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Relationships of decomposability and C/N ratio in different types of organic matter with suppression of *Fusarium oxysporum* and microbial communities during reductive soil disinfestation

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HIGHLIGHTS

- The decomposability of plant residuals was positively related to DE of RSD.
- C/N of plant residuals had a significant negative correlation with DE of RSD.
- Clarifying the changes of dominant bacteria during RSD and their main functions.

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ABSTRACT

Although reductive soil disinfestation (RSD) is an effective method for suppressing *Fusarium oxysporum*, there is limited information on the relationships among types of organic matter, disinfestation efficiencies (DEs), and changes in soil functional microorganisms during the RSD process. Hence, we performed laboratory experiments using nine different organic materials and used quantitative real-time PCR and MiSeq pyrosequencing to investigate the suppression of *F. oxysporum* and changes in soil microbial communities during the RSD process. The results showed that the RSD treatments using different organic matters significantly decreased *F. oxysporum* populations in the soil, but DEs of the RSD treatments were dramatically different. Contents of easily oxidized organic carbon (EOC) in these plant residuals (sugarcane leaf, banana leaf, alfalfa, rice husk, wheat bran, reed, and sugarcane residue) had a significant positive correlation with the DEs and that in all of the organic matters had a significant positive correlation with total organic acid contents. The C/N values of these plant residuals had a significant negative correlation with DEs. Furthermore, the soil bacterial community during the alfalfa RSD process was significantly changed. In the early stage of the RSD treatment, relative abundances of *Coprococcus* (*Firmicutes*), UC-*Clostridiaceae* (*Firmicutes*), and *Klebsiella* (*Proteobacteria*) increased significantly and had a significant positive correlation with organic acids. In contrast, relative abundances of UC-*Cytophagaceae* (*Bacteroidetes*) and UC-*Xanthomonadaceae* (*Proteobacteria*) decreased significantly. In the later stage of the RSD treatment, the number of *Opitutus* (*Verrucomicrobia*) and UC-*Clostridiales* (*Firmicutes*) increased significantly. During the entire RSD process, relative abundances of decomposers of less-degradable organic carbon, such as UC-*Chitinophagaceae* (*Bacteroidetes*), *Clostridium* (*Firmicutes*), UC-*Ruminococcaceae* (*Firmicutes*), *Pseudoxanthomonas* (*Proteobacteria*), and *Flavisolibacter* (*Bacteroidetes*), were always higher than their initial relative abundance values. Overall, types of organic matter with lower C/N could induce higher DEs; organic matters with higher EOC could stimulate organic acid producers to produce more toxic organic acids and consequently induce higher DEs during the RSD process. Dominant functional microorganisms found during the alfalfa RSD treatment belonged to *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*.

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1. Introduction

Soil-borne diseases mainly caused by highly intensive farming and continuous monocultures have become a worldwide problem and seriously threaten agricultural sustainability (Borrero et al., 2006; Ehrmann and Ritz, 2014). Fusarium wilt, caused by *Fusarium oxysporum* (*F. oxysporum*), has been reported as one of the most serious soil-borne diseases that can infect many types of plants, such as spinach, tomato, banana, and cucumber (Correll et al., 1994; Larkin and Fravel, 1998; Ploetz et al., 1990; Singh et al., 1999). Since *F. oxysporum* chlamydospores have a thick wall and a strong ability to resist environmental fluctuations, it can survive in the soil for a long time and cause extensive damage to many crops and huge economic losses (Momma et al., 2013).

For years, the most common strategy for controlling *F. oxysporum* has been the use of chemical pesticides such as methyl bromide and chloropicrin (Porter et al., 1999). However, these chemical pesticides do not prevent all types of diseases and often create a microbial vacuum that causes the pathogens to recur. In addition, accumulated toxic chemical residues in the soil are harmful to human health, environment, and the ozone layer (Borriss, 2011; Gamliel et al., 2000). The soil fumigant methyl bromide is gradually banned or restricted in agriculture (Ristaino and Thomas, 1997). Methods such as soil steaming require complex technology and investment, and soil flooding (3–4 months) and solarization (4–6 weeks) are restricted by climate conditions, long periods, and disinfestation effects (DE) (Goud et al., 2004). Therefore, it is imperative to look for a non-chemical and effective method for suppressing soil-borne pathogens.

Reductive soil disinfestation (RSD) is an effective method for suppressing soil-borne pathogens that incorporates easy decomposition of organic carbon; irrigating fields to the maximum capacity; and covering the soil surface with a plastic film, which was independently developed in the Netherlands (Blok et al., 2000) and Japan (Shinmura, 2000). Furthermore, RSD as an alternative to chemical pesticides has become popular because it is environment-friendly and has a broad spectrum for disinfestation that can not only control many soil-borne pathogens but also nematodes and weeds (Blok et al., 2000; Goud et al., 2004).

To date, two main types of organic matter are used in RSD treatment. The first type is molasses and ethanol, which are simple and easily degradable compounds (Butler et al., 2012; Momma et al., 2010); the second type is agricultural wastes such as plant residuals in which the primary component is cellulose, for example, crop straw reported in our previous study (Wen et al., 2015). However, management of the large amount of agricultural wastes and the environmental problems caused by them has become an urgent issue in China (Hrynchuk, 1998). Agricultural wastes are a better option for used in RSD treatment than molasses and ethanol, which need a higher investment.

Previous studies have indicated that DE due to RSD treatments with various types of organic matter are different. Currently, there is little information on the relationship between the organic matter used in RSD treatments and disinfestation efficiencies (DEs). Moreover, limited research has been performed on changes in soil functional microorganisms during the RSD process (Mowlick et al., 2012). Therefore, to establish standards for selecting appropriate organic matter and understand and investigate the changes in soil functional microorganisms during the entire RSD process, we performed laboratory experiments with nine different organic matters and used quantitative real-time PCR to count the number of *F. oxysporum*, bacteria, and fungi in the soil. In addition, we used MiSeq pyrosequencing to analyze the changes in bacterial community structure. Finally, we used correlation analyzes to study the physicochemical properties of the organic matters and DE as well as toxic organic acids.

2. Materials and methods

2.1. Soil and organic matters

The soil used in this study was collected from Natong Town (23°12′ N, 107°50′ E), Guang Xi Province, China, where banana has been planted for many years; however, the banana plants were seriously infected by *Fusarium oxysporum* f. sp. *cubense*. Physicochemical properties were as follows: pH 7.43; total organic carbon (TOC), 19.56 g kg^{-1}; and total nitrogen (TN), 1.83 g kg^{-1}. Seven local plant residuals and two pure organic materials were used in this study: sugarcane (*Saccharum officinarum Linn.*) leaf, banana (*Musa nana Lour.*) leaf, alfalfa (*Medicago sativa Linn.*), rice (*Oryza sativa Linn.*) husk, wheat (*Triticum aestivum Linn.*) bran, reed (*Phragmites australis Cav. Trin. Ex Steu*), sugarcane residue, glucose, and cellulose. Physicochemical properties of these organic matters were listed in Table 1 and the detection methods were as follows.

Before the treatment, the soil and organic matters were sieved (mesh size, 2 mm). TOC in the soil and organic matters was measured by wet digestion with H_2SO_4–$K_2Cr_2O_7$ (Bremner and Jenkinson, 1960), and TN was determined using semi-micro-Kjeldahl digestion (Bremner, 1960). The easily oxidized organic carbons (EOCs) in the organic matters were measured with 333 mmol L^{-1} of $KMnO_4$ by shaking for 1 h, centrifuging for 5 min at 4000×*g*, diluting to 10,00 times with deionized water, and using a spectrophotometer to detect absorbance at 565 nm (Blair et al., 1995; Skjemstad et al., 2006; Tirol padre and Ladha, 2004). The $KMnO_4$ standard curve and calculation method were according to Blair et al. (1995).

2.2. Experimental design

In this study, eleven treatments were conducted: initial soil as CK, for anaerobic CK treatment, 200 g of soil was placed in a valve bag with 45% (v/w) flooding; for sugarcane leaf, banana leaf, alfalfa, rice husk, wheat bran, reed, sugarcane residue, glucose and cellulose treatments, 200 g of soil was placed in a valve bag with 45% (v/w) flooding and incorporation of 2% (w/w) organic matter, respectively. These treatments were established randomly, and three replications were performed. The cultivation period was 16 days, and temperature was maintained at 35 °C. Soil samples and solutions were collected every 4 days and then stored at −20 °C for further analyses. Changes in the soil bacterial

Table 1
Physicochemical properties of the types of organic matter used in this study.

Types of organic matter	TOC[a] (g kg^{-1})	EOC[b] (g kg^{-1})	TN[c] (g kg^{-1})	C/N[d]
Sugarcane leaf	425.1	122.4	7.05	60.29
Banana leaf	406.9	99.4	13.94	29.19
Alfalfa	399.1	104.1	13.45	29.67
Rice husk	406.0	81.8	4.59	88.45
Wheat bran	439.3	110.0	13.01	33.76
Reed	427.5	102.5	6.80	62.86
Sugarcane residue	442.4	84.2	4.66	94.93
Glucose	385.0	276.7	0.00	–
Cellulose	432.3	7.1	0.00	–

[a] Total organic carbon.
[b] Easily oxidized organic carbon.
[c] Total nitrogen.
[d] "–" Cannot be calculated.

community during the entire alfalfa-amended RSD treatment were investigated using MiSeq pyrosequencing.

2.3. Measurement of soil physicochemical properties

Soil pH was measured using an S220 K pH meter (Mettler-Toledo International Inc., Shanghai, China) with 1:2.5 (v/v) soil: water. NH_4^+-N and NO_3^--N were extracted with 2 mol L^{-1} of KCl solution (1:5 m/V) by shaking at 250 r min^{-1} for 1 h, and the concentrations were measured using a continuous flow analyzer (San++, Skalar Analytical B.V., Breda, The Netherlands).

2.4. Detection of toxic organic acids

Soil water samplers (Momma et al., 2006) were used to collect soil solutions (1.5 mL) from each valve bag on the 0th, 4th, 8th, 12th, and 16th day. Then, the soil solutions were filtered using a 0.22-μm membrane filter, and the organic acids in the soil solutions were quantified using high-performance liquid chromatography (HPLC; Waters eAlliance 2695, USA), according to the method described by Huang et al. (2015b) The column used was XDB-C18 (4.6 × 250 mm, Agilent, USA), and the standards for the organic acids were purchased from Sigma (USA).

2.5. Real-time PCR quantification of F. oxysporum, bacteria, and fungi

The PowerSoil® DNA Isolation kit (MO BIO Laboratories, Inc., USA) was used to extract DNA, according to the manufacturer's instructions. Quantitative PCR amplification was performed in 8-well tubes by using the CFX96™ Real-Time System (Bio-Rad Laboratories Inc., Hercules, CA, USA). Each tube contained 20-μL of the reaction volume: 2 μL of the DNA target, 10 μL of SYBR Green premix EX Taq (2×, TaKaRa, Japan), 1 μL of forward and reverse primers (ITS1-F and AFR308 for ITS of *F. oxysporum*; Eub338 and Eub518 for the 16S rDNA gene of bacteria; and ITS1-f and 5.8 s for ITS of fungi; Table 2), and 6 μL of sterile distilled water. The PCR conditions were as follows: 2 min at 95 °C, followed by 40 amplification cycles of 10 s at 95 °C, 15 s at 58 °C, and 20 s at 72 °C for the quantification of *F. oxysporum* and 10 s at 95 °C, 20 s at 53 °C, and 30 s at 72 °C for the quantification of bacteria and fungi. Fluorescence was recorded in the third stage of each cycle. To assess the specificity of the amplifications, melt curves were detected at the end of each PCR run. The standard curves were determined according to López-Mondéjar et al. (2010) and the slopes of the standard curves for *F. oxysporum*, bacteria, and fungi were −3.368, −3.003, and −3.359, respectively.

2.6. MiSeq pyrosequencing

The soil DNA samples were sent to Personal Biotechnology Co., Ltd. (Shanghai, China) and studied using an Illumina MiSeq instrument (USA) to investigate changes in the soil bacterial community.

Table 2
Primers used in the experiments.

Primers[a]	Sequence (5′–3′)	Reference
ITS1-f (F)	TCCGTAGGTGAACCTGCGG	Gardes and Bruns (1993)
5.8s (R)	CGCTGCGTTCTTCATCG	Vilgalys and Hester (1990)
338 (F)	CCTACGGGAGGCAGCAG	Dorsch et al. (1992)
518 (R)	ATTACCGCGGCTGCTGG	Muyzer et al. (1993)
ITS1-F (F)	CTTGGTCATTTAGAGGAAGTAA	Lievens et al. (2005)
AFR308 (R)	CGAATTAACGCGAGTCCCAAC	Lievens et al. (2005)
520	AYTGGGYDTAAAGNG	Claesson et al. (2009)
802	TACNVGGGTATCTAATCC	Claesson et al. (2009)

[a] F, forward primer; R, reverse primer.

The V4 hypervariable regions of bacterial 16S rDNA in the soil samples were amplified using individual bar-coded reverse primers 520 and 802 (Table 2). The paired-end FASTQ sequences of original pyrosequencing data were quality-filtered by slip window sampling with the average quality of alkaline bases ⩾20 and sequence length ⩾150 bp; then, FLASH software (version 1.2.7) (Magoč and Salzberg, 2011) was used to link these sequences. These linked sequences were quality-controlled using Qiime software (version 1.7.0) (Caporaso et al., 2010), and mothur software (version 1.71.2) was used to remove chimeric sequences (Edgar, 2010; Schloss et al., 2009). Finally, the sequences were clustered into operational taxonomic units (OTUs), according to 97% similarity by using the Uclust method of Qiime software (Edgar, 2010), and then compared and annotated using BLAST in the RDP databases (Altschul et al., 1990). Sequence data have been uploaded in the NCBI Sequence Read Archive (SRA) database (accession number, SRA296668).

2.7. Analysis of sequence data

The Shannon diversity index was calculated on the basis of the OUT results, mothur software was used to evaluate changes in soil bacterial diversity during the alfalfa RSD process. The relationship between microorganism communities and environmental factors was analyzed using Canoco software (redundancy analysis [RDA]).

2.8. Statistical analysis

Microbial count data were $\log_{10}$-transformed, and one way ANOVA and the LSD test ($p \leqslant 0.05$) in SPSS 19.0 (SPSS Inc., Chicago, USA) were used to detect variances. Bivariate correlation and Excel 2013 were used to analyze the relationship between the physicochemical properties of the organic matters and organic acids and between organic acids and dominant bacterial genera.

3. Results

3.1. Soil pH

When compared with initial soil pH (7.43), both anaerobic CK and RSD treatments significantly decreased soil pH ($p < 0.01$). The pH values of the glucose treatment were always less than 5.5. For the cellulose treatment, the lowest pH was observed on the 12th day; for the other anaerobic treatments, the lowest pH was detected on the 4th day, followed by a slow increase in soil pH (Fig. 1).

3.2. Soil NH_4^+-N and NO_3^--N contents

During the entire cultivation process, soil NO_3^--N content decreased significantly ($p < 0.01$) in all the anaerobic treatments when compared with the concentrations on the initial day (51 mg kg^{-1}), and the rate of decrease in NO_3^--N content was significantly higher ($p < 0.05$) in all the RSD treatments than in the anaerobic CK treatments (Fig. 2a). Conversely, soil NH_4^+-N content increased in all the RSD treatments when compared with the concentrations on the initial day (3 mg kg^{-1}); alfalfa (31 mg kg^{-1}) and wheat bran (23 mg kg^{-1}) treatments in particular showed a significant increase in soil NH_4^+-N content on the 16th day (Fig. 2b).

3.3. Organic acid contents

Four types of organic acids (acetic, propionic, butyric, and isovaleric acid) were detected during the RSD treatments, but they were not detected during the anaerobic CK treatments. The highest

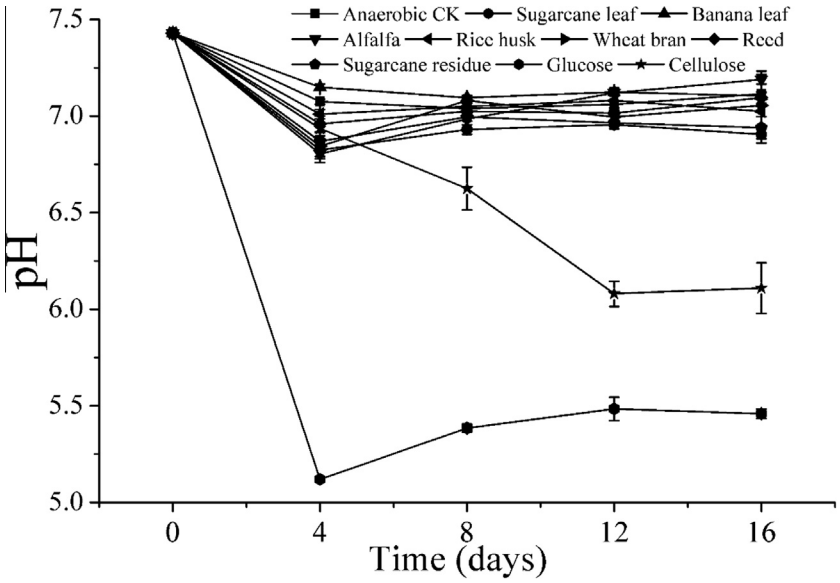

Fig. 1. Soil pH values during the different treatments in the entire RSD process. Anaerobic CK: 200 g of soil placed in a valve bag with 45% (v/w) flooding; Sugarcane leaf, Banana leaf, Alfalfa, Rice husk, Wheat bran, Reed, Sugarcane residue, Glucose, and Cellulose: 200 g of soil placed in a valve bag with 45% (v/w) flooding and incorporation of 2% (w/w) organic matter. Error bars indicate standard errors (SEs).

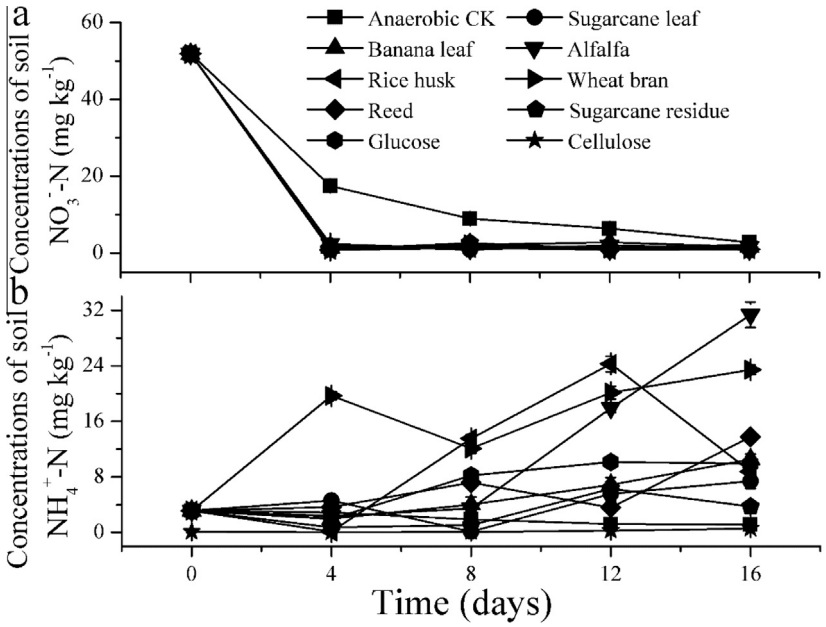

Fig. 2. Concentrations of soil NO_3^--N (a) and NH_4^+-N (b) during the different treatments in the entire RSD process. Error bars indicate SEs.

production of acetic acid (1.67 mg mL^{-1}) and isovaleric acid (0.34 mg mL^{-1}) was found on the 4th day in the sugarcane leaf treatment (Fig. 3a), and the highest production of propionic acid (0.182 mg mL^{-1}) and butyric acid (2.63 mg mL^{-1}) was found on the 16th day in the cellulose treatment (Fig. 3b) and 8th day in the glucose treatment (Fig. 3d), respectively. The highest content of total organic acids in the sugarcane residue (0.43 mg mL^{-1}), glucose (2.81 mg mL^{-1}), and cellulose (0.78 mg mL^{-1}) treatments were found on the 12th, 8th, and 16th day, respectively; the highest content of total organic acids in the other RSD treatments were found on the 4th day, followed by a gradual decrease in total organic acid content (Fig. 3e). The ranks for total organic acids (sum of the four organic acids during the entire period) in all RSD treatments were as follows: Glucose > Sugarcane leaf > Wheat bran > Alfalfa > Cellulose > Reed > Sugarcane residue > Banana leaf > Rice husk.

3.4. Quantification of *F. oxysporum*

At the end of the anaerobic treatments, the populations of soil *F. oxysporum* in all anaerobic treatments were significantly decreased ($p < 0.01$) compared with the initial soil (4.57×10^7 gene copies g^{-1} dry soil). Although DEs for most RSD treatments were more than 90%, they were dramatically different and the lowest DE for the cellulose treatment was only 62.2%. From the perspective of incubation time, the number of *F. oxysporum* in all the anaerobic treatments gradually declined when compared with soil *F. oxysporum* populations on the 0th day. Specifically, the greatest decrease

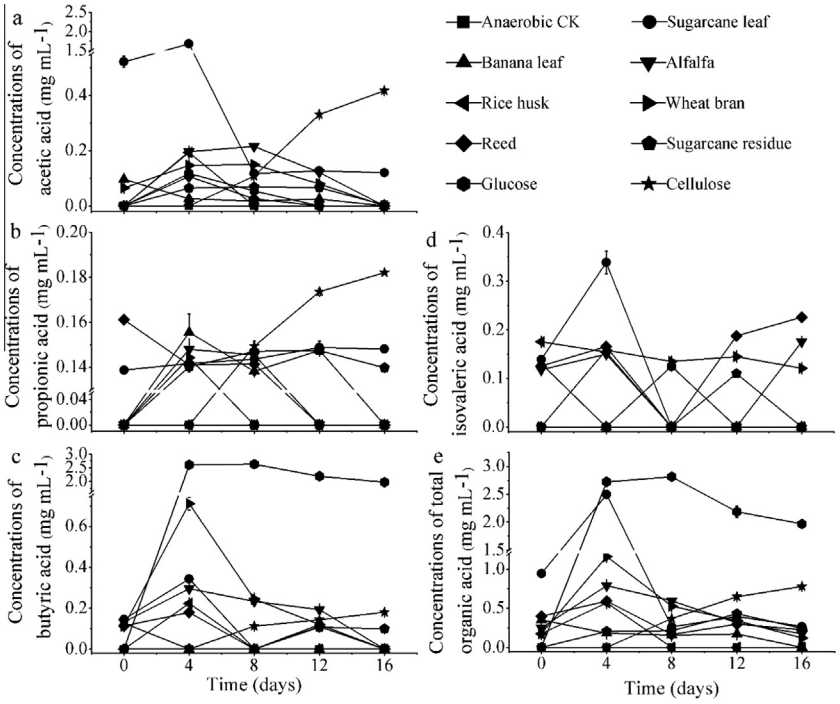

Fig. 3. Concentrations of acetic acid (a), propionic acid (b), butyric acid (c), isovaleric acid (d), and total organic acids (the sum of the four organic acids at the same time, e) in different soil solutions during the entire RSD process. Error bars indicate SEs.

in *F. oxysporum* populations was found in the glucose treatment on the 4th day (DE more than 90%), whereas soil *F. oxysporum* populations in the anaerobic CK and cellulose treatments decreased more slowly than those in the other RSD treatments during the entire RSD process (Fig. 4c).

3.5. Quantification of bacteria

Compared with the populations of bacteria in the soil at the start of the experiment (1.26×10^{10} copies g^{-1} dry soil), significantly increased ($p < 0.05$) populations of bacteria were observed after all the anaerobic treatments; however, the populations of soil bacteria after the glucose treatment (1.44×10^{10} copies g^{-1} dry soil on the 16th day) were only 0.13 times that in the soil at the start of the experiment. The highest populations of soil bacteria in all the RSD treatments were found on the 12th day (Fig. 4a).

3.6. Quantification of fungi

Populations of soil fungi in the glucose (6.91×10^{7} copies g^{-1} dry soil on the 16th day) and cellulose (3.08×10^{8} copies g^{-1} dry soil on the 16th day) treatments were significantly decreased ($p < 0.01$) at the end of the anaerobic treatments when compared with those in the soil at the start of the experiment (9.47×10^{8} copies g^{-1} dry soil). Populations of soil fungi in anaerobic CK, sugarcane leaf, and rice husk treatments showed no significant changes, and those in the rest of the RSD treatments were significantly increased ($p < 0.05$) (Fig. 4b).

3.7. Composition of the bacterial communities

The dominant bacterial phyla found during the alfalfa RSD treatment were *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Gemmatimonadetes*, *Planctomycetes*, *Acidobacteria*, *Verrucomicrobia*, *Chloroflexi*, and *Chlorobi*, accounting for more than 94.5% of the total bacterial sequences (Fig. 5a). Relative abundances of some dominant bacterial phyla were significantly different during the entire alfalfa RSD process, for example, the relative abundance of *Firmicutes* (36.9%, 36.5%, 31.9%, and 32.6% on the 4th, 8th, 12th, and 16th day, respectively) was significantly higher ($p < 0.01$) than the initial relative abundance (1.2%). Besides, the dominant bacterial genera found during the alfalfa RSD treatment were UC-*Ruminococcaceae*, UC-*Clostridiaceae*, *Flavisolibacter*, *Clostridium*, UC-*Chitinophagaceae*, *Pseudoxanthomonas*, *Opitutus*, UC-*Clostridiales*, UC-*Cytophagaceae*, *Klebsiella*, *Coprococcus*, UC-*Xanthomonadaceae*, and UC-*Ignavibacteriaceae* (Fig. 5b). The total relative abundance of UC-*Ruminococcaceae*, UC-*Clostridiaceae*, *Clostridium*, UC-*Clostridiales*, and *Coprococcus* was 26.1%, 23%, 22.1%, and 23.1% on the 4th, 8th, 12th, and 16th day, respectively, accounting for more than 63% of *Firmicutes*.

In the early stage of the alfalfa RSD treatment, relative abundances of UC-*Cytophagaceae* (*Bacteroidetes*) and UC-*Xanthomonadaceae* (*Proteobacteria*) decreased significantly ($p < 0.01$). In contrast, relative abundances of *Coprococcus* (*Firmicutes*), UC-*Clostridiaceae* (*Firmicutes*), and *Klebsiella* (*Proteobacteria*) increased significantly ($p < 0.01$). In the later stage of the alfalfa RSD treatment, relative abundances of *Opitutus* (*Verrucomicrobia*) and UC-*Clostridiales* (*Firmicutes*) increased significantly ($p < 0.01$). Besides, relative abundances of UC-*Chitinophagaceae* (*Bacteroidetes*), *Clostridium* (*Firmicutes*), UC-*Ruminococcaceae* (*Firmicutes*), *Pseudoxanthomonas* (*Proteobacteria*), and *Flavisolibacter* (*Bacteroidetes*) were always high during the entire alfalfa RSD treatment (Fig. 6). The Shannon diversity index calculated on the basis of OTUs during the entire alfalfa RSD process was significantly lower than ($p < 0.01$) the initial value (Fig. 7).

3.8. Correlations between physicochemical properties of the organic matters and DEs as well as organic acids

Except for TOC of these plant residuals (sugarcane leaf, banana leaf, alfalfa, rice husk, wheat bran, reed, and sugarcane residue), TN and EOC values showed significant positive correlations ($p < 0.01$)

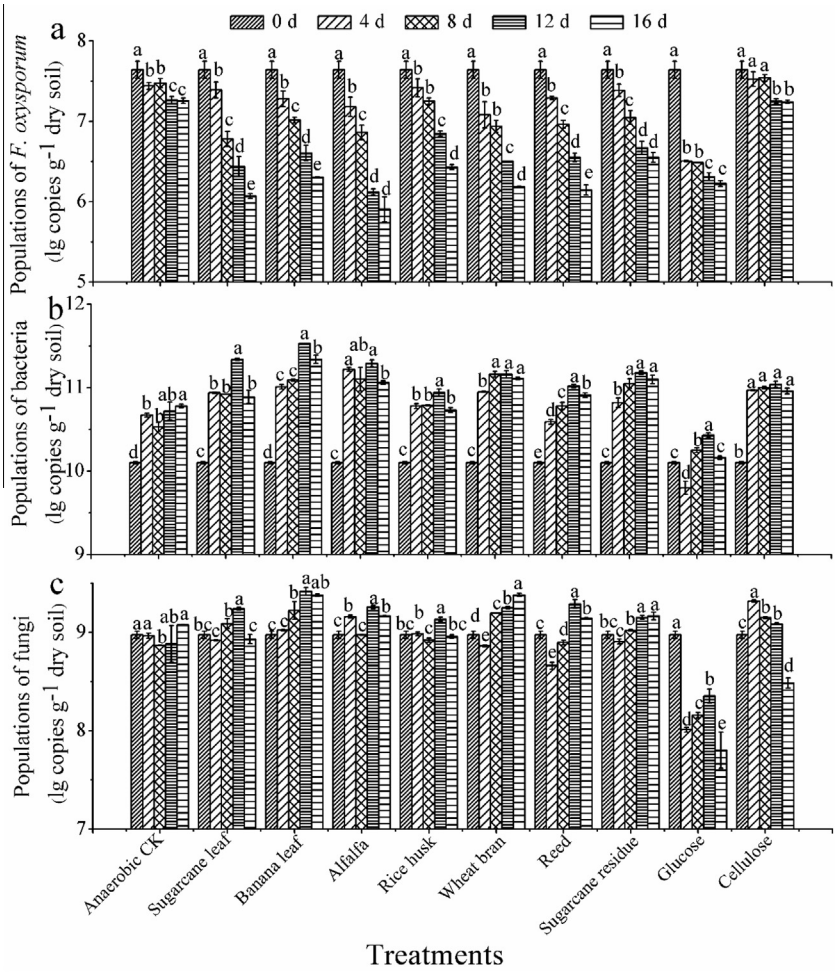

Fig. 4. Populations of soil *F. oxysporum* (a) bacteria (b), and fungi (c) found during the different treatments in the entire RSD process. Bars with different letters represent significant differences among the same treatments at the different time through LSD tests ($p < 0.05$). Error bars indicate SEs.

with DE, and C/N showed a highly negative correlation ($p < 0.01$) with DE (Fig. 8). With respect to the correlations between physicochemical properties and organic acid contents, only EOC showed a significant positive correlation ($p < 0.01$) with organic acid contents during the entire RSD process (Table 3).

3.9. Relationships between microbial communities and environmental factors

RDA results for the bacterial species and environmental factors showed that total organic acid contents, anaerobic days, and TOC had a significant positive correlation with *Firmicutes*, while pH had a negative correlation with *Firmicutes* (Fig. 9). Results of the correlations between dominant bacterial genera and organic acid contents in the alfalfa RSD treatment revealed that UC-*Clostridiaceae* (*Firmicutes*) and *Klebsiella* (*Proteobacteria*) had a significant positive correlation with acetic, propionic, and butyric acid and *Coprococcus* (*Firmicutes*) had a significant positive correlation with acetic and butyric acid (Table 4).

4. Discussion

Many studies (Blok et al., 2000; Huang et al., 2015b) had demonstrated that the application of organic matter is crucial for the success of RSD, and in this study the same results were obtained that the DEs in the RSD treatments were much better than flooding sole. Although the populations of *F. oxysporum* decreased significantly after all RSD treatments, DEs of the RSD treatments were dramatically different. Thus, to understand the key factors of the used organic matters, it is necessary to investigate the relationships between the physicochemical properties of the organic matters and DEs. Because the TOC values of the different plant residuals were almost the same, there was no significant correlation between TOC and DE. In contrast, the higher TN values of these plant residuals, that is, the lower C/N values in terms of similar TOC values of the different plant residuals, could induce higher DEs during the RSD treatments, which was consistent with the findings of previous studies (Oka et al., 2007; Rodriguez-Kabana et al., 1987) that RSD incorporated with organic matter with lower C/N induced higher mortality of nematodes. The possible reasons were that lower C/N in organic matter could induce higher generation of nitrogenous compounds (such as ammonia and nitrous acid) (Tenuta and Lazarovits, 2002) or stimulate bacterial growth and improve their gross activity (Rousk and Bååth, 2007) and have a better effect on the suppression of nematodes and fungal pathogens during the RSD process. Shrestha et al. (2013) have reported that C/N in organic matter had a negative correlation with soil anaerobic conditions during the RSD treatment. Therefore, organic matter with lower C/N amended into the soil could induce the highest soil anaerobic conditions. Many studies (Blok et al., 2000; Shinmura, 2000) have reported that the

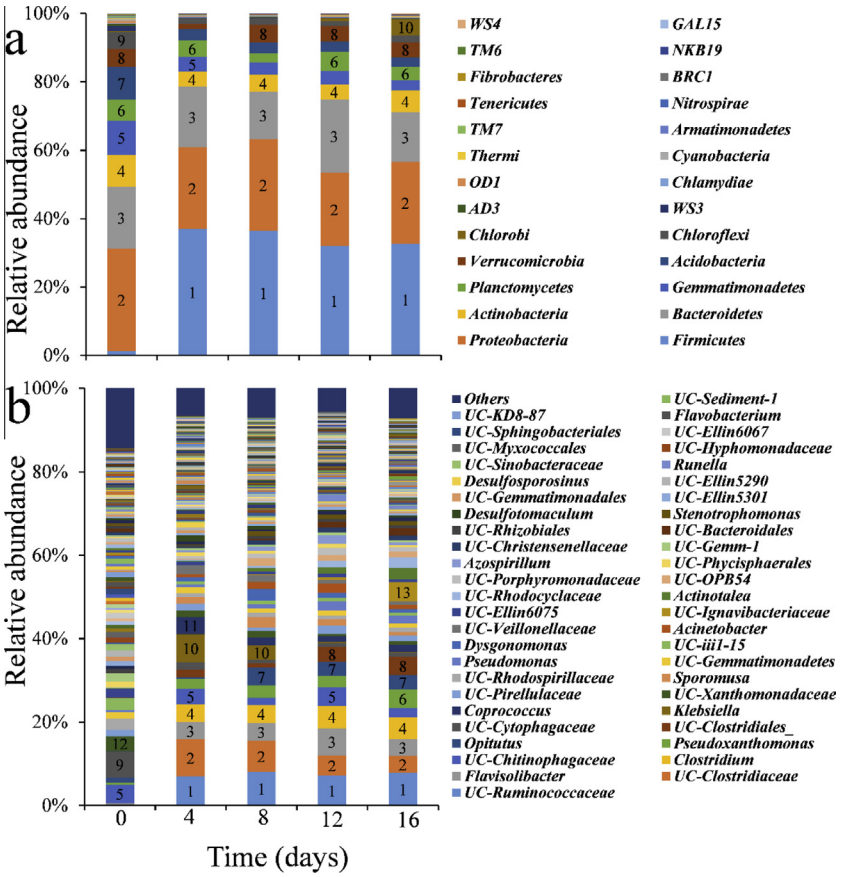

Fig. 5. Relative abundances (%) of the bacterial phyla (a) and genera found (b) in the soil during the alfalfa RSD treatment from the 0th to 16th day. Phyla (a) with relative abundances higher than 0.1% during the entire alfalfa RSD process, and the numbers 1–10 represent phyla with relative abundances higher than 4%, i.e., *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Gemmatimonadetes*, *Planctomycetes*, *Acidobacteria*, *Verrucomicrobia*, *Chloroflexi*, and *Chlorobi*. Genera (b) with relative abundances higher than 1% are listed, and those lower than 0.3% are copolymerized in "Others" during the entire alfalfa RSD process. The numbers 1–13 represent the genera with relative abundances higher than 3%, i.e., UC-*Ruminococcaceae*, UC-*Clostridiaceae*, *Flavisolibacter*, *Clostridium*, UC-*Chitinophagaceae*, *Pseudoxanthomonas*, *Opitutus*, UC-*Clostridiales*, UC-*Cytophagaceae*, *Klebsiella*, *Coprococcus*, UC-*Xanthomonadaceae*, and UC-*Ignavibacteriaceae* (letters followed by "UC-" represent the most detailed classification of the genus).

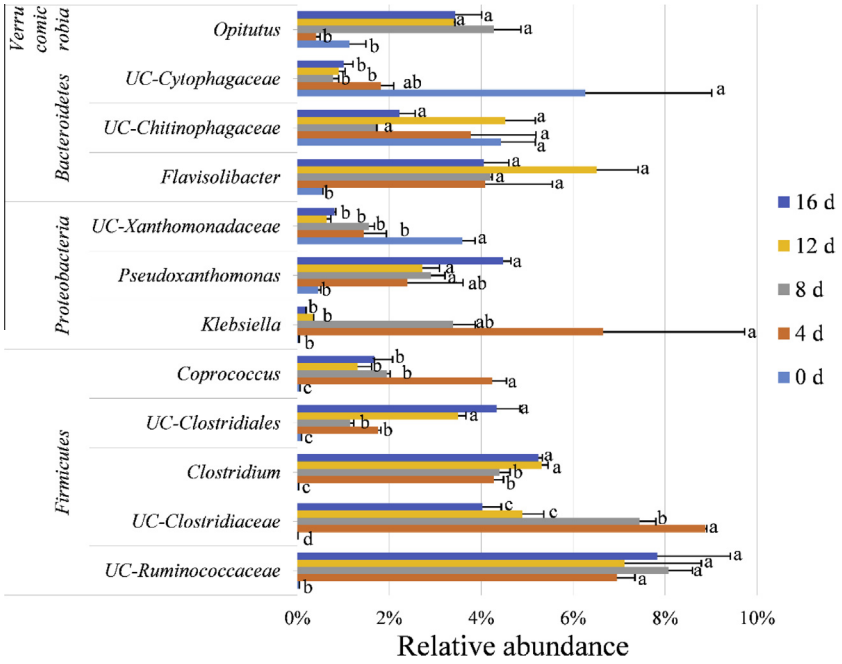

Fig. 6. Relative abundance (%) of dominant bacterial genera present (Top 12) in the soil microbial communities during the alfalfa RSD treatment from the 0th to 16th day. Bars with different letters represent significant differences among the different days through LSD tests ($p < 0.05$). Error bars indicate SEs.

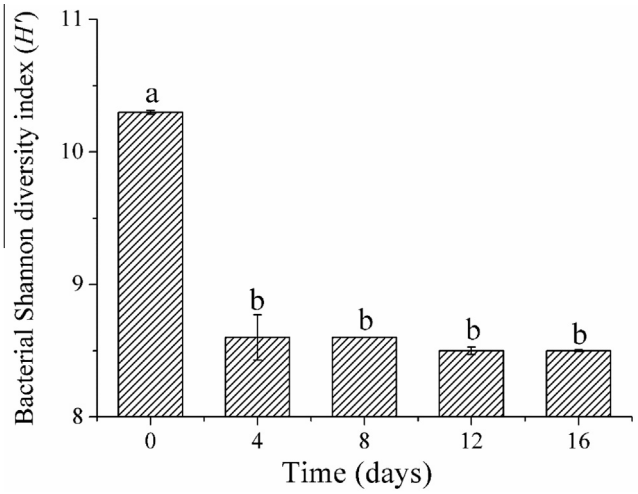

Fig. 7. The Shannon diversity index for the bacterial communities found during the alfalfa RSD treatment investigated using MiSeq pyrosequencing. Bars with different letters represent significant differences among the different days through LSD tests ($p < 0.05$). Error bars indicate SEs.

anaerobic condition during the RSD treatment is a mechanism of disinfestation. Thus, organic matter with low C/N in the RSD treatment could result in better DE.

TOC in organic matter could be divided into easily degradable organic carbon and less-degradable organic carbon; the primary component of the former is EOC, which can be decomposed quickly in the early stages of anaerobic treatment, and the latter mainly includes cellulose, which are fermented slowly (Heal et al., 1997; Puget and Drinkwater, 2001). A previous study (Rui et al., 2009) showed that the different contents of easily degradable organic carbon in organic matters could induce the disparity in organic acid production. In this study, EOC in the organic matters had a significant positive correlation with organic acids during the entire RSD process. The highest organic acid contents in the soils amended with the plant residues with higher EOC, such as sugarcane leaf, alfalfa, wheat bran, reed were observed in the early stage of the RSD treatments, probably because of the fast decomposition of the added EOC. It was demonstrated in the pure carbon source of glucose treatment that produced the highest organic acid among all of the organic matters in the early stage mainly for its highest EOC contents. Then, organic acid content decreased rapidly, probably because the rest of TOC in the organic matter might contain less-degradable organic carbon, as observed by the organic acids were mainly produced in the later stage of the cellulose treatment. On the basis of EOC and less-degradable organic carbon, the trends of organic acids during the RSD treatment could be divided into two stages: fast accumulation in the early stage and rapid consumption in the later stage; this is consistent with the results of previous studies in which organic acids accumulated rapidly after the establishment of a reductive and anaerobic environment and then rapidly decreased with an increase in incubation time (Momma et al., 2006). Furthermore, our previous study had demonstrated that the production of toxic organic acids, such as acetic, butyric, propionic, and isovaleric acid, during the RSD process could kill soil-borne pathogens (Huang et al., 2015b). In this study, similar to TN, EOC values of the plant residuals had a significant positive correlation with DE. Therefore, decomposition of EOC in the early stage of the RSD treatment is very important for the production of organic acids and the suppression of pathogens.

Additionally, there was a huge divergence of soil pH between the pure carbon source (glucose and cellulose) and plant residuals amended soils at the 4th day. The pH change during RSD may be associated with the organic matters, microbial communities, the production of organic acids and reduction substances. The significant negative correlation between soil pH and organic acids (Table 3) in all RSD treatments was consistent with our previous study (Huang et al., 2015b), which indicated that the organic matters with higher EOC values could stimulate the TOC decomposers and organic acid producers to produce higher toxic organic acids and thus induced the lower soil pH. Besides, many studies (Blok et al., 2000; Shinmura, 2000) had demonstrated that RSD treatment could decrease soil oxidation-reduction potential greatly to create a strict reductive soil environment and promote the reduction of NO_3^-, SO_4^{2-}, MnO_2, and ferric iron (Rosskopf et al., 2015) accompanied by the microbial decomposition of liable

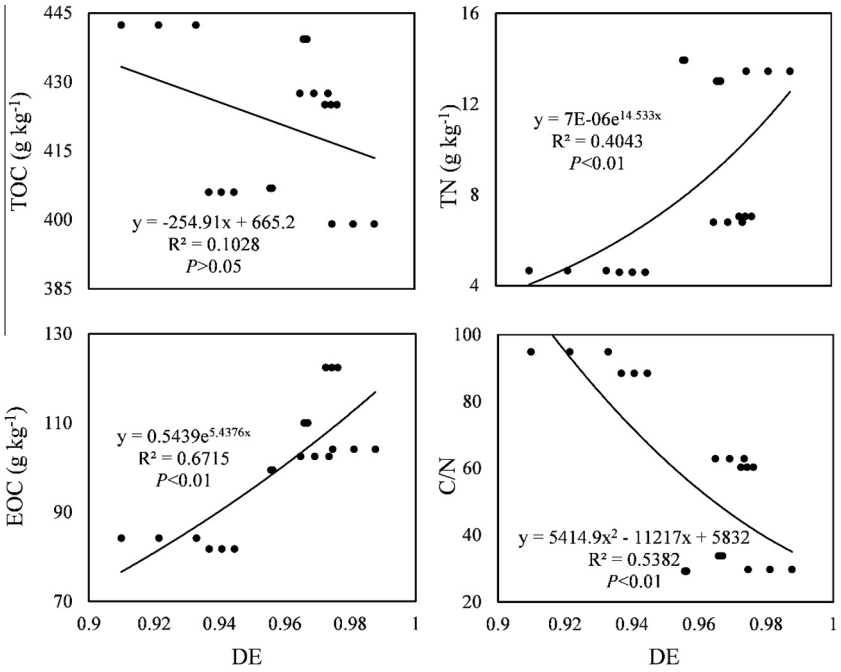

Fig. 8. Relationships between physicochemical properties of the plant residuals added into the soil during RSD process and the disinfestation efficiency (DE). TOC, TN, EOC, and C/N were the total organic carbon, total nitrogen, easily oxidized organic carbon, and toc/tn of the seven plant residuals.

Table 3
Correlations between the types of organic matter and total organic acids during the entire RSD process.

	Total organic acids[a]				
	0th day	4th day	8th day	12th day	16th day
TOC	N	N	N	N	N
TN	N	N	N	N	−0.67*
EOC	N	0.81**	0.87**	0.78**	0.69**
C:N	N	N	N	N	N
pH	N	−0.73*	−0.95**	−0.92**	−0.96**

* $p < 0.05$.
** $p < 0.01$.
[a] "N" represents no significant correlation.

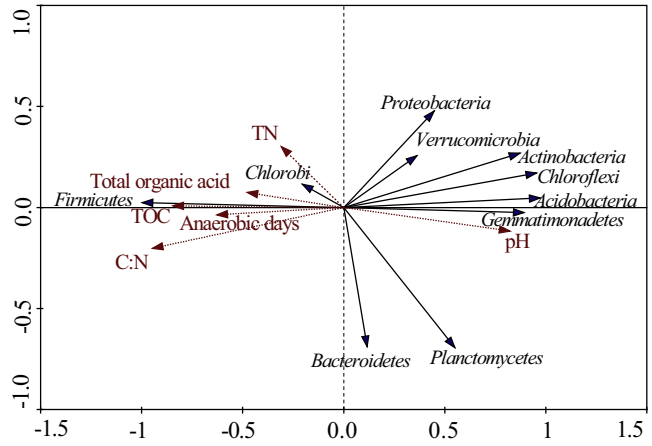

Fig. 9. Redundancy analysis (RDA) for the bacterial phyla and environmental factors in the alfalfa soil during the RSD treatment.

Table 4
Correlations between dominant bacterial genera and organic acid contents during the alfalfa RSD process.

Dominant bacteria genera	Acetic acid	Propionic acid	Butyric acid[a]
UC-Clostridiaceae	0.87**	0.83**	0.64*
Klebsiella	0.70*	0.81**	0.70*
Coprococcus	0.67*	0.74*	N

* $p < 0.05$.
** $p < 0.01$.
[a] "N" represents no significant correlation.

organic matter. Therefore, soil pH in the RSD treatment could be increased by consuming protons and releasing basic cations during the chemical nature of reduction processes (Rosskopf et al., 2015). In this study, due to the decomposition of EOC and the producing of organic acids at the 4th day, soil pH in all of the RSD treatments decreased significantly. Specifically, the lowest pH was found in the glucose treatment mainly for the highest production of organic acids. Then, soil pH increased slowly, probably because the consumption of organic acids and the generation of reduction substances with the increasingly reductive soil environment. As a pure carbon, the effects of glucose on stimulating the growth of microorganisms and improving their gross activity were lower than the plant residuals (Fig. 4), which might induce the lower production of reduction substances which needing the participation of microorganisms in glucose amended treatment than in plant residuals. Hence, although the organic acids were approximate between the treatments of glucose and sugarcane leaf, the pH in glucose treatment still significant lower than sugarcane leaf treatment at the 4th day.

As we known, RSD is driven by anaerobic microorganisms; however, functional and dominant microorganisms and their changes during the entire RSD process are poorly understood (Mowlick et al., 2013). A few studies (Huang et al., 2015b; Momma et al., 2006; Mowlick et al., 2012) used denatured gradient gel electrophoresis and clone libraries and indicated that *Clostridium* spp. significantly increased and may play an important role in the RSD process. In this study, MiSeq pyrosequencing results showed some dominant microorganisms (Fig. 6). For instance, in the early stage of the alfalfa RSD treatment, relative abundances of UC-*Cytophagaceae* (*Bacteroidetes*) and UC-*Xanthomonadaceae* (*Proteobacteria*) decreased significantly. Although UC-*Cytophagaceae* acted as a cellulolytic bacteria, it is a completely aerobic bacterium (Reichenbach, 1992; Xie et al., 2007); therefore, the significant decrease was mainly caused by the anaerobic condition. Besides, UC-*Xanthomonadaceae* has been reported as a phytopathogen and often causes diseases in plants (Pieretti et al., 2009); therefore, a decrease in its population would maintain soil health. Relative abundances of *Coprococcus* (*Firmicutes*), UC-*Clostridiaceae* (*Firmicutes*), and *Klebsiella* (*Proteobacteria*) increased significantly, and the three genera had a significant positive correlation with the produced organic acids. These results are consistent with those of previous studies (Holdeman and Moore, 1974; Patel et al., 2010; Wüst et al., 2011) in which these microorganisms produced a lot of organic acids by fermenting EOC. When EOC was decomposed completely and only less-degradable organic carbon remained, these organic acid producers were significantly decreased in the later stage of the RSD treatment. Meanwhile, *Opitutus* (*Verrucomicrobia*) and UC-*Clostridiales* (*Firmicutes*) were significantly increased. Many studies (Chin and Janssen, 2002; Van Passel et al., 2011) have reported that *Opitutus* can ferment carbohydrates to produce propionate; however, a significant correlation between *Opitutus* and propionic acid was not detected in this study. The dormant spores of UC-*Clostridiales* can germinate rapidly when nutrients (Paredes-Sabja et al., 2011) are absorbed in the RSD treatment and then produce enzymes to decompose the less-degradable organic carbon (Kamada et al., 2013). During the entire RSD treatment, relative abundances of UC-*Chitinophagaceae* (*Bacteroidetes*), *Clostridium* (*Firmicutes*), UC-*Ruminococcaceae* (*Firmicutes*), *Pseudoxanthomonas* (*Proteobacteria*), and *Flavisolibacter* (*Bacteroidetes*) were always high and previous studies (Bhat and Bhat, 1997; Biddle et al., 2013; Del Rio et al., 2010; Eichorst et al., 2013; Kumar et al., 2015) have indicated that they also can break down less-degradable organic carbon. Therefore, these TOC decomposers and organic acid producers could collaborate to control *F. oxysporum* (Huang et al., 2015a).

In general, populations of soil microorganisms, especially bacteria, were significantly increased during the RSD process, and these soil microorganisms mutually participated into the transformation of soil carbon. In the plant residuals amended RSD treatments, approximately 16.91% (the lowest percentage observed in the sugarcane residue treatment) to 57.09% (the highest percentage observed in the alfalfa treatment) (data was not shown) of added organic carbon was degraded, and it is expected that the rest of the organic carbon would continuously stimulate the aerobic microorganisms and improve soil microbial activity after RSD treatment. Besides, quick removal of NO_3^--N could improve the problem of soil salinization caused by nitrate accumulation. However, it may also lead to nitrogen loss, especially when an RSD treatment with high C/N organic matter is used for soil with low N content.

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