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Pharmaceutical exposure changed antibiotic resistance genes and bacterial communities in soil-surface- and overhead-irrigated greenhouse lettuce

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ABSTRACT

New classes of emerging contaminants such as pharmaceuticals, antibiotic resistant bacteria (ARB), and antibiotic resistance genes (ARGs) have received increasing attention due to rapid increases of their abundance in agroecosystems. As food consumption is a direct exposure pathway of pharmaceuticals, ARB, and ARGs to humans, it is important to understand changes of bacterial communities and ARG profiles in food crops produced with contaminated soils and waters. This study examined the level and type of ARGs and bacterial community composition in soil, and lettuce shoots and roots under soil-surface or overhead irrigation with pharmaceuticals-contaminated water, using high throughput qPCR and 16S rRNA amplicon sequencing techniques, respectively. In total 52 ARG subtypes were detected in the soil, lettuce shoot and root samples, with mobile genetic elements (MGEs), and macrolide-lincosamide-streptogramin B (MLSB) and multidrug resistance (MDR) genes as dominant types. The overall abundance and diversity of ARGs and bacteria associated with lettuce shoots under soil-surface irrigation were lower than those under overhead irrigation, indicating soil-surface irrigation may have lower risks of producing food crops with high abundance of ARGs. ARG profiles and bacterial communities were sensitive to pharmaceutical exposure, but no consistent patterns of changes were observed. MGE *intl1* was consistently more abundant with pharmaceutical exposure than in the absence of pharmaceuticals. Pharmaceutical exposure enriched Proteobacteria (specifically *Methylophilaceae*) and decreased bacterial alpha diversity. Finally, there were significant interplays among bacteria community, antibiotic concentrations, and ARG abundance possibly involving hotspots including *Sphingomonadaceae*, *Pirellulaceae*, and *Chitinophagaceae*, MGEs (*intl1* and *tnpA_1*) and MDR genes (*mexF* and *oprJ*).

1. Introduction

Consumption of fresh produce (fruits and vegetables) is important to human health, and national and international dietary guidelines call for more dietary intake of fresh produce (Slavin and Lloyd, 2012; WHO, 2019). For example, World Health Organization (WHO) recommends daily consumption of > 400 g of fresh produce to decrease risk of certain noncommunicable diseases and to improve overall health (WHO, 2019). As a result, global average vegetable supply increased from 66 kg per capita in 1979 to 102 kg per capita in 2000 with substantial regional variations (WHO, 2003). Actual vegetable consumption also varies significantly with region, age and gender groups of human populations, and in fact vegetable intake in the US has declined from 136 kg per person in 2003 to 123 kg per person in 2013 (Kearney, 2010; Lin and Morrison, 2016; WHO, 2003). To improve the dietary vegetable intake for human health benefits, it is critical to ensure microbial safety of vegetables as microbial contamination of vegetables often resulted in disease outbreaks and costly product recalls (Dewey-Mattia et al., 2018). Recently attention is being given to diverse microbiomes in vegetables (specifically opportunistic pathogens) rather than only to

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obligate pathogens (Berg et al., 2014a; Fogler et al., 2019; Leff and Fierer, 2013; Marti et al., 2013; Rahube et al., 2014; Wang et al., 2015), as it is believed that plant microbiomes could impact human gut microbiome and thus human health (Berg et al., 2014b; Wassermann et al., 2017).

It is important to assess the changes of vegetable microbiomes in extensively managed agricultural production settings and/or in stressed conditions due to water shortage and/or environmental contamination. Crop irrigation with reclaimed water (e.g., treated wastewater effluents and agricultural wastewater) has become increasingly popular for alleviating water shortage in many regions in the world (Hamilton et al., 2007). In fact, globally about 359,000 km^2 of croplands are irrigated with urban wastewater (Zhu et al., 2017a). Reclaimed water often contains trace level of pharmaceuticals (including antibiotics) (Oulton et al., 2010; Petrie et al., 2015), due to the extensive and imprudent use of pharmaceuticals in animal production and human healthcare (Michael et al., 2013; Sarmah et al., 2006). For example, the concentrations of sulfamethoxazole, caffeine, acetaminophen, carbamazepine, and trimethoprim were up to 22.0, 15.2, 11.7, 3.1, and 2.5 μg/L in wastewater effluents, respectively (Franklin et al., 2016; Oulton et al., 2010; Petrie et al., 2015). Thus, vegetables can be exposed to low levels of pharmaceuticals when irrigated with reclaimed water. As many pharmaceuticals are bioactive to microorganisms, it is important to examine possible changes in microbiomes and antibiotic resistance genes (ARGs) of vegetables resulted from pharmaceutical exposure via crop irrigation. Alarmingly, antibiotic resistant bacteria (ARB), including antibiotic resistant pathogens, have recently been isolated from vegetables produced in greenhouses, open fields, and household farms, even when no animal production was in their proximity (Araújo et al., 2017; Holvoet et al., 2013; Marti et al., 2013). Two studies confirmed that antibiotic resistant *Escherichia coli* isolates were more prevalent in vegetables (e.g., lettuce [*Lactuca sativa*]) than in soils and waters used for vegetable production (Araújo et al., 2017; Holvoet et al., 2013). If pathogens are resistant to antibiotics, or could acquire ARGs via horizontal gene transfer under selection pressure of antibiotics accumulated in vegetables, any associated food safety risks could be substantially greater.

Indeed, a number of studies have shown that exposure to pharmaceuticals and heavy metals in animal manures, wastewaters and biosolids could change ARGs and bacterial communities in soil and water environments (Han et al., 2016; Li et al., 2015; Peak et al., 2007; Wang et al., 2014; Zhu et al., 2013; Zhu et al., 2017b). In soils irrigated with reclaimed water the abundance of ARGs and mobile genetic elements (MGEs) could be increased by 99–8655 folds (Negreanu et al., 2012; Wang et al., 2014), and are influenced by levels of salinity, pharmaceutical residues, nutrients, and heavy metals, as well as varying wastewater treatment and soil characteristics (Gatica and Cytryn, 2013; Graham et al., 2011; Zhu et al., 2017b). Interestingly, it was reported that overhead sprinkler irrigation caused more persistent *E. coli* in harvested lettuce than soil-surface irrigation after washing with chlorine solution (Solomon et al., 2002). Thus, irrigation method may have profound impact on the microbiome and ARGs in lettuce, which has been rarely investigated.

Therefore, this study aimed to assess the impact of overhead and soil-surface irrigation on the diversity and abundance of microbiomes and ARGs in lettuce through a well-controlled greenhouse experiment, using 16S rRNA amplicon sequencing and high throughput qPCR, respectively. Lettuce was selected as a model vegetable crop because it is the most popular fresh vegetable consumed with minimal processing (Berg et al., 2014a). This study may help better utilize reclaimed water while minimizing food safety risks associated with microbial pathogens and ARGs.

2. Materials and methods

2.1. Lettuce growth experiment and sample collection

Lettuce growth experiment was previously described in detail (Bhalsod et al., 2018). Briefly, Burpee® Black Seeded Simpson Lettuce (Burpee, Warminster, PA) were grown for 5 weeks in nursery pots (14.6-cm top diameter and 10.8-cm high) each packed with a loamy sand soil to a depth of 9 cm. The loamy sand soil had pH of 7.4, organic matter of 2.5%, 81.3% sand, 10.5% silt, 8.2% clay, 71 mg/kg Bray P1 extractable phosphorus, and 7.0 meq/100 g cation exchange capacity. The soil did not contain any pharmaceutical tested in this study. The lettuce plants were irrigated daily with fertilizer solution in the absence or presence of 8 antibiotics (carbadox, lincomycin, monensin sodium, oxytetracycline, sulfadiazine, sulfamethoxazole, trimethoprim, and tylosin) and 3 other pharmaceuticals (acetaminophen, caffeine, and carbamazepine) at 30 μg/L each. The selected 11 pharmaceuticals are widely used in human medicine and/or animal production, and vary in physiochemical properties such as molecular weight, charge speciation (pKa), water solubility, and hydrophobicity, which were described in detail by Bhalsod et al. (2018). Three non-antibiotic drugs were selected because pharmaceuticals were often present as mixture in waters. As explained in Bhalsod et al. (2018), the concentration of each pharmaceutical (30 μg/L) was at the high end of typical pharmaceutical concentrations in reclaimed water (Oulton et al., 2010; Petrie et al., 2015), and was selected because it allowed for the detection of pharmaceutical residues in lettuce.

Irrigation water was applied via either overhead irrigation or soil-surface irrigation. Samples of lettuce shoots, roots, and soils were collected weekly, as detailed in Bhalsod et al. (2018). Lettuce shoot and root samples were washed with deionized (DI) water to remove pharmaceuticals and bacteria loosely associated with lettuce shoots and roots, and to remove soil particles from lettuce roots. The concentrations of each pharmaceutical were measured in lettuce shoot, root and soil samples, which were already published in Bhalsod et al. (2018). Therefore, this study focused on the analyses of microbiomes and ARGs for the lettuce shoot, root and soil samples collected on the final week 5, which represented cumulative impact of pharmaceutical exposure over 5 weeks. It is noted that the sample collection procedure could not separate bacteria on the shoot and root surfaces from those within the shoots and roots (i.e., endophytes). Thus, it should be understood that the microbiomes and ARGs measured in this study were shoot- and root-associated, including those on the shoot and root surfaces and inside the shoots and roots.

Prior to DNA extraction, all lettuce shoots and root samples were stored in a −20 °C freezer (Northland, Greenville, MI), and soil samples were air-dried and stored at room temperature. Each pharmaceutical exposure treatment had triplicate samples named as 1, 2, and 3 following the abbreviation of sample names (Supplementary Table S1). No replication was included for the pharmaceuticals-free control treatment. SO, SS, SOC, and SSC refer to lettuce shoot samples under overhead irrigation with pharmaceuticals (SO), soil-surface irrigation with pharmaceuticals (SS), overhead irrigation without pharmaceuticals (i.e., control, SOC), and soil-surface irrigation without pharmaceuticals (i.e., control, SSC), respectively. RO, RS, and ROC denote lettuce root samples under overhead irrigation with pharmaceuticals (RO), soil-surface irrigation with pharmaceuticals (RS), overhead irrigation without pharmaceuticals (i.e., control, ROC), respectively. The root sample under the control treatment of soil-surface irrigation was exhausted in the earlier work (Bhalsod et al., 2018) and thus not available in this study. Soil samples under soil-surface irrigation were similarly named as STS, SMS, SBS, STSC, SMSC, and SBSC, referring the top (0–3 cm), middle (3–6 cm), and bottom (6–9 cm) layers with and without pharmaceuticals, respectively (Supplementary Table S1). The majority of irrigation water under overhead irrigation eventually drained to soils. No differences in pharmaceutical concentrations in

lettuce roots and soils were found between overhead and soil-surface irrigations (Bhalsod et al., 2018). Thus, the soil samples under overhead irrigation was not selected because no major difference was expected from that of surface-irrigated soil samples.

2.2. DNA extraction and analyses

Lettuce shoot and root samples were thawed and placed in the tared PowerBead tubes, weighed, and extracted for DNA following the manufacturer's instruction using PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA). Absolute DNA concentration was measured using Qubit® dsDNA BR Assay Kit (Life Technologies, Eugene, OR). All DNA samples were stored in −20 °C freezer before high throughput quantitative polymerase chain reaction (qPCR) analysis and 16S rRNA amplicon sequencing. WaferGen SmartChip Real-time PCR System (WaferGen Bio-systems, Fremont, CA) was used to quantify ARGs and MGEs in the lettuce and soil samples. The system has 5184 individual SmartChips nanowells that provide high throughput reactors for multiple primers. We first tested SS, RS, STS, and STSC with 384 primers targeting 382 ARGs and MGEs and two 16S rRNA genes. This preliminary test detected genes targeted by 178 primer sets. For the same genes targeted by multiple primer sets, the primer sets producing the lower cycle number (C_T) were selected. As a result, 144 primer sets were chosen for further analyses, including 2 primer sets for 16S rRNA and142 primer sets for ARGs and MGEs (Supplementary Table S2). The initial enzyme was activated at 95 °C for 10 min. The DNA samples were then amplified by 30 s denaturation at 95 °C and 30 s annealing at 60 °C for 40 cycles. All qPCR runs were conducted in triplicates in the WaferGen system.

The amplicon sequencing of 16S rRNA were conducted for all DNA samples from the 27 lettuce and soil samples (Supplementary Table S1). DNA samples were first amplified with cycling conditions as follows: 95 °C for 2 min, 95 °C for 20 s (30 cycles), 55 °C for 15 s, 72 °C for 1 min, and 72 °C for 10 min. PCR products were then purified, followed by normalization with the SequalPrep Normalization Plate Kit (Invitrogen, Carlsbad, CA). Sample library was prepared by pooling 5 μL of each sample. Illumina dual-indexed compatible primers 515f/806r were used to amplify the V4 hypervariable region of 16S rRNA gene to minimize cost of long customized primers and produce more high quality sequences (Kozich et al., 2013). Batch normalization of amplicon libraries were performed using Invitrogen SequalPrep DNA Normalization Plates. Products eluted from the plates were then pooled, followed by quality control and quantification using Qubit dsDNA HS, Caliper LabChipGX HS DNA, and Kapa Illumina Library Quantification qPCR assays. The amplicon pool was then loaded onto an Illumina MiSeq v2 standard flow cell and sequenced in a 2 × 250 bp paired-end format using a v2 500 cycle MiSeq reagent cartridge. Primers complementary to the 515f/806r sequences were added to appropriate wells of the reagent cartridge to serve as sequencing and index primers (Kozich et al., 2013). Base calling was performed by Illumina Real Time Analysis (RTA) v1.18.54 and RTA output was demultiplexed and converted to the format of FastQ with Illumina Bcl2fastq v2.19.0.

2.3. Data analyses

Cycle numbers (C_T) measured by the WaferGen qPCR were used to calculate copy number of genes via Copy Number $= 10^{(30-C_T)/(10/3)}$ (Stedtfeld et al., 2008), using the cutoff threshold of $C_T < 30$. Relative abundance of detected genes was computed by dividing the estimated gene copy number with the gene copy number of 16S rRNA. Then patterns and characteristics in profiles of ARGs and MGEs of all the samples were analyzed by heatmap, chord diagram, and ordination analysis using R packages. Bacteria community analysis was first pre-processed using the MacQIIME pipelines v. 1.9. following online tutorial for operational taxonomic unit (OTU) picking based on a 97% similarity threshold with default uclust to cluster to Greengenes reference database (Caporaso et al., 2010; DeSantis et al., 2006). Bacteria belonging to mitochondria and chloroplast were removed because they are from contamination by small subunit ribosomal RNA genes of plant organelles (mitochondria and chloroplast). Top 10 phyla and families were selected to plot the composition of bacterial communities, and top 10 phyla were selected for principal coordinates analysis (PCoA). Alpha diversity was calculated by the Chao1 estimator (Chao, 1984; Hughes et al., 2001). Finally, network analysis among antibiotics concentrations, ARGs/MGEs relative abundance, and percentages of family-level bacterial communities were conducted based on correlation tests (correlation coefficient > 0.6 or < -0.6 and p-value < 0.05) and plotted using Gephi v0.9.1 software. Detailed data analysis procedures are provided in Supplementary Material.

3. Results and discussion

3.1. Profiles of ARGs

In total 53 subtypes of ARGs and MGEs were detected with the greatest detection in the pharmaceutical-free top soil (STSC) and the lowest detection in the surface-irrgated lettuce shoots (SS), as shown in Supplementary Fig. S1. Overhead-irrigated lettuce shoots had a greater number of ARGs and MGEs than the soil-surface-irrigated shoots in the presence and absence of pharmaceuticals. Interestingly, the number of ARGs and MGEs was greater in the pharmaceuticals-free lettuce and soil samples than in the samples exposed to pharmaceuticals except for the bottom soil (Supplementary Fig. S1). Close examination of Fig. S1 and Fig. 1 revealed that only four MGEs were found in all soil samples (6 samples in total) (*tnpA_1*, *intl1*, *ISSps*, and *repA*), and only one ARG (*mexF*) and one MGE (*ISPps*) were found in all lettuce shoot samples (4 samples in total), likely suggesting different ARG/MGE profiles in various soil and shoot samples. However, the greater number of shared ARGs and MGEs between root samples (*rarD*, *tnpA_1*, *mexF*, *oleC*, *merA*, *intl1*, *ISPps*, and *ISSm2*) might be due to root defense mechanisms to external changes (Raaijmakers et al., 2009; van Loon et al., 1998). Four ARGs/MGEs were found in all soil, lettuce shoot and root samples (*tnpA_1*, *merA*, *intl1*, and *ISPps*). Lettuce roots and shoots shared multidrug resistance (MDR) gene (*mexF*), whereas lettuce root and soil samples shared *oleC* and *ISSm2* gene (Fig. 1).

To have a clearer picture about frequently detected ARGs and MGEs, we removed the genes detected in less than half of our samples to produce a condensed heatmap (Supplementary Fig. S2). The genes in Fig. S2 included 5 MGEs (*ISPps*, *ISSm2*, *intl1*, *repA*, and *tnpA1*), 3 MDR genes (*oprJ*, *mexE*, and *mexF*), 2 beta-lactam resistance genes (*blaPDC* and *blaFOX*), 1 macrolide-lincosamide-streptogramin B (MLSB) resistance gene (*oleC*), and 1 mercury resistance gene (merA), suggesting the high prevalence of these genes in these settings. MLSB, beta lactam, amphenicol, and aminoglycoside are widely used in veterinary or human medicine, which corroborated their prevalence in this study. Specifically, as beta-lactam rings are often found in many antibiotics, this resistance mechanism can be troublesome when developing alternative drugs to replace ineffective ones (Demain and Elander, 1999). The high prevalence of MGEs and MDR genes are also alarming. Class 1 integron (*intl1*) gene was detected in all samples (except for SSC) (Fig. 1). Its abundance was increased in the bottom soil, top soil, lettuce root and overhead-irrigated lettuce shoot with pharmaceutical exposure. Class 1 integrons are one of the five classes of mobile integrons (part of MGEs) that facilitate resistance to multiple antibiotics and are significantly correlated with anthropogenic activities (Gillings et al., 2015; Ma et al., 2017; Mazel, 2006; Zhu et al., 2013; Zhu et al., 2017b). Its high prevalence may result from its genetic function that integrate exogenous gene sequences into functional genes using integron gene (*intI*), recombination site (*attI*), and an outward-orientated promotor (Mazel, 2006). Our observation supported the proposed strategy to use *intl1* as an indicator gene for the ARG surveillance (Chen et al., 2019; Gillings et al., 2015; Zhu et al., 2017b).

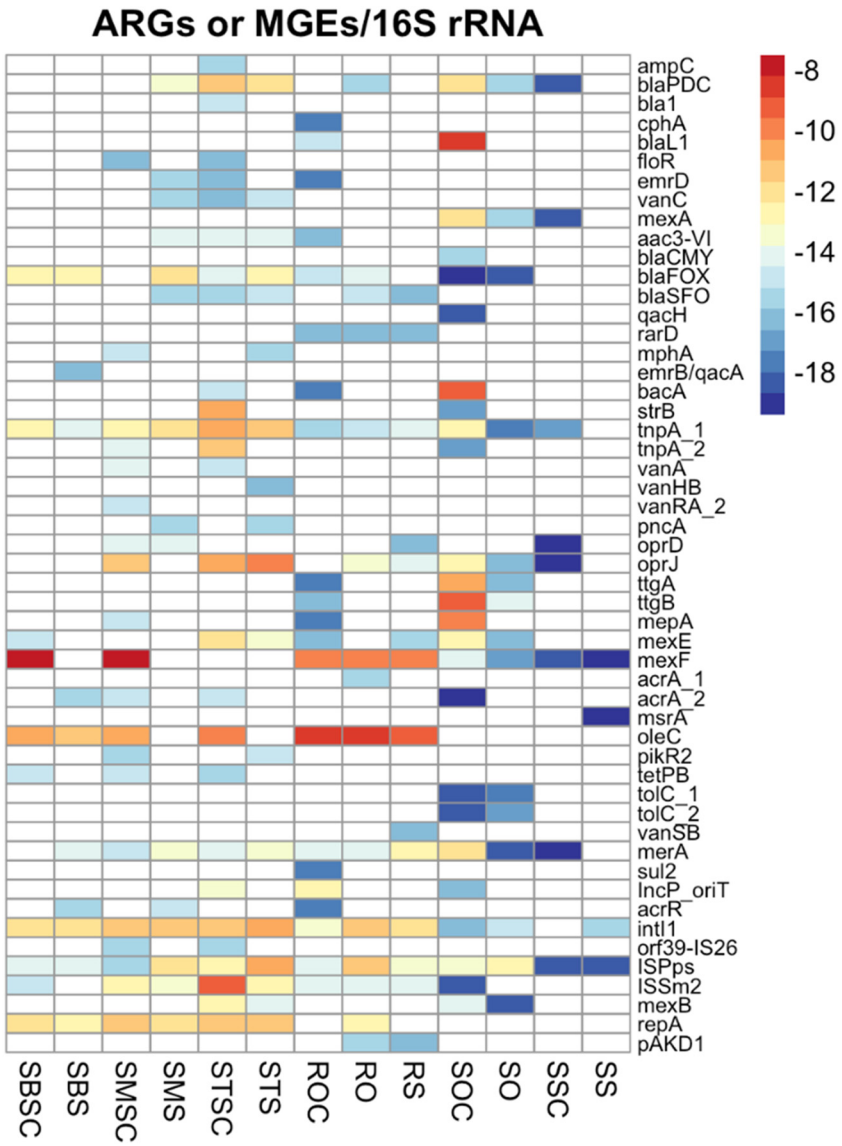

Fig. 1. Relative abundance (gene copy number/16 s rRNA gene copy number) of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs). Data were Log 2 transformed. Blank cells represent genes that were either not detected or below detection limit. Color bar on the right means relative abundance from low (blue) to high (red) levels. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

As shown in Fig. 1, the ARGs/MGEs in the soils and lettuce roots were more abundant than in the lettuce shoots. Pharmaceutical exposure altered the profiles of ARGs and MGEs in the soil, lettuce root and shoot samples (Fig. 1 and Supplementary Fig. S3). With pharmaceutical exposure, the relative abundance of some genes increased, whereas other genes decreased (Fig. 1). Among the most detected genes (Supplemental Fig. S2) no consistent patterns of changes in ARGs and MGEs with pharmaceutical exposure were found. For example, in soils receiving pharmaceuticals *ISSm2* and *oleC* decreased, *merA* increased, and no consistent trend was found for other genes. This observation deviated from previous field studies reporting increased relative abundance of ARGs and MGEs in environmental samples under the influence of antibiotics residues (Wang et al., 2014; Zhu et al., 2013; Zhu et al., 2017b). Thus, it appears that pharmaceutical exposure did not always increase the abundance of any given ARG/MGE subtype in this short-term (35 days) study in the greenhouse. Instead, the applied antibiotics may inhibit the growth of some susceptible bacteria harboring non-targeted or non-functional ARGs and MGEs, thus decreasing their abundance. On average, MGEs (*intl1* and *ISPps*) were more abundant in samples with pharmaceutical exposure. Lettuce shoots with pharmaceutical-free overhead irrigation (SOC) had several highly enriched genes (i.e., *bacA*, *blaL1*, *ttgB*, and *mepA*) that may originate from bacteria in dusts. Since these genes were not detected in the surface-irrigated lettuce shoots (SSC and SS), they may come from bacteria non-native to lettuce shoots. In addition, pharmaceutical exposure resulted in greater abundance of ARGs (*blaPDC*, *mexA*, *mexB*, *mexE*, *mexF*, *tnpA_1*, *ttgA*, *oprJ*, *tolC_1*, and *tolC_2*) and MGEs (*intl1*, *ISPps*) associated with lettuce shoots under overhead irrigation than under soil-surface irrigation, likely due to greater availability of pharmaceuticals, water and nutrients for bacteria associated with overhead irrigated shoots. We did not expect the difference in the root uptake and translocation of pharmaceuticals and resultant effect on ARGs and bacterial communities between overhead and soil-surface irrigations, as the pharmaceutical concentrations in soils and roots were similar under these two irrigation practices (Bhalsod et al., 2018). For the same reason, the difference of ARGs between overhead-irrigated and soil-surface irrigated lettuce shoots was unlikely caused by the changes of ARGs and bacteria occurring in soils and roots. The abundance and diversity of ARGs and MGEs visually appeared to decrease with increasing soil depth (Fig. 1), which may be due to greater nutrient

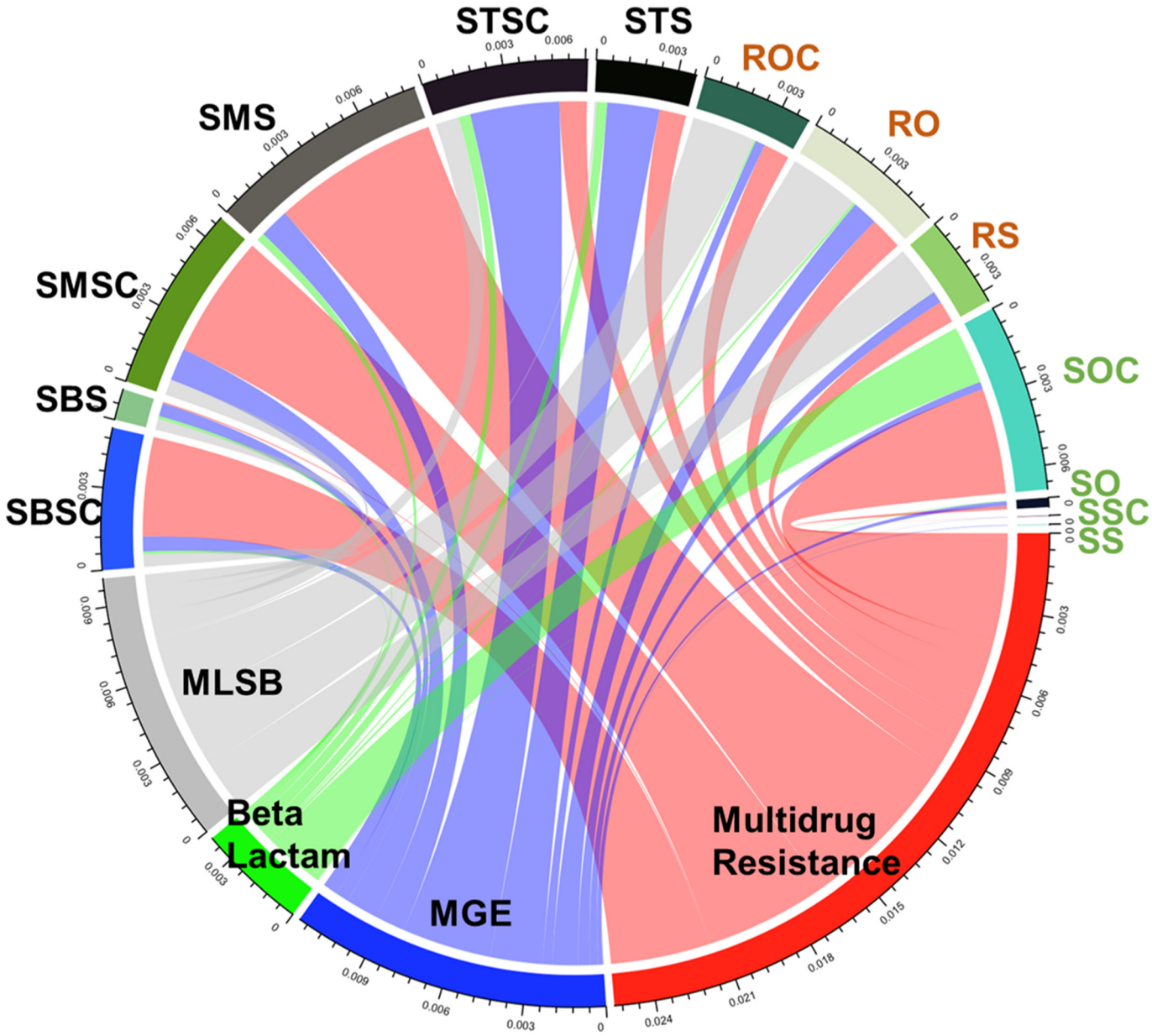

Fig. 2. Total relative abundance (gene copy number /16s rRNA gene copy number) of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) among various categories. Top four categories include MLSB, Beta Lactam, MGEs, and Multidrug Resistance. The width of the circular bar represents the total abundance of ARGs and MGEs in each sample.

concentrations and thus bacterial growth and activity in the top soils (Sun et al., 2016; Zhu et al., 2013).

As shown in Fig. 2, MGEs, and MLSB, and MDR genes were dominant resistance types. However, there was no consistent patterns of changes in each of the resistance mechanisms in response to pharmaceutical exposure, suggesting again that pharmaceutical exposure did not lead to a cross-board increase in the abundance of ARGs and MGEs among all the lettuce and soil samples. It is worth to note that the lettuce shoots had less overall abundance of ARGs and MGEs than the lettuce root and soil samples. Most of the soil and lettuce root samples appeared to have greater total abundance of MGEs, and MLSB-resistant, beta-lactam-resistant and MDR genes than the lettuce shoot samples. Similar to the diversity of AGRs/MGEs, the overhead-irrigated lettuce shoot (SOC and SO) had much more abundance of MDR and beta-lactam resistant genes than the soil-surface-irrigated lettuce shoots (SSC and SS). Thus, soil-surface irrigation with reclaimed water may help decrease the diversity and abundance of ARGs in lettuce.

3.2. Soil and lettuce microbiomes

On both bacterial phylum and family levels pharmaceutical exposure changed bacterial community structures (Fig. 3). Soil samples had the highest bacterial community diversity, followed by the lettuce root and shoot samples. Proteobacteria was the most abundant bacterial phyla in all samples, followed by Actinobacteria, Bacteroidetes, Acidobacteria, and Firmicutes (Fig. 3A). Proteobacteria were slightly increased in proportion with pharmaceutical exposure (Fig. 3A). This is interesting as some strains in Proteobacteria can actually grow on various antibiotics and confer various resistance mechanisms (Allen et al., 2010). Moreover, Proteobacteria carry many mobile integrons and integron genes even in very ancient times (Davies and Davies, 2010; Mazel, 2006; Rowe-Magnus et al., 2001). As a result, Proteobacteria were also found to be the most mobile phylum associated with the transfer of ARGs and MGEs, followed by Firmicutes, Bacteroidetes, and Actinobacteria (Hu et al., 2016). Actinobacteria are known for

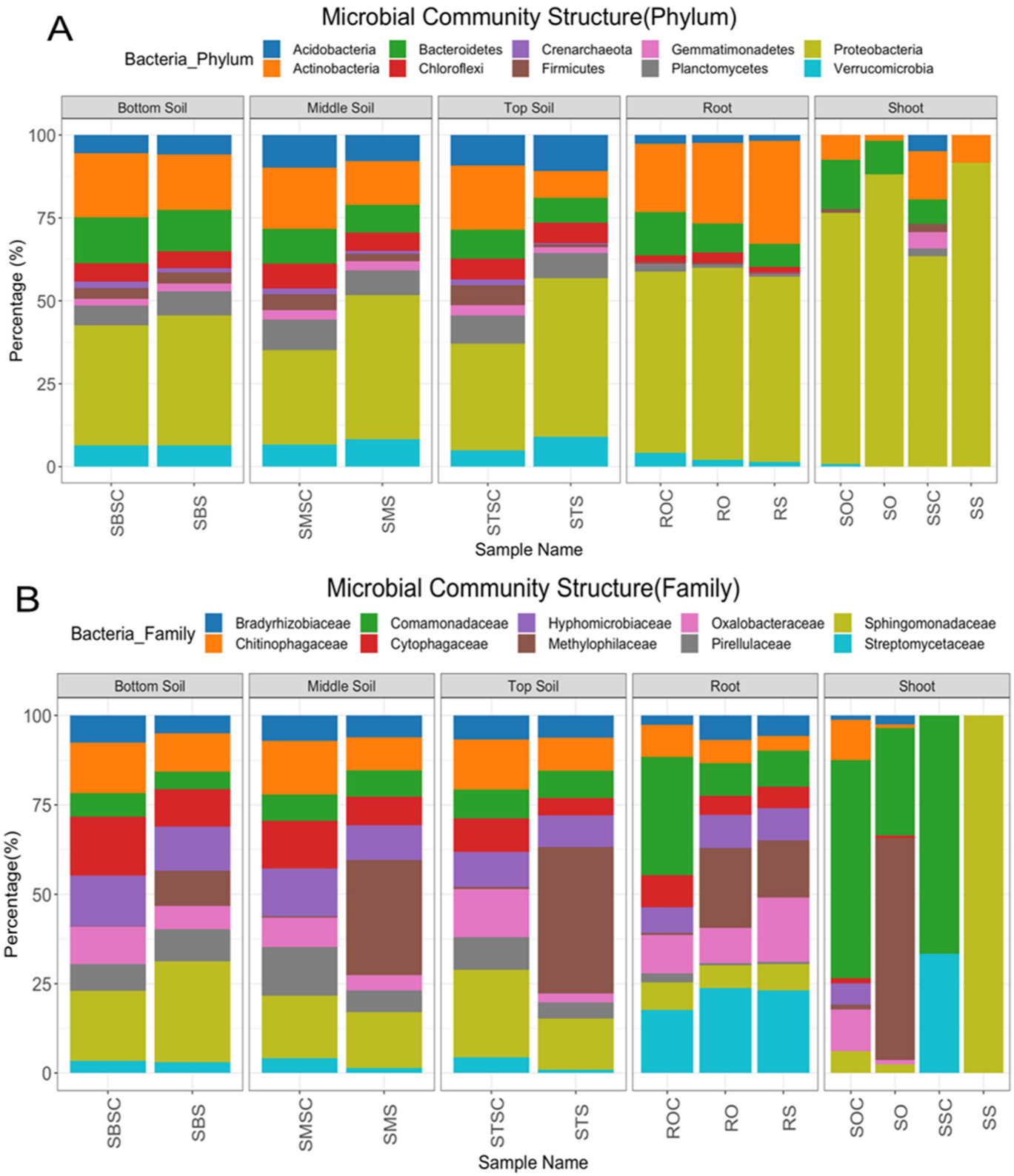

Fig. 3. Bacterial community composition on the phylum (A) and family (B) levels. Total percentages of the total 10 phyla or families were 100%. Each bar represents the fraction of each bacteria phylum and family.

harboring ARGs through their ability to synthesize various secondary metabolites (D'Costa et al., 2011). They are ubiquitous in soil and plant environments and are often associated with root symbiosis. Bacteroidetes were also abundant and present in all samples (except for SS), but Firmicutes had a very small fraction in the lettuce roots. It is interesting to observe that with pharmaceutical exposure Proteobacteria increased but Bacteroidetes decreased in proportions. This observation was in agreement with studies on human gut microbiome where antibiotic treatment increased the percentage of Proteobacteria from < 1% to 71%, but decreased the percentage of Bacteroidetes

(Antonopoulos et al., 2009; Sommer et al., 2009). It is worth to mention that Bacteroidetes, Firmicutes, and Beta Proteobacteria were found to be highly correlated with some antibiotic resistant strains such as ciprofloxacin-resistant heterotrophs, ciprofloxacin-resistant enterococci, and sulfamethoxazole-resistant enterobacteria (Novo et al., 2013).

On the family level, *Methylophilaceae* was not observed in the pharmaceuticals-free samples, but became abundant with pharmaceutical exposure (Fig. 3B). *Methylophilaceae* is a family in the order of *Methylophilales* in Proteobacteria and may contribute to the increase of Proteobacteria with pharmaceutical exposure shown in Fig. 3A. Species of *Methylophilaceae* have been isolated or sequenced from various ecological niches including aquaculture, wastewater, activated sludge, soil, rhizosphere, and phyllosphere (Colombo et al., 2016; Doronina et al., 2014; Fogler et al., 2019; Hultman et al., 2018; Kalyuzhnaya et al., 2006). However, no *Methylophilaceae* bacteria were found to cause opportunistic infections in humans and animals (Doronina et al., 2014). It is unknown why *Methylophilaceae* was increased as a result of pharmaceutical exposure, which should be further investigated. One possible explanation may be related to biodegradation properties of bacteria in the order of *Methylophilales* (Liu et al., 2015). Conversely, with pharmaceutical exposure *Chitinophagaceae* (part of Bacteroidetes) was slightly decreased in the soil and lettuce root samples, but was sharply decreased in the overhead-irrigated lettuce shoots. There was also a slight decrease in *Pirellulaceae* (part of Planctomycetes) in the middle and bottom soil layers. In fact, six of the top ten bacteria families belong to Proteobacteria (i.e., *Bradyrhizobiaceae*, *Comamonadaceae*, *Hyphomicrobiaceae*, *Oxalobacteraceae*, *Sphingomonadaceae*, and *Methylophilaceae*). The richness of Proteobacteria may explain some of the interplays in the ARGs and MGEs, because MGEs are transferrable between bacteria in the same phyla and cross-phyla transfer are often difficult (Hu et al., 2016).

The bacterial beta diversity analysis showed the ordination position of top ten phyla (Fig. 4A). Compared with the other methods (Fig. 4A and Supplementary Fig. S4), the PCoA analysis is a simpler ordination method that can separate groups with less underlying assumptions (Ramette, 2007). The top left panel is the sample panel showing each sample's position of ordination, with the soil samples densely clustered on the left, the lettuce root samples in the middle, and the lettuce shoot samples at the right. The following ten panels showed the leading bacteria phylum that caused the ordination difference (Fig. 4A). All top 10 phyla were present in soil samples, whereas Crenarchaeota, Gemmatimonadetes, and Firmicutes were not present in the lettuce roots. In the lettuce shoots, Crenarchaeota, Gemmatimonadetes and Planctomycetes were not present. As the dominant bacteria of Verrucomicrobia were discovered in soils, fresh water, and marine water, no contact of lettuce shoots with irrigation water in the soil-surface irrigation treatment may have resulted in the absence of Verrucomicrobia (Lee et al., 2009). Finally, pharmaceutical exposure decreased bacterial alpha diversity with each sample measured by the Chao 1 diversity index (Fig. 4B). The Chao1 diversity index is useful when the dataset is more skewed toward the low-abundance species, especially species only captured once (singleton) or twice (doubleton) (Chao, 1984; Hughes et al., 2001). Pharmaceutical exposure may suppress some low-abundance susceptible bacteria in the community to an undetectable level, resulting in decreases in overall species richness and evenness. This decreased bacterial diversity may have a negative impact on the ability of native microbiomes to defend against the invasion of non-native pathogens and thus extend the survival of pathogens in soils and lettuce (Berg et al., 2014a; van Elsas et al., 2012; Yao et al., 2014). It is interesting to note that bacterial alpha diversity of lettuce shoots was much greater for overhead irrigation than for soil-surface irrigation (Fig. 4C), again likely due to greater bacterial growth and activity resulted from greater water and nutrient availability in overhead-irrigated lettuce shoots.

3.3. Interplays among antibiotics concentrations, ARGs/MGEs relative abundance, and bacterial families

The network analysis (Fig. 5) was performed based on correlation tests as described in Supplementary Material and the correlation results are summarized in Supplementary Table S4. The MGEs (*intI1*, *ISSm2*, *ISPps*, *repA*, and *tnpA_1*) were clustered together and linked with tylosin, sulfadiazine, sulfamethoxazole, and total antibiotic concentrations. It was suggested that increased concentrations of mixed antibiotics, even at the sub-inhibitory level, caused an increase in ARGs and MGEs in waters and soils (Berglund, 2015; Zhu et al., 2013). Interestingly, the insertion sequence genes (*ISPps*) were positively correlated with tylosin, sulfadiazine, sulfamethoxazole, and total antibiotics. Insertion sequences are class of MGEs that are incorporated into transmissible plasmids and promote horizontal gene transfer. Clearly, pharmaceutical exposure promoted the abundance of MGEs. More interestingly, MGEs (*intI1*, *repA*, or *tnpA_1*) were positively correlated with MDR genes (*mexF*, *oprJ*, or *mexE*) (Fig. 5), indicating that MGEs may facilitate the proliferation of MDR genes. Future study is needed to reveal molecular mechanisms on the connection of MGEs and MDR genes. *OprJ* was positively correlated with certain antibiotics (sulfadiazine and sulfamethoxazole), bacterial families (*Sphingomonadaceae*, *Pirellulaceae*, and *Chitinophagace*ae), and MGEs (*tnpA_1* and *repA*). This observation is a clear example of close interactions among MDR genes, bacteria, antibiotic stress, and MGEs.

Indeed, *Pirellulaceae*, *Chitinophagace*ae, and *mexF* may be hotspots for bacteria community interactions and ARG exchanges. *Pirellulaceae* in Planctomycetes had been found in soils, plant roots, and lake sediments (Hermans et al., 2017; Signori et al., 2014; Weigel and Erwin, 2017). One species (*Rhodopirellula baltica* SH1) in Planctomycetes was found to harbor integrons (Mazel, 2006). However, previous studies have not emphasized the importance of *Pirellulaceae* in ARGs and MGEs exchanges, and its possible relationship with MDR genes. *Chitinophagace*ae were found to be positively correlated with three MDR genes and one MGE gene (Fig. 5). Recently Liu et al. (2018) also found a positive correlation between *Chitinophagace*ae and MDR genes. Mechanistic studies on *Chitinophagace* association with MDR genes are needed in the future.

The network analysis revealed that the positive impact of antibiotics are mainly acted on the gene level instead of the bacterial family level, which was expected as genes may be more sensitive to external stress than the whole bacterial populations. Oxytetracycline and trimethoprim were primarily negatively correlated with ARGs (*mexF*, *oleC*, and *oprJ*), MGEs (*tnpA_1*) and bacterial families (Fig. 5). These two antibiotics classes are commonly used in veterinary medicine and have been detected in wastewater effluents (Dodgen and Zheng, 2016; Li et al., 2008; Liu et al., 2012). *Methylophilaceae* was positively related to tylosin, and total antibiotics concentration. The abundance of this family may increase MGEs (*ISPps*). Also, it was found that tylosin consistently increased the abundance of ARGs in manured soils (Zhang et al., 2017). Although *Methylophilaceae* family is not well known for their pathogenicity and antimicrobial resistance in current clinical and environmental samples, future studies are needed to investigate why *Methylophilaceae* are sensitive to antibiotics, and the potential risks associated with the increased abundance of MGEs (*ISPps*).

Finally, the results of this study have several important implications to food safety of vegetables and human health. MDR genes (*OprJ*, *mexE*, *mexF*) detected in the soil and lettuce samples are found in *Pseudomonas aeruginosa* through NCBI whole genome sequencing database (NCBI Blast). *Pseudomonas aeruginosa* is an opportunistic pathogen that belongs to Proteobacteria. Indeed, the family of *Pseudomonadaceae* ranked in top 20 detected bacterial families, and *Pseudomonas* was the most detected genus (Supplemental Table S3). Although the genus was not specified through the match with Greengenes database, Proteobacteria did have a greater chance of horizontal gene transfer and more potential risks for the spread of antibiotic resistance (Hu et al., 2016).

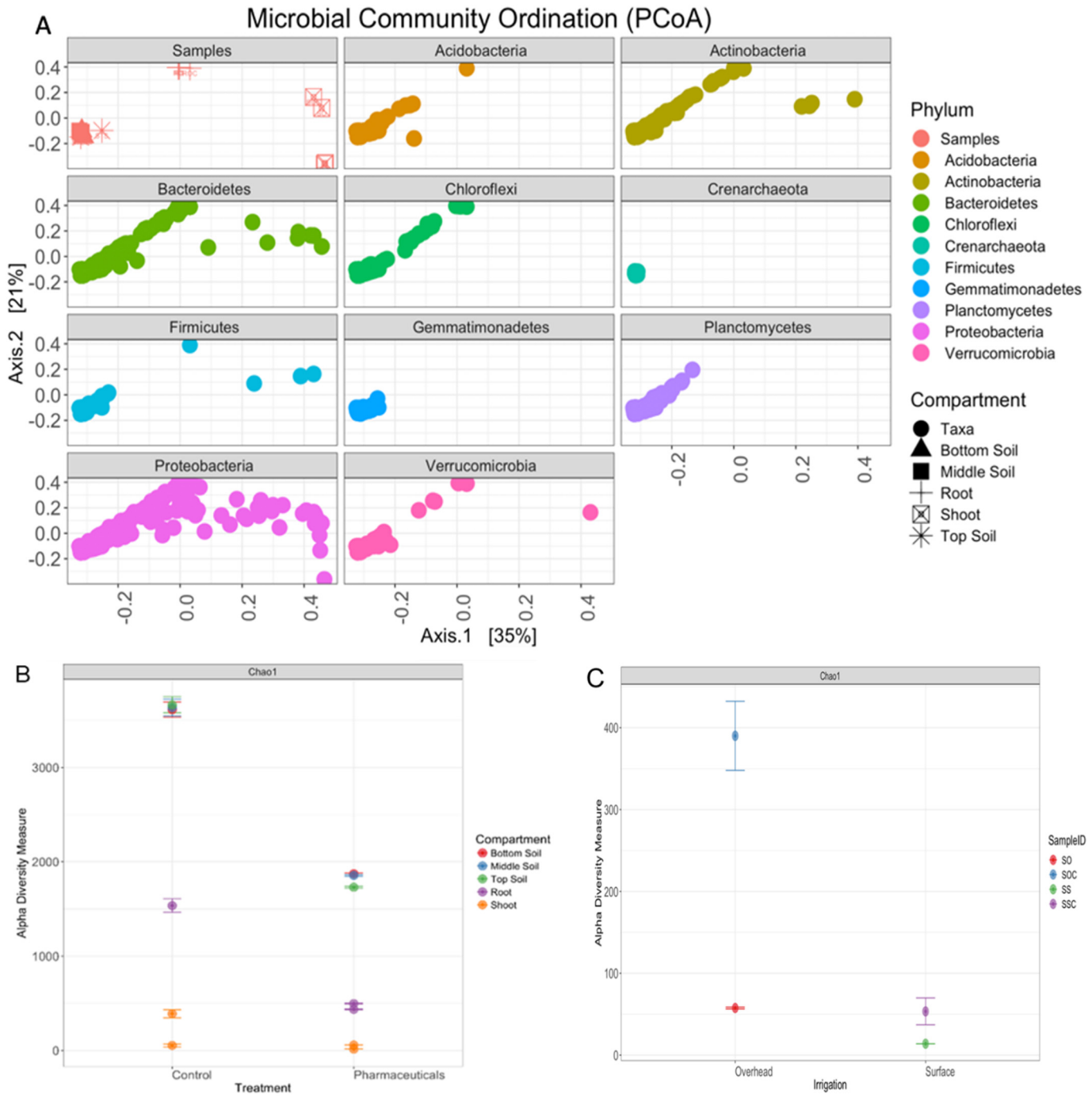

Fig. 4. Bacterial community alpha diversity based on species level and beta diversity based on phylum level. A. Beta diversity biplot based on principal coordinates analysis (PCoA). Sample panel shows the ordination position of sample type, and the remaining ten panels showed the ordination position of each bacterial phylum. *B. alpha* diversity plot based on Chao1 estimator. Error bars represent the 95% confidence interval. C. Bacterial alpha diversity of lettuce shoots based on Chao1 estimator. Error bars represent the 95% confidence interval.

MLSB-resistant gene *OleC* was reported to be isolated from *Streptomyces antibioticus* and confers resistance to Oleandomycin because *Streptomyces antibioticus* prodcues natural antibiotics (Rodriguez et al., 1993). We found that all genus of *Streptomycetaceae* is *Streptomyces* that is often found in plant roots even after disinfection, suggesting the internalization of bacteria into roots (Cao et al., 2004; Coombs and Franco, 2003; Sardi et al., 1992). *OleC* was especially abundant in lettuce roots (Fig. 1), which may be linked to the abundance of the rhizosphere *Streptomyces* (Fig. 5), supported by the high fraction of *Streptomycetaceae* in the root samples (Fig. 3B). This observation is interesting not only from the human heath perspective, but also for the plant protection (Berg et al., 2014b), as *Streptomyces* have been proposed as effective biological control agents against pathogen infection of plant roots (Ezziyyani et al., 2007; Xiao et al., 2002). In fact, understanding true implications of ARGs and microbiomes to food safety and human health is very challenging as the framework for food safety and human health risk assessment of ARGs and microbiomes has not been well established. Large-scale public health and epidemiological studies are needed to advance this important research direction.

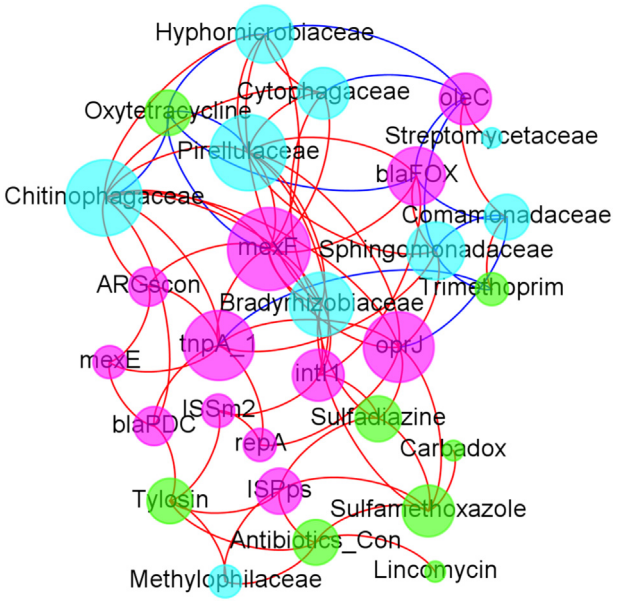

Fig. 5. Network analysis diagram of the correlations between ARGs, antibiotic concentrations and bacterial families for the lettuce shoot, root and soil samples with pharmaceutical exposure. Green nodes represent the concentrations of antibiotics, blue nodes represent bacterial families, and pink nodes represent ARGs and MGEs. Red lines indicate positive correlations (Correlation coefficient > 0.6, $p < 0.05$). Blue lines indicate negative correlations (Correlation coefficient < -0.6, $p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

This study addressed the shifts in lettuce ARGs and microbiomes influenced by pharmaceuticals-containing irrigation water via overhead or soil-surface irrigation. Overhead irrigation resulted in a greater abundance and diversity of ARGs/MGEs and bacteria in lettuce shoots than soil-surface irrigation, regardless pharmaceutical exposure, suggesting that soil-surface irrigation has lower risks of producing food crops enriched with ARB and ARGs and thus could be adopted for crop irrigation with reclaimed water. MGEs (*ISPps*, *ISSm2*, *intl1*, *repA*, and *tnpA_1*) and MDR genes (*oprJ*, *mexE*, and *mexF*) were most frequently detected genes with high abundance. Pharmaceutical exposure to soils and lettuce did not result in consistent patterns of change with regard to the abundance of ARGs/MGEs. Class1 integrons (*intl1*) gene mostly increased with pharmaceutical exposure, demonstrating that anthropogenic activities can enrich the abundance of this classic mobile integron. Proteobacteria was the most abundant bacterial phyla and its abundance increased with pharmaceutical exposure. A clear increase in the abundance of *Methylophilaceae* (a family of Proteobacteria) was observed with pharmaceutical exposure (specifically the exposure to tylosin), suggesting that more studies are needed to explore if *Methylophilaceae* could be used to monitor the impact of pharmaceutical exposures to soil and plant microbiomes. Finally, network analysis revealed that MGEs (*mexF*, *intl1*, and *tnpA_1*) and MDR gene *oprJ* are possible hotspots for bacteria community interactions and ARGs exchanges. This study was intentionally performed in a well-controlled greenhous condition, thus limiting sample size and environmental variables (e.g., soil type, plant species, field practices and climatic factors). However, its results may provide useful information for designing and implementing future large-scale field stuides by focusing on indicator ARGs/MGEs and bacterial phyla or families that are hotspots for bacterial interactions and ARG movements.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.105031.

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