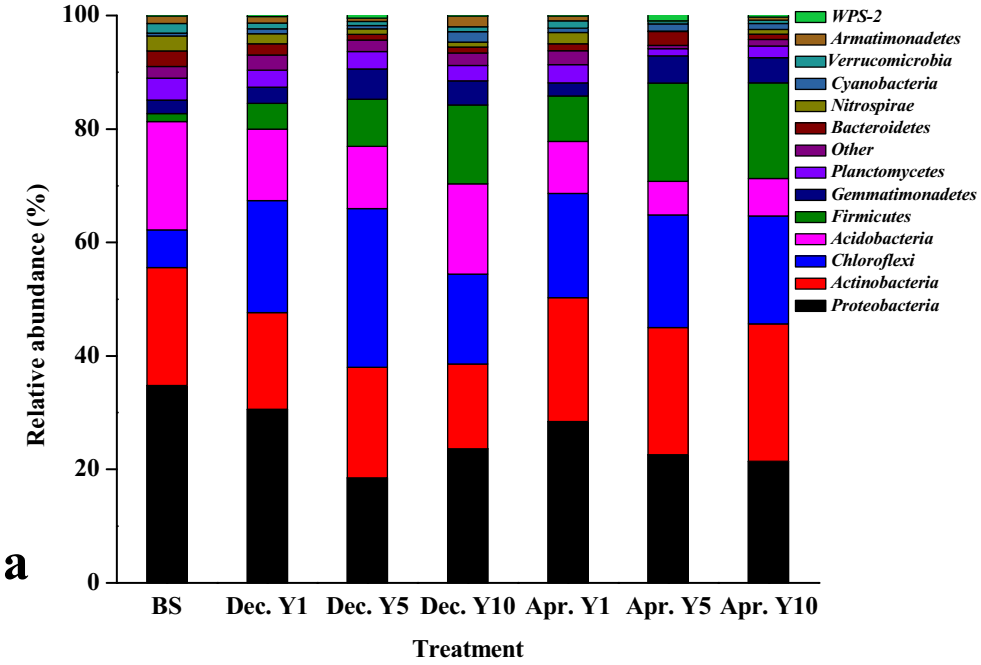
After quality-filtering steps, 697,760 and 698,547 high-quality sequences were obtained in total for bacteria and fungi, respectively (SI Table S3). A sequencing depth of 28,062 and 29,693 sequences for bacteria and fungi was selected from each sample. Above all, bacterial OTUs (3126 to 6974 g^{-1} dry soil) were ten times higher than those in fungal community (615 to 1181 OTUs g^{-1} dry soil). For bacteria, Chao1 and Shannon index showed a significant ($p < 0.05$) higher richness and diversity under 1-year field across two seasons. Simpson index showed an opposite tendency with Shannon index. For fungi, the values of Chao1 significantly increased under a 1-year field at both seasons whereas the lowest Simpson index was observed under bare soil and 1-year field. Shannon index was neither influenced by soil age or by seasons. In addition, the rarefaction analysis confirmed that the numbers of observed OTUs in both bacterial and fungal communities significantly increased under a 1-year field but decreased after monoculture of strawberry for more than 5 years across two seasons (SI Fig. S2).

The bacterial and fungal community structures across diverse soil samples at the phylum level were compared and at least 13 bacteria phyla and 5 fungal phyla were identified (Fig. 1). Continuous cropping in strawberry fields resulted in significant ($p < 0.05$) changes of soil bacterial and fungal community structure. The most abundant bacterial phyla were *Proteobacteria* (25.7% ± 5.6%), *Actinobacteria* (20.1% ± 3.6%), *Chloroflexi* (18.2% ± 6.4%), *Acidobacteria* (11.4% ± 4.7%), *Firmicutes* (10.0% ± 5.8%), *Gemmatimonadetes* (3.7% ± 1.2%), *Planctomycetes* (2.7% ± 0.8%), *Bacteroidetes* (1.6% ± 0.8%), and *Nitrospira* (1.3% ± 0.8%) which accounted for more than 94% of the sequences in all the treatments (Fig. 1a). The relative abundances of some bacterial phyla significantly ($p < 0.05$) increased (*Chloroflexi*, *Firmicutes*, *Nitrospira*) or decreased (*Proteobacteria*, *Bacteroidetes*, *Gemmatimonadetes*, *Planctomycetes*) over soil age while *Actinobacteria* remained constant across all the soil samples (Fig. 1a).

The fungal community was mainly composed of *Ascomycota* (64.8% ± 18.6%), unclassified fungi (18.7% ± 11.9%), *Zygomycota* (10.1% ± 5.3%), and *Basidiomycota* (3.6% ± 2.0%). The relative abundance of *Basidiomycota* significantly increased with soil age while *Ascomycota* showed a noticeably higher abundance at spring samples. The relative abundance of *Glomeromycota* phylum was significantly higher under a 1-year field across two seasons, ranging from 14 to 235 times higher than the rest of the treatments (Fig. 1b).

Network analysis of bacterial and fungal communities

The co-occurrence patterns in microbial communities revealed which taxonomic groups were significantly influenced by cropping years and seasons (Fig. 2). At the genus level, a total of 56 bacterial and 43 fungal phylotypes, representing 82.0 and 78.2% of the most abundant (> 1%) genus taxa, respectively, were significantly influenced by cropping years and seasons. Bacterial taxa associated with bare soil were assigned to *Acidobacteria*, *Actinobacteria*, *Alpha-*, *Beta-*, and *Deltaproteobacteria*, *Gemmatimonadetes*, *Nitrospirae*, or *Planctomycetes* (Fig. 2a). Members in 1-year cropping soils were assigned to *Chloroflexi*, *Firmicutes*, *Actinobacteria*, *Planctomycetes*, *Beta-* and *Deltaproteobacteria*. *Bacillus*, *Anaeromyxobacter*, *Arthrobacter*, *Ellin6529*, and *Anaerolinea* increased significantly in 1-year field at winter or spring samples and negatively related with BS, Apr.Y5, and Dec.Y10. Members in 5-year cropping fields were affiliated to *Actinobacteria*, *Chloroflexi*, *Gemmaproteobacteria*, *Firmicutes*, and *Bacteroidetes*. The gemmaproteobacterial *Rhodanobacter* increased significantly in 5-year field at spring sample. The *Acidobacteria-*, *Armatimonadetes-*, *Chloroflexi-*, *Firmicutes-*, *Betaproteobacteria-*, and *Actinobacteria*-related taxa were primarily retrieved from 10-year cropping soils. The



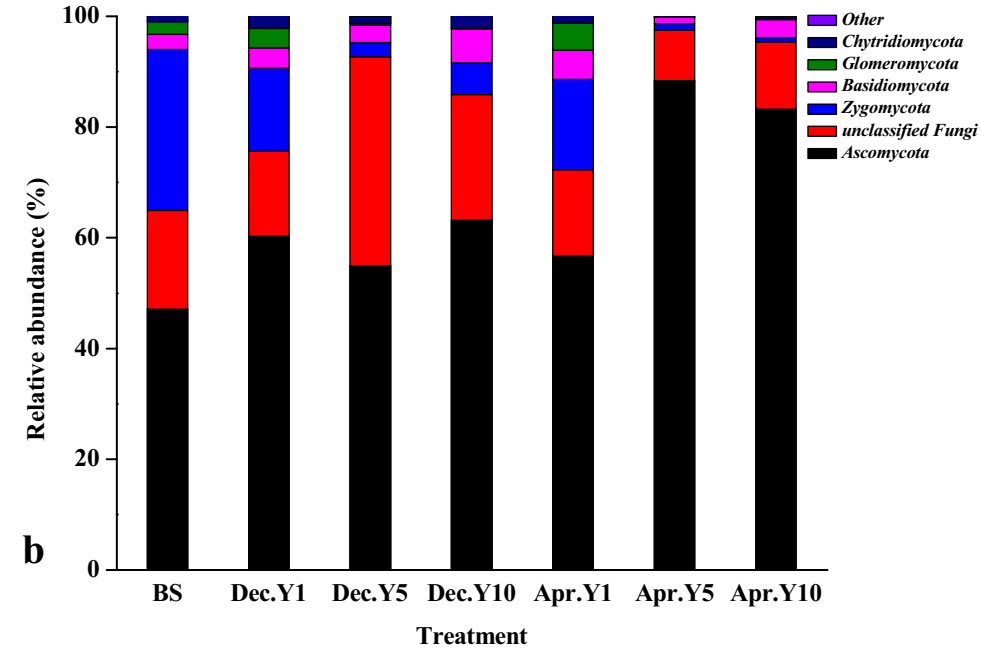
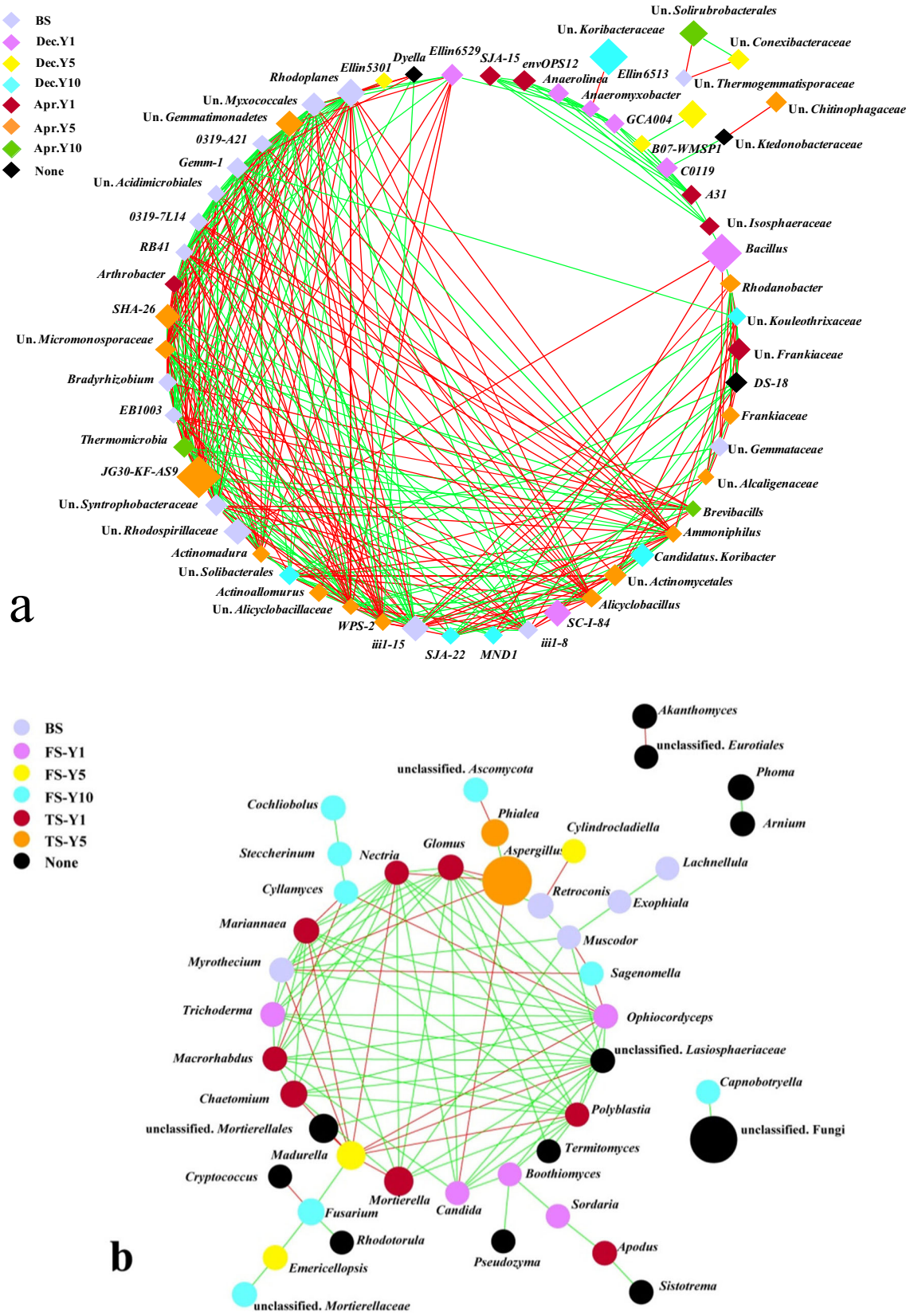


Fig. 1 Relative abundances of bacterial (**a**) and fungal (**b**) community composition at the phylum level determined in different soils

acidobacterial genera *Candidatus* and *Koribacter* revealed the optimum correlation with 10-year field in winter sample.

Fungal taxa that were primarily associated with bare soil were assigned to Ascomycota (Fig. 2b). In a 1-year field, fungal taxa affiliating to Ascomycota, Chytridiomycota, Glomeromycota, and Zygomycota increased significantly and negatively associated with 5-year and 10-year fields. Ascomycota genera *Trichoderma* and *Chaetomium* dominated in a 1-year field at winter and spring samples, respectively. Members that were primarily affected by 5-year cropping

Fig. 2 Network of abundant (≥ 1%) bacterial (**a**) and fungal (**b**) taxa at the genus level. The size of the nodes represents the relative abundance of the taxonomic group. Colored nodes represent group that were significantly ($p < 0.05$) affected by cropping years and growth stage between treatments. The color gradient represents the degree of association with BS (light gray), Dec.Y1 (pink), Dec.Y5 (yellow), Dec.Y10 (blue), Apr.Y1 (red), Apr.Y5 (orange), and Apr.Y10 (green), that is, exhibited the highest abundance in those corresponding treatments. Nodes in black represent taxa with no significant treatment relation. Green and red solid lines represent significantly positive ($r > 0.7$) and negative ($r < -0.7$) linear relationships between taxa, respectively. The prefix "Un." represented unclassified species



were assigned to Ascomycota. The Ascomycota genera *Aspergillus* increased significantly under 5-year field at spring sample and negatively related to samples under bare soil and 1-year field. Ascomycota, Basidiomycota, Chytridiomycota,

and Zygomycota occupied the most abundant fungal taxa and *Fusarium* significantly increased in 10-year field and positively associated with 5-year field at winter sample. None of the species showed significant differences under a 10-year field at spring sample.

Abundance of beneficial and pathogenic species and their correlation with soil properties

Compared with bare soil, the abundances of beneficial bacterial species *Bacillus subtilis* first increased significantly in 1-year field and then decreased with cropping years across two seasons, ranging from 0.04 to 2.53% (Fig. 3b). On the other hand, the relative abundance of bacterial pathogen *Ralstonia solanacearum* or *Xanthomonas fragariae* showed no significant difference in different conditions (data not shown). For fungi, the relative abundance of beneficial *Trichoderma harzianum* was higher in a 1-year field at both seasons, whereas no significant ($p > 0.05$) difference was observed among the rest treatments (Fig. 3a). On the contrary, the relative abundance of fungal pathogen *Fusarium oxysporum* accumulated significantly ($p < 0.05$) with increasing cropping years (Fig. 3c). Higher abundance of *Fusarium oxysporum* was observed in 5-year and 10-year fields at winter, achieving 2.88 and 3.44%, respectively, and two seasons displayed a similar trend over cropping years. The abundance of *Colletotrichum acutatum* showed no significant difference between treatments (data not shown). Pearson's correlation analysis suggested that soil MBC showed significantly ($p < 0.01$) positive correlations with the relative abundances of beneficial microorganisms (*Bacillus subtilis* and *Trichoderma harzianum*) (SI Table S4). Soil EC was positively correlated with abundance of *Fusarium oxysporum* while soil moisture showed significantly ($p < 0.05$) positive correlations with abundance of both *Bacillus subtilis* and *Fusarium oxysporum*.

Bacterial and fungal communities in response to environmental variables

The CCA consisted of the important edaphic factors (TOC, pH, NH_4^+, MBC, and soil moisture) and dominant microbial communities (bacteria and fungi) at the order level (Fig. 4). The soil bacterial community structure was significantly ($p < 0.05$) affected by soil age and sampling seasons based on ADONIS analysis. The first two axes of the CCA of the bacterial communities explained 54.8% of the total variance in

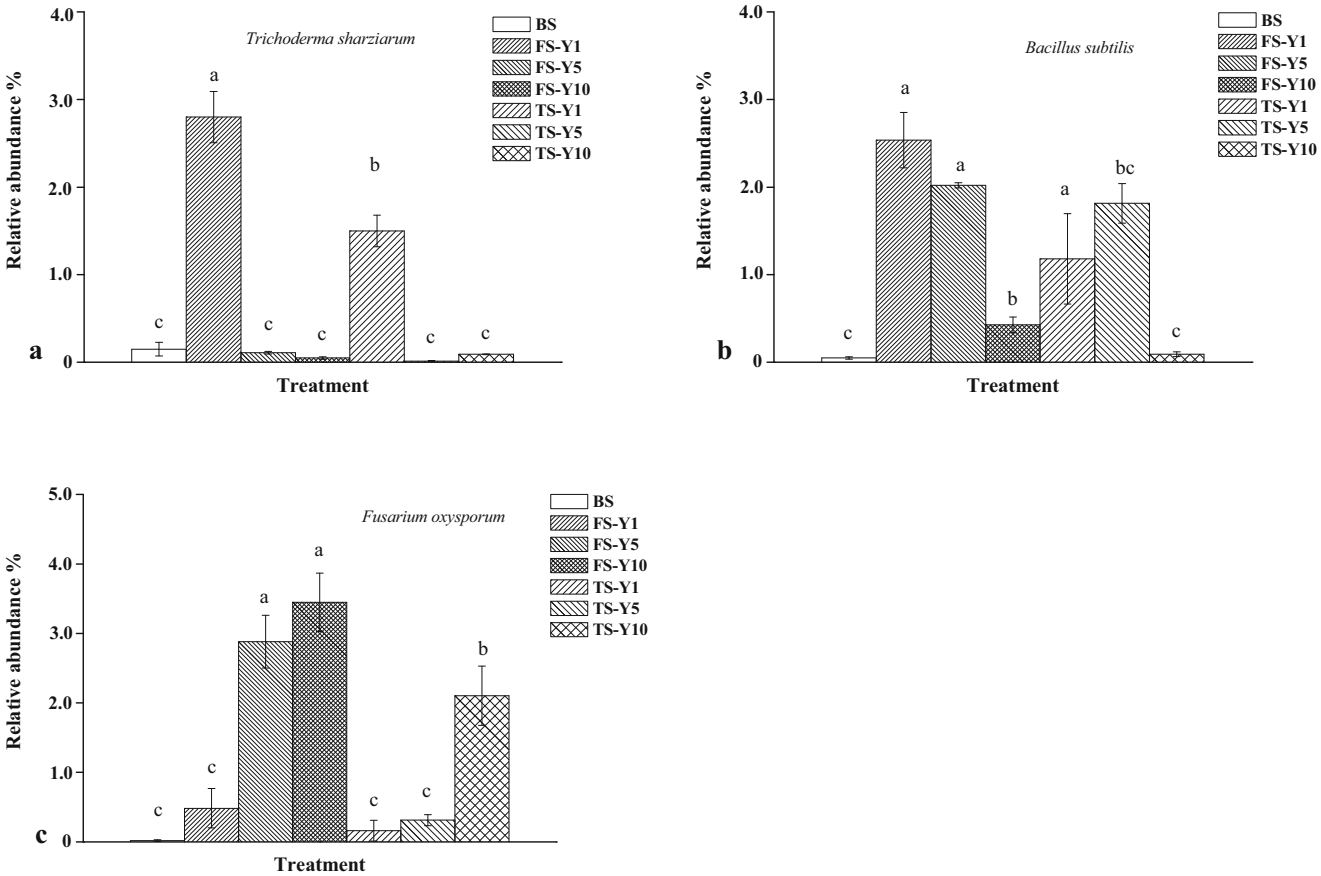


Fig. 3 Relative abundances of beneficial fungal species *Trichoderma harzianum* (**a**), bacterial species *Bacillus subtilis* (**b**), and *Fusarium oxysporum* pathogen (**c**) under seven conditions. Different letters represent statistical significance at $p < 0.05$

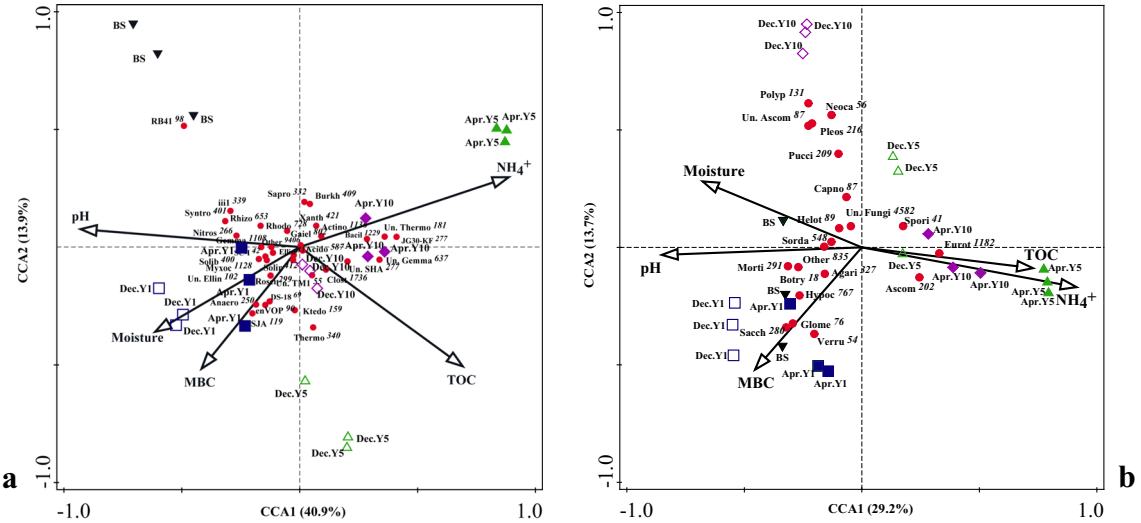


Fig. 4 Canonical correspondence analysis (CCA) of bacterial (**a**) and fungal (**b**) taxa (relative abundance of the dominant orders represented by more than 2% in at least one sample) and environmental parameters (TOC, pH, NH 4$^+$, MBC, and soil moisture). Numbers in italic represent the quantity of OTUs that belong to the taxon. Bacterial order: *Acido*, *Acidobacteriales*; *Actino*, *Actinomycetales*; *Anaero*, *Anaerolineales*; *Bacil*, *Bacillales*; *Burkh*, *Burkholderiales*, *Clost*, *Clostridiales*; *Ellin*, *Ellin6067*; *envOP*, *envOPS12*; *Gaiel*, *Gaiellales*; *Gemma*, *Gemmatales*; *iii1*, *iii1-15*; *Ktedo*, *Ktedonobacterales*; *Myxoc*, *Myxococcales*; *Nitros*, *Nitrospirales*; *Rhizo*, *Rhizobiales*; *Rhodo*, *Rhodospirillales*; *Rosei*, *Roseiflexales*; *Sapro*, *Saprospirales*; *Solib*, *Solibacterales*; *Solir*, *Solirubrobacterales*; *Syntr*, *Syntrophobacterales*; *Thermo*, *Thermogemmatisporales*; *Un. Ellin*, unclassified *Ellin6529*; *Un. Gemma*, unclassified *Gemmatimonadetes*; *Un. Thermo*, unclassified *Thermomicrobia*; *Un. TM1*, unclassified *TM1*; *Xanth*, *Xanthomonadales*. Fungal order: *Ascom*, *Ascomycota*; *Agari*, *Agaricales*; *Botry*, *Botryosphaeriales*; *Capno*, *Capnodiales*; *Eurot*, *Eurotiales*; *Glome*, *Glomerales*; *Helot*, *Helotiales*; *Hypoc*, *Hypocreales*; *Morti*, *Mortierellales*; *Neoca*, *Neocallimastigales*; *Pleos*, *Pleosporales*; *Polyp*, *Polyporales*; *Pucci*, *Pucciniales*; *Sacch*, *Saccharomycetales*; *Sorda*, *Sordariales*; *Spori*, *Sporidibolales*; *Verru*, *Verrucariales*; *Un. Ascom*, unclassified *Ascomycota*; *Un. Fungi*, unclassified fungi

the bacterial 16S rRNA genotypes composition (Fig. 4a). The BS samples were clearly separated from other treatments on the first axis due to the effect of soil age. Soil samples under 1-year field across two seasons grouped together and occupied the most bacterial communities. Under 1-year field, soil pH ($F = 11.009$, $p = 0.001$, 1000 permutations) was the most influential factor on the distribution of *Rhodospirillales* and *Rhizobiales* among the samples, followed by MBC ($F = 5.128$, $p = 0.001$, 1000 permutations) and moisture ($F = 3.344$, $p = 0.001$, 1000 permutations). In 5-year or 10-year field, the winter soil samples were clearly separated from spring samples on the second axis. In 5-year field, *Thermogemmatisporales* and *Ktedonobacterales* that belong to *Chloroflexi* phylum were mostly related with higher TOC and NH_4^+at spring samples. *Gaiellales*, *Solibaterales*, *Acidobacteriales* at winter samples were the most related to lower TOC under 10-year field.

Upon ADONIS analysis, the fungal community composition was significantly ($p < 0.05$) affected by soil age and seasons. The first two axes of the CCA explained 42.9% of the total variance in the fungal ITS rRNA genotypes composition (Fig. 4b). The samples in 1-year field and bare soil were grouped together and clearly separated from the rest of the treatments on the first axis. In 1-year field, *Glomerales* were mostly correlated with soil pH ($F = 2.687$, $p = 0.043$, 1000 permutations), followed by MBC ($F = 2.348$, $p = 0.014$, 1000 permutations) and moisture ($F = 1.167$, $p = 0.346$, 1000 permutations). Seasons significantly influenced distribution of fungal genotypes under 5-year or 10-year field. At the winter, *Sporidiobolales* and unclassified fungi were strongly related with higher TOC and NH_4^+in 5-year field. At the spring, *Eurotiales* and *Ascomycota* that belong to *Ascomycota* phylum were mostly correlated with lower TOC in 10-year field.

Discussion

Changes of soil physicochemical properties due to continuous cropping years of strawberry

Our results showed that cropping years of greenhouse strawberry and seasons significantly affected soil properties (SI Table S2). Obviously, prolonged and repeated fertilizer application could lead to accumulation of TC, TOC, TN, EC, NH_4^+-N, NO_3^--N, and available P and K in strawberry fields compared with bare soil. It has been reported that low soil pH could keep ions such as NO_3^-, NH_4^+, $H_2PO_4^-$, and SO_4^{2-} from being absorbed by the plant root (Li et al. 2016). In this study, higher EC, NH_4^+-N, and NO_3^-N were observed in a 5-year field compared with the rest of the fields across two seasons. The significantly ($p < 0.01$) negative relationships between these parameters and soil pH suggested that the lowest pH under 5-year field is partly attributed to the inhibition of

ion uptake and therefore lead to accumulated ion in the soil. Soil MBC represents the labile component of soil organic matter and has been regarded as an important indicator of soil quality (Shukla et al. 2013). In this study, soil MBC decreased significantly after cultivated for more than 1 year. This phenomenon is owing to the fact that long-term continuous cropping decreased some beneficial microorganisms in C turnover and therefore, led to reducing soil MBC (Zhou and Wu 2012). To obtain high yield and quality of strawberry, extra N fertilization was applied to the extent that exceeded the tolerance of soil microorganisms and plant. Therefore, soil pH decreased owing to accumulated N after long-term strawberry cultivation, which was in accordance with previous studies in other cropping systems (Xu et al. 2009; Huang et al. 2015; Li et al. 2016). In particular, significantly lower pH was observed in these strawberry fields at April soil samples. The reasons we speculated were related with the management regime and plant (Lauber et al. 2008). As revealed by Pearson's correlation analysis, soil pH showed a positive correlation with moisture but a negative relationship with NH_4^+. In April, the whole life cycle of strawberry would finish soon so that irrigation or fertilization was scarcely applied by farmers during the last fruiting stage. Lack of irrigation offered environments with higher oxygen availability that not only stimulated root activity and produced more phenolic acids (Li et al. 2016) but also enhanced nitrification activity at higher NH_4^+ concentration, and therefore, led to decreased soil pH along with soil age (de Boer and Knowalchuk 2001).

Response of bacterial communities to continuous cropping years of strawberry

The consecutive cropping years and seasons significantly influenced bacterial communities in this study. For bacteria, significantly higher abundance was observed in a 1-year field across both seasons, which correspond to the results obtained from Illumina sequencing in this study. On the other hand, profound changes in microbial community structure from three time-course strawberry fields across two seasons were observed. For example, *Proteobacteria* and *Actinobacteria* were the most abundant bacterial phyla. This was in accordance with previous studies in which *Proteobacteria* and *Actinobacteria* were the top two phyla in soils with long-term monocultural fertilization (Liu et al. 2015). Mendes et al. (2011) identified several bacterial taxa that are capable of pathogen suppression and found *Proteobacteria* was consistently related with disease suppression. In our study, *Proteobacteria* abundance decreased with cropping years of strawberry monoculture. This may be one of the factors that lead to soil weakness after continuous cropping of strawberry. In addition, network analysis showed members capable of aerobic or anaerobic respiration dominated in a 1-year field at winter or spring samples. These anaerobic taxa contained *Chloroflexi-Anaerolinea* and *Bacillus* (Hartmann et al. 2014), iron reducer like *Anaeromyxobacter* (Treude et al. 2003), N_2-fixing members like *Cloroflexi-Ellin6529* (Dos Santos et al. 2012), and highly aerobic *Arthrobacter* (Jones and Keddie 2006). For fungi, *Ascomycota* genera *Trichoderma* and *Chaetomium* dominated in a 1-year field at either season. Previous studies indicated that bacterial *Arthrobacter* and *Bacillus* and fungal *Trichoderma* and *Glomus* have been shown capable of protecting host plants against pathogens (Zhang et al. 2011; Yuan et al. 2014; Jayasekhaer et al. 2008). In the present study, the abundances of these beneficial taxa increased specifically ($p < 0.05$) in a 1-year field but reduced in 5-year or 10-year fields (Fig. 2a, b), indicating that decreased beneficial microorganisms may be the cause for soil weakness in continuous cropping fields of strawberry.

Response of fungal communities to continuous cropping years of strawberry

Fungal ITS gene abundance had a positive correlation with soil EC, which is in agreement with results from sequencing result. However, higher fungal abundance was detected in 5-year field at spring sample in which soil pH was the lowest between treatments. And, a negative correlation between ITS gene abundance and soil pH was observed, which is in agreement with previous studies showing a higher fungal dominance in soils with lower pH (Strickland and Rousk 2010). The reasons for these differences are summarized as follows: firstly, negative relationship between soil pH and fungal abundance may in part be driven by increased ectomycorrhizal fungi due to a variation in plant species composition (Joergensen and Wichern 2008). Secondly, as ribosomal gene copy number and cellular nucleic acid concentrations change between taxa, quantitative PCR would not provide an evaluation of biomass ratios (Rousk et al. 2010). Afterwards, it does not resolve the dynamics in soil fungal and bacterial communities that were revealed by high-throughput sequencing. Therefore, further studies should be carried out on the basis of metabolically active community at the rRNA level. It has been reported that long-term continuous cropping regime of greenhouse vanilla significantly increased soil fungal diversity and richness (Xiong et al. 2015). In this study, *Fusarium* accumulated under 10-year field at winter or spring samples, similar to results from previous studies showing that *Fusarium* accumulated in long-term continuous cropped cucumber (Zhang et al. 2008) or vanilla (Xiong et al. 2015). To further reveal the variations between species under continuous cropping, the relative abundances of beneficial or pathogenic species between treatments were compared. *Trichoderma harzianum* and *Bacillus subtilis* that have been demonstrated to effectively control *Fusarium* wilt in strawberries (Moon et al. 1995; Nam et al. 2009) showed higher abundances under 1-year field than other fields in our study. On the contrary,

Fusarium oxysporum accumulated in 10-year field across either season may be attributed to the reduction of beneficial microbes (Xiong et al. 2015). Other bacterial and fungal pathogens exhibited lower abundance to the extent that could not be determined by Illumina sequencing or showed no significant difference between treatments (data not shown). The reasons may be related with seasonal regulation of pathogenesis among different pathogens. For example, fungal anthracnose frequently happened during seeding stage and engraftment period from May to September in traditional strawberry cultivation. Therefore, anthracnose pathogen *Colletotrichum acutatum* showed low abundance and no significant difference between treatments during fruiting stages in the present study (data not shown).

The controlling environmental factors in determining beneficial and pathogenic communities

Soil properties have been reported to be a dominant factor in controlling microbial community structure according to a number of reported studies (Lauber et al. 2008; Xu et al. 2014; Freedman and Zak 2015). More specially, soil pH and moisture were generally recognized as the edaphic factors most strongly related to soil microbial community structure (Romanowicz et al. 2016). Here, we provide powerful evidence that soil pH, MBC, and moisture shape the composition of beneficial and pathogenic microbial populations across three time-course strawberry fields at two seasons. *Rhodospirillales*, *Rhizobiales*, and *Glomerales* which have been reported to promote plant growth and control plant pathogens under continuous cropping stress (Chen et al. 2012; Chen et al. 2014) accumulated under a 1-year field at two seasons. The CCA suggested a positive correlation between these beneficial taxa and soil pH, MBC, and moisture, indicating that these taxa may be adapted to a niche of higher soil pH, MBC, and moisture under a 1-year strawberry field. In addition, *Chaetomium* also dominated in a 1-year field based on network analysis and the abundances were positively related with MBC. It has been reported that *Chaetomium* predominated in organic matter degradation and the abundance was strongly positive related with organic matter decomposition in agricultural soils (Banerjee et al. 2016). Therefore, significantly positive correlations ($p < 0.01$) between the abundances of beneficial species (*Bacillus subtilis* and *Trichoderma harzianum*) and soil MBC suggested that *Chaetomium* may serve as an indicator of MBC and beneficial microorganism accumulation. On the other hand, the abundance of *Fusarium oxysporum* was positively ($p < 0.01$) correlated with soil moisture and EC. The result was in agreement with that of previous study, showing that greenhouse fertilization causes increases in EC, and therefore leading to *Fusarium oxysporum* accumulation in a 7-year gradient of potato monocultural soils (Liu et al. 2014). In this study, the variations in soil moisture and EC are mainly associated with management regime between two seasons. As we previously mentioned that the whole vegetative period of strawberries almost finished in April, significantly lower soil moistures and EC were detected among these greenhouse fields owing to lack of irrigation and fertilization. Therefore, enrichment of *Fusarium oxysporum* was observed at winter samples with higher soil moisture and EC.

Based on these analyses, we show that the bacterial and fungal communities, especially the keystone beneficial taxa, are stimulated by the potential of microcosm in a 1-year field. After long-term monoculture, the microbial community diversity decreased as well as ecological balance between soil pathogens and antagonists was broken. This phenomenon was mainly related to changes in soil pH, MBC, and moisture owing to the vegetation type and the management practices through different seasons. To alleviate continuous cropping problems, rotating with other plants, e.g., vegetable, rice, or corn not only successfully controls fungal pathogen on strawberry but also improves soil quality (Fang et al. 2012). In addition, significantly lower pH and moisture were observed during the last fruiting stage of strawberry. Therefore, the appropriate fertilization (e.g., liming, irrigating) during the last fruiting stages should be considered in daily cultivation of strawberry.

In summary, both cropping years and seasons significantly affected bacterial and fungal community compositions in greenhouse strawberry fields. In general, owing to long-term monocropping management, decreased beneficial microorganisms and accumulated fungal pathogen *Fusarium oxysporum* may be the cause for soil weakness and crop disease in continuous cropping fields of strawberry during fruiting stages. The abundances of beneficial microbes dominating under a 1-year field across winter and spring were largely controlled by changes in soil pH, moisture, and MBC and therefore, exhibited contrasting communities among continuous cropping years. Our findings indicated both bacterial and fungal beneficial communities may be important in the suppression of fungal pathogen *Fusarium oxysporum* in strawberry monoculture, providing further insights into understanding how variations in edaphic factors and microbial community structure can contribute to continuous cropping obstacle across different stages and the possible corresponding solution.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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