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Multiple selection of resistance genes in arable soil amended with cephalosporin fermentation residue

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ABSTRACT

Cephalosporin fermentation residue (CFR) is a byproduct of the pharmaceutical industry that may be disposed through land application. While this organic residue can improve soil physico-chemical properties, the possibility of CFR-induced antibiotic resistance in the native soil microbial community still needs to be investigated. In a lab-based incubation study, the CFR-induced selection for antibiotic resistance genes (ARGs), including changes in the structure of native soil bacterial populations, antibiotic-induced selection and lateral transfer potential, was determined with quantitative PCR array and 16S amplicon sequencing. CFR amendment of soil increased the abundance of β-lactam resistance genes over time. There were higher abundance of β-lactam resistance genes in soil receiving raw CFR than treated CFR, indicating that antibiotics or metabolites contained in raw CFR contributed to the selection of resistant microorganisms. There were more mobile genetic elements in the raw CFR-amended soil, which is further evidence that bacterial responses contributed to the dissemination of antibiotic resistance genes within species in the bacterial community. Marked shifts in the native soil bacterial community composition were observed and several specific genera probably contributed to the resistance as representatives of corresponding phyla Bacteroidetes, Actinobacteria, Proteobacteria and Firmicutes, suggesting a phylogenetic basis for the increase of resistance genes in CFR-amended soil. These results imply the selection for β-lactam resistance genes at multiple levels following the application of CFR to arable soil.

1. Introduction

Antibiotics are widely used in the treatment and prevention of bacterial infections (NHS, 2019). China is one of the biggest manufacturers and global exporters of bulk antibiotics (Zhang et al., 2015). Most bulk antibiotics are produced by natural bacteria and fungi with fermentation technology (Chandra and Kumar, 2017), which generates approximately 1.4 million tonnes of bio-fermentation residue each year (Li et al., 2012). More than 50% of the bio-fermentation residue produced in China is cephalosporin fermentation residue (CFR) due to the global market for cephalosporin, which was valued at US$ 11.9 billions with the largest sales among the antibiotics market in 2009 (Hamad, 2010). Disposing of CFR remains a challenge for the biopharmaceutical industry. One solution is to recycle CFR as a soil amendment, since it contains humic acid-like substances that increased soil organic matter (Cai and Liu, 2018) and improved the growth (height and yield) of snap bean (*Phaseolus vulgaris*) in arable soil (Wang et al., 2016).

While recycling CFR as a soil amendment is way to dispose of this residue, it presents an environmental risk due to the fact that CFR contains cephalosporin C (CPC) and desacetyl cephalosporin C (DCPC) (Cai et al., 2017). Land-use of CFR will release antibiotics and antibiotic resistance genes (ARGs) into the environment (soil, air and water). The amending practice may lead to the development of antibiotic resistance in non-pathogenic and pathogenic organisms that are of concern for public health (Bondarczuk et al., 2016; Chen et al., 2016). Besides, injecting or mixing CFR into soil could increase the selective pressure on native soil bacteria, leading to greater occurrence of horizontal gene transfer (HGT: conjugation, transduction and transformation (Ochman et al., 2000)) mediated by mobile genetic elements (MGEs) (Jechalke et al., 2014; Blair et al., 2015). Before large-scale application of CFR to arable soil can be approved, it is essential to evaluate the changes in soil ARGs as a consequence of CFR application.

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There are multiple levels at which the application of CFR may select ARGs in soil (which is defined as "multiple selection" in this study). In general, six scenarios could support our speculation: 1) new ARGs added from the CFR itself, 2) killing off sensitive strains and increasing the relative abundance of ARGs, 3) increase in absolute abundance of ARGs due to success without as many competitors, 4) increase in ARG abundance without concomitant changes in community membership (i.e. via HGT), 5) increase in resistant bacteria due to more mutations, 6) increase in bacteria containing ARGs because of growth rate differences with CFR addition and potentially new nutrients. In all cases, several contributing factors could result in the proliferation of antibiotic resistance in CFR-amended soil, which have been well proven based on the other studies without CFR, e.g., residual antibiotic exposure (Zhang et al., 2017), nutrients input (Udikovic-Kolic et al., 2014), changes in the population structure (Heuer and Smalla, 2007) and HGT (Tenover, 2006; Ghosh and LaPara, 2007). However, we are not aware of any studies that examined effects of CFR application on ARGs in soil and that explored whether a multiple selection could explain the increased soil ARGs following application of CFR.

The aim of this study was to investigate the impacts of raw and treated CFR on the abundance of ARGs and on the soil microbial community structure in arable soil. The different CFR-amended soils were incubated in the laboratory and sampled six times during a 50 d period to assess the response of 50 ARGs that potentially confer resistance to β-lactams. The occurrence of three MGEs were investigated as indicators of HGT in the soil bacterial community. We hypothesized that: (i) CFR amendment of soil would increase the abundance of β-lactam resistance genes over time, (ii) more pronounced effects of resistance would be detected in the soil bacterial community exposed to raw CFR than treated CFR, (iii) CFR amendment would enhance the possibility of HGT, and (iv) bacterial phylogeny would be the driver in shaping the β-lactam resistance genes in CFR-amended soil.

2. Materials and methods

2.1. Materials and experimental design

Soil for this experiment was collected in October 2016 from a fertilized, arable field used for cabbage (*Brassica oleracea* L.) production at Hongli (126°87′E, 45°75′N), Harbin, China. Surface soil (0–15 cm) was collected, transported to the laboratory, thoroughly homogenized and sieved (< 2.0-mm mesh). Raw CFR with 88%–90% water content was obtained from the Chuanning Biotechnology Co. (Yili, China) and was stored in sterilized plastic bucket at 4 °C in the refrigerator until use. The raw CFR was adjusted to the moisture rate of 95%, followed by microwave treatment at 100 °C for 20 min using the customized apparatus reported by Cai et al. (2017), and hereafter referred to as treated CFR. The raw CFR and treated CFR were then centrifuged (5000 rpm for 20 min) and the solid portion (dewatered CFR with moisture of 67%) was retained for the incubation experiment. Initial physico-chemical properties of soil and dewatered CFR are provided in Table S1.

The experiment was a repeated measures design with three treatments (unamended control, raw CFR, treated CFR), each replicated 3 times. The experimental unit was a pot (20 cm high, 15 cm diam.) filled with 1.0 kg of sieved arable soil that was unamended (control), mixed with 30 g (wet weight) raw CFR kg^{-1} soil or mixed with 30 g (wet weight) treated CFR kg^{-1} soil. The CFR amendment rate was based upon oven dry-weight and equivalent to a field application of 30 t ha^{-1} (surface horizons with about 15–20 cm depth), the suggested agronomic rate for comparable organic residues such as cattle manure and compost (Gou et al., 2018). The soil or soil-CFR mixture was moistened to 20% gravimetric moisture content with deionized water and incubated at 25 °C and 60% relative humidity in the dark for 50 d. Soil moisture was maintained at 65% of water-holding capacity by adding deionized water as necessary.

2.2. Sampling for physico-chemical analyses

Each pot was sampled six times during the incubation period, at 1, 2, 4, 8, 20 and 50 d after the pots were prepared. Each time, a subsample (~20 g soil from the 0–10 cm depth) was collected using a sterilized cylinder (30 mm diam.) from a unique zone in the pot to minimize sampling bias. The remaining soil (0–10 cm depth) was homogenized using a medicine spoon that was surface-sterilized with 70% ethanol. Each soil sample point was uniformly mixed to achieve high representativeness, and then it was stored at −20 °C with approx. 20 g for analysis. Each subsample (5 g) underwent physico-chemical analyses for soil pH and electrical conductivity (PHS-3G acidity meter and DDS-307 conductivity meter, Leici, China), and another subsample (5 g) was measured for organic carbon and nitrogen content (with a CHNOS Elemental Analyzer, Elementar Co., Germany) (Cai and Liu, 2018). Concentrations of antibiotics (e.g., CPC and DCPC) present in soil subsamples (2 g) were measured by the modified method of Cai and coauthors (Cai et al., 2017). Briefly, this involved extracting CPC and DCPC by repeated sonication with an extraction solution of 50:50 (*v:v*) acetonitrile: H_2O. Acetonitrile was removed by adding dichloromethane, and the aqueous phase was filtered through a 0.22 μm nylon syringe filter, then injected into an Agilent 6460 liquid chromatograph (Agilent, Palo Alto, CA, USA) interfaced with triple quad liquid chromatograph tandem mass spectrometer multiple reaction monitoring mode and positive ion scan mode.

2.3. DNA extraction

Microbial DNA was extracted from each soil subsample (0.5 g) with a Fast DNA® Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. The concentration of DNA was determined using the QuantiFluor® dsDNA system (Promega Corporation, USA) and the DNA was stored at −20 °C for qPCR analysis. The DNA concentrations varied between 30 and 50 ng μL^{-1} for the tested soil samples.

2.4. Quantitative PCR

The high-throughput qPCR reactions were conducted using an Applied Biosystems ViiA™ 7 Real-time PCR System (Wcgene Biotechnology, Shanghai, China) reported elsewhere (Pu et al., 2017). In total, 54 qPCR arrays (primer sets) were measured, including 50 β-lactam resistance gene primer sets, 3 MGEs primer sets and one bacterial universal 16S rRNA gene (Su et al., 2015; Muurinen et al., 2017). The β-lactam resistance gene primer group is categorized into "antibiotic deactivation" (Class A, Class B, Class C and Class D) and "cellular protection" (Mayers, 2009) and MGEs primer sets include one transposase gene and two class 1 integrase genes (Table S2). The qPCR mixture (10 μL) consisted of 5 μL Master (2 ×), 0.75 μL of each primer (10 μM), 3 μL ddH_2O and 0.5 μL template. Initial enzyme activation was performed at 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 s and annealing at 60 °C for 30 s. The specificity of amplification was determined with a melting process at 60 °C–95 °C, as described previously (Pu et al., 2017). The detection limit of the threshold cycle (CT = 31) was considered the limit for genes with no amplification, according to the sensitivity of the Real-Time PCR System. All three technical replicates of each sample were above the detection limit, which was the criteria used for a positive detection of the ARGs. The $2^{-\Delta CT}$ method assumed $\Delta CT = CT_{(detected\ ARG)} - CT_{(16S\ rRNA\ gene)}$ and was used to calculated the relative gene abundances, normalized to the 16S r RNA gene according to Schmittgen and Livak (2008).

2.5. Illumina sequencing

The V3–V4 regions of the bacterial 16S rRNA gene were amplified with universal primers 341F: CCCTACACGACGCTCTTCCGATCTG

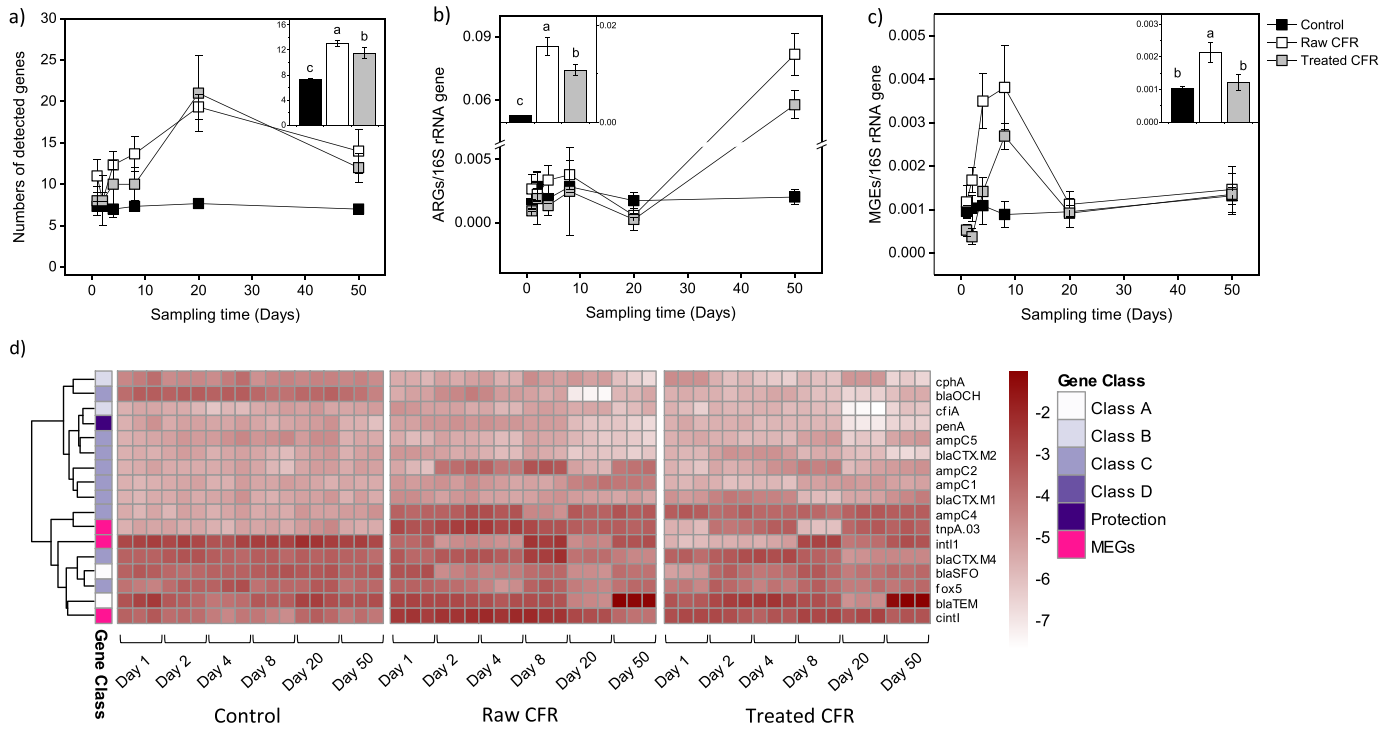

Fig. 1. Change in β-lactam resistance genes and mobile genetic elements (MGEs) during the incubation of unamended soil (Control) and soil amended with raw cephalosporin fermentation residue (CFR) or treated CFR. a) Temporal changes and averages in numbers of β-lactam resistance genes in the control and CFR-amended soils. **b)** Temporal changes and averages in relative abundance of β-lactam resistance genes in the control and CFR-amended soils. **c)** Temporal changes and averages in relative abundance of MGEs in the control and CFR-amended soils. Error bars indicate standard deviations. Different letters above the bars indicate a significant difference ($P < 0.05$). **d)** Heat map showing temporal changes in the relative abundance of β-lactam resistance genes and mobile genetic elements (MGEs) in the control and CFR-amended soils. Each row gives the results from a single primer set and data are the logarithm transformed relative abundance values. Primer sets (17) with amplification in at least three samples are shown. Assays are grouped as "Gene Class" according to resistance mechanisms of β-lactam resistance genes and MGEs. Rows were clustered based on Bray-Curtis distance.

(barcode) CCTACGGGNGGCWGCAG and 805R: GACTGGAGTTCCTTGGCACCCGAGAATTCCAGACTACHVGGGTATCTAATCC. After the enzyme activation at 94 °C for 3 min, amplification was conducted as followed: 94 °C for 30 s, 45 °C for 20 s, 65 °C for 30 s with 5 cycles; 94 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s with 20 cycles. Amplification products were purified and sequenced on an Illumina Miseq™ platform (Sangon Biotech, Shanghai, China). The obtained sequences were filtered and analyzed using Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso et al., 2010). The default method was used for picking the open-reference operational taxonomic unit (OUT) that was defined at the 97% identity level using a chimera filtering approach (UPARSE) (Edgar, 2010).

2.6. Statistical analysis

Descriptive statistics (mean and standard deviation) of the resistance genes were calculated using Excel 2013 (Microsoft, USA). The effect of CFR treatments on the detected numbers and relative abundances of resistance genes and MGEs was calculated with repeated measures analysis of variance (rANOVA) using SPSS 20 (IBM, USA). Heat maps of patterns of resistance genes and bacterial taxa were produced using the package pheatmap 1.0.8 (Kolde, 2015) in R Studio Version 1.0.136 (Team, 2017). Spearman's correlation test detected correlations between the relative abundance of resistance genes and MGEs.

To analyze co-occurrences, network analysis of the abundance of β-lactam resistance genes, MGEs and bacterial taxa at genera levels for all samples (54 samples: three treatments, six sampling points and three replicates) was calculated using the package psych in the R software (Revelle, 2017) with false discovery rate control (Glickman et al., 2014). A statistical correlation was remained when it met the conditions of the Spearman's correlation coefficient ($\rho > 0.6$) and $P < 0.05$. The pairwise correlations that were imported into Gephi or Cytoscape (Halary et al., 2010) for network visualization. Co-occurrence among β-lactam resistance genes and MGEs was explored using the Frucherman Reingold algorithm in Gephi platform. Close connections between β-lactam resistance genes and bacteria genera were displayed using Cytoscape with the circular layout algorithm.

Structural equation models (SEMs) were developed to evaluate the hypothetical response of β-lactam resistance genes (direct and indirect responses) to CFR amendment, bacterial abundance and diversity, introduced antibiotics and MGEs. SEM is an a priori approach with the capacity to identify causal relationships between variables by fitting data to the models representing causal hypotheses. The pairwise correlations were determined among these variables using Mantel test with the package vegan 2.4–3 (Oksanen et al., 2017). The data from all time points used for the Mantel test included CFR amendment (categorical variable), the bacterial 16Sr RNA gene abundance, the bacterial diversity (Shannon index, Chao1 index and OTU numbers), and the relative abundance of MGEs and β-lactam resistance genes. The theoretical model assumptions were as follows (Fig. S3). The obtained correlation matrix for model fitting was imported into AMOS software (SPSS, IBM, USA) to construct SEMs using the maximum-likelihood estimation method. Non-significant chi-square test ($P < 0.05$), high goodness-of-fit index (GFI > 0.90) and low root-mean-square errors of approximation (RMSEA < 0.05) were used to evaluate the goodness of fit for SEMs. The standardized total effects of tested variables on resistance genes were also calculated.

3. Results

3.1. Abundance of β-lactam resistance genes during the incubation

Prior to the incubation, three experimental materials in this study (tested soil, raw CFR and treated CFR) contained nine β-lactam resistance genes in total (Table S3). There was four shared β-lactam resistance gene (*blaSFO*, *blaTEM*, *ampC4* and *blaCTX-M4*) in soil, raw CFR and treated CFR, and each of these materials had from four to nine resistance genes. Following both CFR amendment, the number of detected β-lactam resistance genes increased significantly from 8 to 11 genes on day 1 to 19–21 genes on day 20 and remained significantly greater with around 2-fold more β-lactam resistance genes in the both CFR-amended soils than the control soil after 50 d of incubation ($P < 0.05$, rANOVA, Bonferroni test, Fig. 1a). The relative abundance of β-lactam resistance genes in both CFR-amended soils also increased significantly during the incubation ($P < 0.05$, rANOVA, Bonferroni test), while there were no significant difference in the relative abundance of β-lactam resistance genes in the control soil ($P > 0.05$, rANOVA, Fig. 1b). When it comes to different treatments, there were greater average relative abundance of β-lactam resistance genes in the raw CFR > treated CFR > control with the values of 0.015, 0.010 and 0.001 ARGs/16S rRNA gene, respectively ($P < 0.01$, Table S4, Fig. 1b insert panel).

The overall patterns of β-lactam resistance genes were altered with a distinct clustering ($P < 0.05$, Adonis test). PCoA analysis showed that the control samples clustered together and were separated from the other samples (Fig. S2). The individual subtype distribution of β-lactam resistance genes showed different temporal patterns during the incubation (Fig. 1d). Two β-lactam resistance genes (*cphA* and *blaOCH*) decreased over time in both CFR-amended soils, but β-lactam resistance gene *ampC4* (Class C) and *tnpA-03* (MGE) became more abundant. Specially, the relative abundance of *blaTEM* gene declined by 20 d and thereafter increased to the extremely high value by 50 d of the incubation (Fig. 1d).

3.2. Abundance of MGEs during the incubation

The relative abundance of MGEs increased in both CFR-amended soils from day 1 to day 8 of the incubation, and then declined to the initial level ($P < 0.05$, rANOVA, Bonferroni test), while there was no significant difference in MGE abundance of control soil during the incubation period ($P > 0.05$, rANOVA, Fig. 1c). The average abundance of MGEs in raw CFR-amended soil was two times as high as the control, while there was no significant difference of average MGEs abundance between control and treated CFR treatment (Fig. 1c insert panel). The co-occurrence pattern among β-lactam resistance genes was separated into five modules (Fig. 2a). Each module consisted of different types of genes, and the most densely connected node was defined as the 'hub'. For example, *tnpA-03* gene (MGEs) was the 'hub' in module IV, and could be regarded as an indicator of the presence of other co-occurring β-lactam resistance genes in the same module. Spearman's correlation analysis revealed that the total abundance of β-lactam resistance genes was significantly correlated with abundance of MGEs although not strongly ($\rho = 0.40$, $P < 0.01$, Table S5). The abundance of genes from Class C was also correlated with the abundance of MGEs ($\rho = 0.38$, $P < 0.01$, Table S5).

3.3. Co-occurrence patterns among β-lactam resistance genes, MGEs and bacterial taxa

To illustrate the co-occurrence patterns, the bacterial community was investigated. A total of 3,057,262 high quality sequences were obtained from all 54 samples, with sequences per sample ranging from 50,657 to 76,121. These sequences were clustered into 29,020 OTUs at the 3% dissimilarity level. Rarefaction curves of OTUs approached a plateau at the sequencing depth of 32,800 (Fig. S3). Soil bacterial community was initially dominated by Actinobacteria, Proteobacteria, Bacteroidetes, Firmicutes, Acidobacteria, Gemmatimonadetes, Chloroflexi, and Saccharibacteria (Fig. 3e). After CFR amendment, the bacterial 16S rRNA gene abundance increased significantly ($P < 0.05$, rANOVA, Bonferroni test, Fig. 3a), and marked shifts in native soil bacterial community composition were observed (Fig. S4) with three phyla (Actinobacteria, Proteobacteria, Bacteroidetes) accounting for more than 95.0% of the total bacterial 16S rRNA gene sequences by day 50 of the incubation (Fig. 3e). The bacterial diversity indexes (Shannon, Chao1 and OTU numbers) significantly decreased following both raw CFR and treated CFR amendment ($P < 0.05$, rANOVA, Bonferroni test, Fig. 3b, c and d).

The co-occurrence patterns among β-lactam resistance gene types and bacterial genera were explored using network analysis. Significant ($\rho > 0.6$, $P < 0.05$) correlations in Fig. 2b reveal that several genera with > 2% 16S rRNA abundance belonging to four phyla (Bacteroidetes, Actinobacteria, Proteobacteria and Firmicutes) were associated with resistance genes, suggesting these genera are potential contributor for the resistance genes (Fig. 2b and Fig. S5). For instance, *Arachidicoccus* was the potential contributor of *ampC1*, *fox5* and *ampC2* genes. It must be noted that seven bacterial genera (*Arachidicoccus*, *Actinomadura*, *Flexivirga*, *Kribbella*, *Rhodanobacter*, *Rhizobium* and *Mesorhizobium*) were found to be the candidates of *blaTEM* gene in 'Class A' category which contributed most to the increased relative abundance of β-lactam resistance genes. In addition, we found that nine bacterial genera (*Shimazuella*, *Arachidicoccus*, *Actinomadura*, *Flexivirga*, *Kribbella*, *Rhodanobacter*, *Dyella*, *Rhizobium* and *Mesorhizobium*) probably harbored MGEs, presenting the horizontal gene transfer potential.

3.4. Factors influencing the patterns of resistance genes

Hypothesized causal relationships among CFR amendment, bacterial abundance and diversity, introduced antibiotics, MGEs and β-lactam resistance gene patterns from all the soil samples were explored with SEMs (Fig. 4). The best-fit model explained 70% of the total variance of resistance gene patterns in the both CFR-amended and control soils. CFR amendment had significant influence on bacterial diversity ($\lambda = 0.27$, $P < 0.05$) and MGEs ($\lambda = 0.32$, $P < 0.01$). Strong impacts of bacterial abundance ($\lambda = 0.55$, $P < 0.001$), bacterial diversity ($\lambda = 0.17$, $P < 0.05$) and MGEs ($\lambda = 0.38$, $P < 0.001$) on resistance gene abundance were also observed (Fig. 4a). The standardized total effects from SEMs showed that bacterial abundance contributed most to resistance gene patterns, followed by MGEs and introduced antibiotics (Fig. 4b).

4. Discussion

4.1. Temporal succession of resistance genes in amended soil

Our first hypothesis stating that CFR amendment of soil would increase the abundance and diversity of β-lactam resistance genes with time was confirmed. Raw and treated CFR were the likely source of resistance genes in soil microbial communities because a certain number (Table S3) and relative abundance of resistance genes were detectable (Relative abundance of β-lactam resistance genes in the control soil, raw CFR and treated CFR were 0.002, 0.132 and 0.101 ARGs/16S rRNA gene, respectively). Considering that antibiotic-producing mycelia (*Acremonium chrysogenum* in this study) were fungi, the resistance genes tested in CFR might be derived from the proliferation of extraneous bacteria that developed resistance to antibiotics throughout the post-processing period (collection, dewatering, etc.). Despite the detection of β-lactam resistance genes in CFR, it was not significant ($P > 0.05$, rANOVA Bonferroni test, Fig. 1b) that these genetic materials contributed directly to the increase of resistance levels measured in soil immediately following the application of CFR

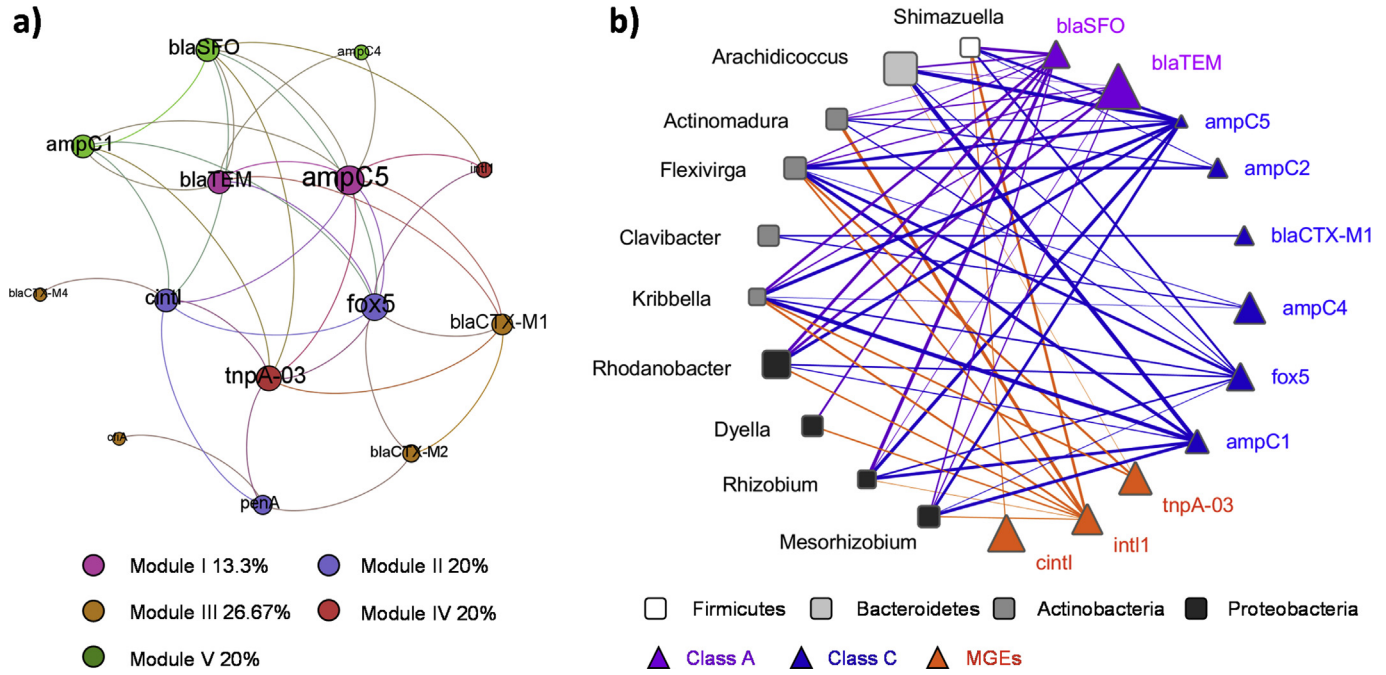

Fig. 2. a) Network co-occurrence patterns among β-lactam resistance genes in all control and both CFR-amended soils (n = 54). Only significant correlations ($\rho > 0.6$, $P < 0.05$) were shown. The nodes with different colors represent distinct modularity classes. Node size weighted according to the number of connections between nodes (degree). **b)** Network of predicted bacterial genera associated with each β-lactam resistance gene and MGE in control and both CFR-amended soils (n = 54). Nodes with different colors represent different classes of β-lactam resistance genes, MGEs (triangles), and bacterial genera (rounded squares). Genera are denoted with squares and their bacteria phyla are identified. Edge thickness is proportional to the correlation coefficient and node size is proportional to the abundance of each bacterial genus. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

when injecting ratio of CFR to soil was 1/100 (based on dry weight).

A significant temporal succession of β-lactam resistance genes was observed following CFR amendment ($P < 0.05$, rANOVA). This may occur because of the proliferation of resident bacteria carrying specific genes (Su et al., 2015). The absolute abundance of resistance genes increased on 20 d and 50 d in both CFR-amended soils compared to the control (Fig. S1), with the significant shifts in bacterial communities ($P < 0.05$, Adonis test). The lowest diversity of bacteria and the elevated abundance of bacterial 16S rRNA genes occurring after 20 d (Fig. 3a, b, c and d) suggested that the increased β-lactam resistance genes abundance may be due to the proliferation of bacteria that harbored specific resistance genes. After that, the 16S copy numbers dropped but the relative abundance of β-lactam resistance genes was still high, indicating that genes could have been transferred or microorganisms carrying those genes increased by the end of the incubation. It is noted that the decline in the Acidobacteria after 20 d of the incubation was observed (Fig. 3e), while this phyla was not main candidate potentially contributing to β-lactam resistance genes in the CFR-amended soil environment (Fig. 2b).

The dominance of *blaTEM* gene among β-lactam resistance genes on 50 d is noteworthy in both CFR-amended soils (Fig. 1d), causing the speculation that nutrients addition could favor bacteria carrying β-lactam resistance genes with different grow rates. This broad-spectrum β-lactam resistance gene was first reported in 1963 in clinical isolates (Graham et al., 2016). The *blaTEM* gene encodes resistance by the production of β-lactamases to hydrolyze the four membered β-lactam ring (Bush et al., 1995), and is naturally prevalent in soil bacteria according to culture-dependent and -independent measures (for example, in *Pseudomonas* genus and *Stenotrophomonas* sp.) (Demanèche et al., 2008). The control soil in this study already contained the *blaTEM* gene, indicating an intrinsic resistance pathway for native soil microorganisms. Among the seven potential contributors for *blaTEM* gene, *Arachidicoccus* was the dominant genus (Fig. 2b) that might account for the proliferation of the *blaTEM* gene in CFR-amended soil. A future hypothesis about the performance of this genus should be tested. Chitin, which is a major component of the cell walls of most fungi, could be potentially introduced into the soil following the CFR amendment, while *Arachidicoccus* containing *N*-acetyl-*β*-glucosaminidase activities could drive the cleavage of the chitin into monomers (*N*-acetyl-*β*-glucosamine) (Siddiqi et al., 2017).

4.2. Additional selection pressure on resistance genes

Although the abundance of β-lactam resistance genes increased in both CFR-amended soils, there were more pronounced effects on β-lactam resistance genes when exposed to raw CFR than treated CFR ($P < 0.05$, Table S4, Fig. 1a and b), indicating that the introduced antibiotics, potential degradation products or other substrates in raw CFR posed an additional pressure on the β-lactam resistance genes. Antibiotics induce selective pressure on sensitive or antibiotic-resistant microorganisms (mutants carrying resistance genes or pre-existing genotypes), and the vertical transmission of this trait through specific lineages allows for population expansion. Despite the low potency (Morin and Jackson, 1970) and short half-life ($t_{1/2} < 4$ d) of CPC and DCPC in soil (Fig. S6), we stress that adding significant quantities of broad-spectrum antibiotics to soil could potentially interfere with the soil microbial environment. On the another hand, residual antibiotics and metabolites in raw CFR can indirectly select for resistance through modification of the bacterial community composition, which could partially explain the difference between the raw CFR- and treated CFR-amended soils.

4.3. CFR amendment enhanced the potential of HGT in amended soil

We examined MGEs because resistance dissemination and subsequent acquisition of resistance by human pathogens depend on the HGT from natural-clinical environments (Martínez, 2008). In this study, CFR amendment led to the enrichment of *cintl1* and *tnpA-03* genes

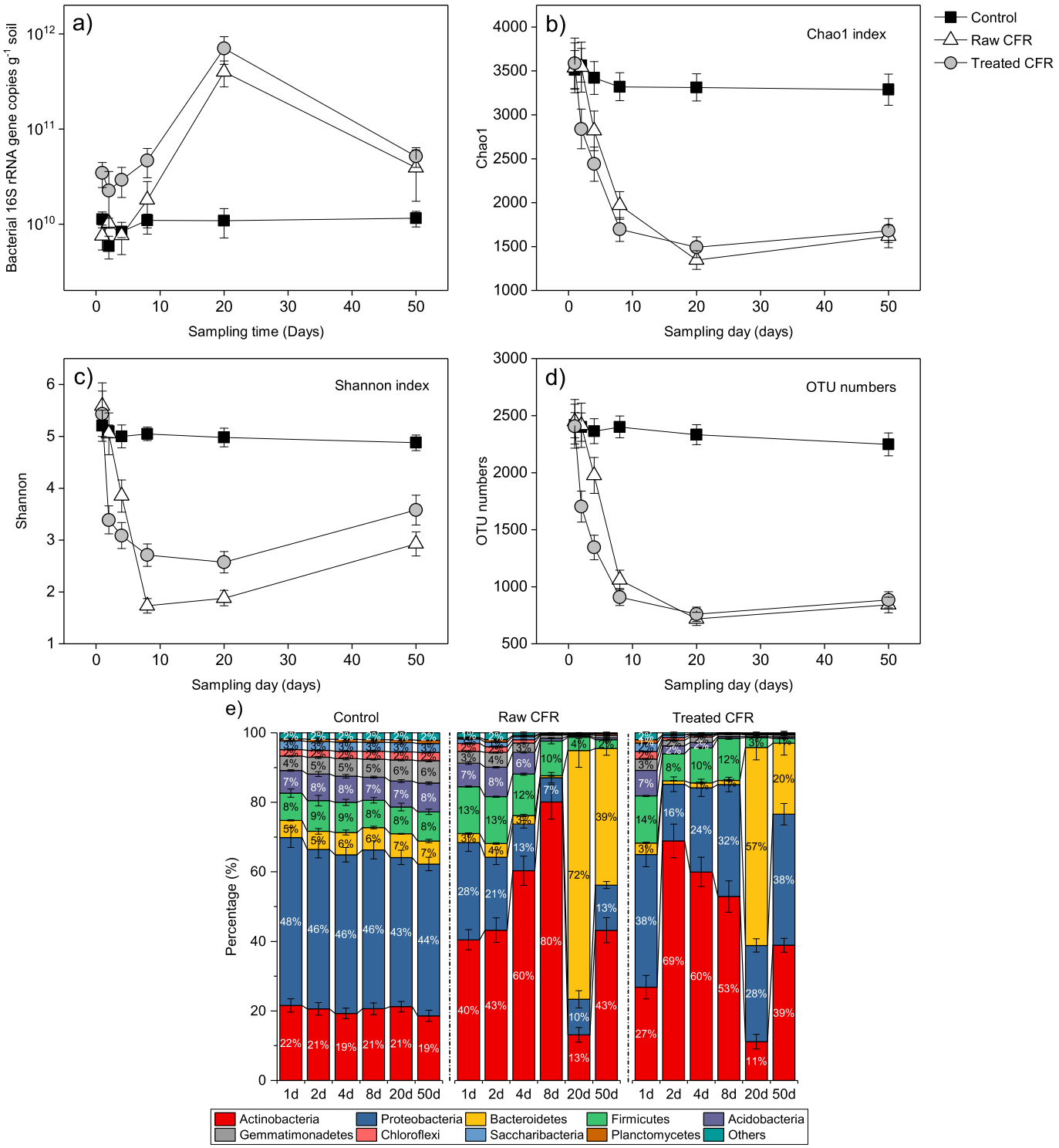

Fig. 3. a) Changes in the bacterial 16S rRNA gene abundance in the control and different CFR-amended soils. **b), c)** and **d)** alpha-diversity of bacterial community in the control and CFR-amended soils. **e)** average percentages of total 16S rRNA gene sequences in each bacterial phylum of control and CFR-amended soil. The category 'Others' contains the bacteria taxa whose abundance was < 1% of the 16S rRNA sequences in the soil sample. Error bars indicate standard deviations (n = 3).

(P < 0.05, rANOVA, Fig. 1d), indicating the increased activity of transposases and integrases, and thereby enhanced transposition rates of resistance gene cassettes. In addition, we found that MGEs were significantly correlated with resistance genes (P < 0.05, Table S5, Fig. 2a), and were associated with specific bacterial genera (Fig. 2b). This suggested that MGEs may play important roles in dissemination of the active ARGs (Gillings et al., 2015).

In addition, more MGEs were detected in the raw CFR-amended soil than treated CFR-amended soil (P < 0.05, rANOVA, Fig. 1c insert panel), indicating greater possibilities of HGT with raw CFR than treated CFR. In fact, certain concentration of antibiotics was introduced by the raw CFR addition (Fig. S6). Under the pressure of residual antibiotics, the potential for HGT mediated by MGEs could be enhanced with the increase of β-lactam resistance gene abundance. Zhu et al. (2013) have reported a high correlation (r^2 = 0.96) between ARG and transposase abundance at swine farms. In addition, the changes in

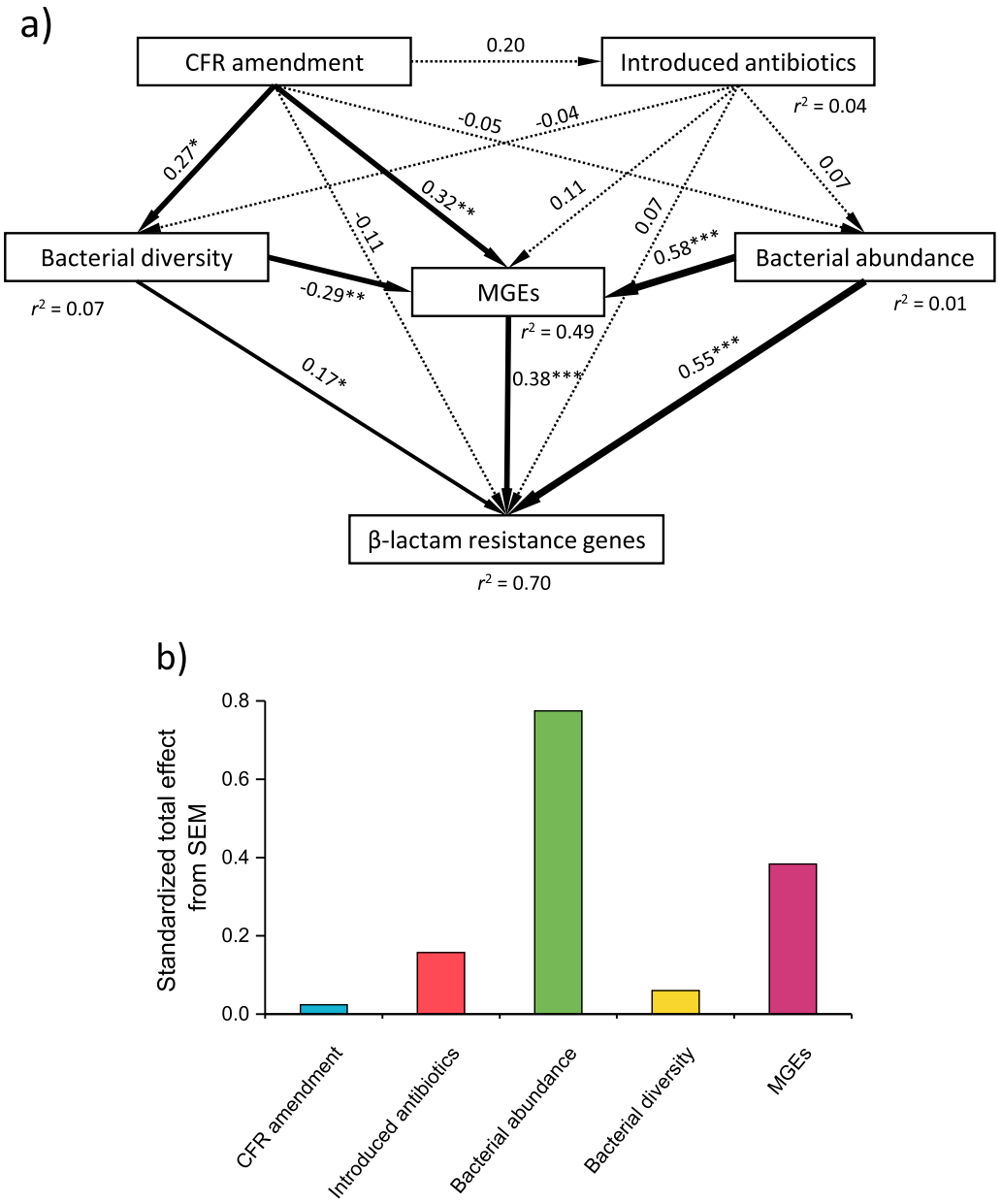

Fig. 4. Structural equation models showing the direct and indirect effects of CFR amendment, bacterial abundance, bacterial diversity, introduced antibiotics and mobile genetic elements (MGEs) on β-lactam resistance genes patterns in soil. Continuous and dashed arrows indicate significant and non-significant relationships, respectively. Numbers adjacent to arrows are path coefficients, and width of the arrows is proportional to the strength of path coefficients. r^2 values denote the proportion of variance explained for each variable. Significance levels are indicated: $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. The hypothetical models show the good fitness indicated by $\chi^2 = 4.64$, $P = 0.46$, GFI = 0.96 and RMSEA = 0.00.

bacterial community composition could potentially account for the difference of MGE abundance. For example, certain species within nine potential genera (Fig. 2b) might become competent in antibiotic-bearing environments (Charpentier et al., 2012), facilitating the occurrence of natural transformation (Thomas and Nielsen, 2005).

4.4. Linkages between bacterial community and antibiotic resistome

A previous study has found that soil resistomes were correlated with bacterial phylogenetic composition (Forsberg et al., 2014), prompting speculation that bacterial phylogeny is the driver in shaping the β-lactam resistance genes in CFR-amended soil. Our work supported this hypothesis. Network analysis has been widely used to explore the underlying interaction/association among microbial taxa/ARGs in complex microbial communities (Forsberg et al., 2014; Hu et al., 2017). In this study, network analysis revealed non-random co-occurrence patterns between β-lactam resistance genes and microbial genera, with greater presence of β-lactam resistance genes within the Bacteroidetes, Actinobacteria, Proteobacteria and Firmicutes phyla (Fig. 2b). In addition, changes in the soil bacterial community composition can favor the propagation of resistance genes in the CFR-amended soils, and this is in line with other findings in which the ARGs succession in the copper-contaminated soil was mainly driven by changes in bacterial community composition and MGEs (Hu et al., 2016). Bacteria differ in their susceptibility to inhibitory substances and toxicants (Piddock, 2006; Olivares Pacheco et al., 2013), implying that CFR amendment partially impacted the presence and abundance of β-lactam resistance genes in the soil environment.

A secondary dominant factor affecting soil resistance was the HGT mediated by MGEs, potentially shaping the β-lactam resistance genes profiles in the CFR-amended soils (Fig. 4b). MGEs could be the critical role in the adaptation of bacterial communities against soil environment interfered by CFR addition. These results supported the findings obtained from another recent study (Zhang et al., 2017), in which the relative abundance of ARGs had significantly positive correlations with integrase and transposase genes in manure and tylosin treated soils. Additionally, we also showed that the antibiotic-induced effects on bacteria were not strong (Fig. 4). The residual antibiotics (CPC and its main metabolite DCPC) contained in CFR have relatively low antibacterial activity (e.g., CPC has only about 0.1% of the activity of penicillin G against *Staphylococcus aureus* (Loder et al., 1961)), and they may be regarded as potential inhibitors or signaling compounds that can impact bacteria functions (Brandt et al., 2015; Abeles et al., 2016). However it has been proposed that antibiotics can increase the evolvability of bacterial populations which is related to the mutation and the gene transfer (Úbeda et al., 2005). Future work could test longer-term exposure to repeated applications of CFR to investigate its phylogenetic composition that will be helpful in determining the susceptibility of soil microbial communities to respond to selective pressure from antibiotics, and the impact on soil ARGs.

In conclusion, the laboratory-based evidence from this study confirmed the temporal succession of β-lactam resistance genes in both CFR amended soils. Raw and treated CFR amendments significantly enhanced the average relative abundance of β-lactam resistance genes by around 15 folds and 10 folds, respectively. MGEs abundance was also increased by a factor of two following the raw CFR addition. These results suggest that addition of antibiotics or their degradation products with raw CFR amendment creates a selective pressure for more antibiotic-resistant microorganisms. Moreover, the co-occurrence pattern and SEM model imply that bacterial phylogeny and MGEs are both drivers in shaping the β-lactam resistance genes in CFR-amended soil. These findings confirm that a multiple selection process is responsible for the increase in β-lactam resistance genes following the application of CFR amendments. Our work also highlights the necessity to consider that land application of CFR promotes antibiotic resistance in the soil environment, and consider the consequences and risks to public health of this proposed practice.

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Appendix A. Supplementary data

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