ANTIBIOTIC RESISTANCE AND BACTERIAL MICROBIOME IN LETTUCE-SOIL SYSTEMS

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ABSTRACT

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Food safety challenges from emerging contaminants such as antibiotics and antibiotic resistance genes (ARGs) have received increasing attention due to rapid increases in their abundance in agroecosystems. This is particularly true in soil-vegetable systems as microbiomes and antibiotic resistomes of vegetables are important to their quality and safety and could be influenced by crop production with contaminated soil and water. Additionally, the food safety of vegetables may also drive consumers' preference and demand for certain food products (especially for labeled products such as USDA Organic, Raised Without Antibiotics, etc.). Using a soil-lettuce (*Lactuca sativa*) model system, the first study in this dissertation assessed how irrigation with antibiotics-contaminated water via overhead or soil-surface irrigation could influence bacterial communities and ARG profiles in lettuce shoots, roots, and soil, using 16S rRNA amplicon sequencing and high throughput qPCR techniques, respectively. The overall abundance and diversity of ARGs and bacteria associated with soil-surface irrigated lettuce shoots 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. The second study examined the fate and transport of selected antibiotics through bulk soil, rhizosphere soil, and lettuce roots and shoots under soil-surface irrigation. Root concentration factors based on the antibiotic concentrations in bulk soil (RCF_{bs}) were significantly higher than those based on antibiotic concentrations in rhizosphere soil (RCF_{rs}) for

ciprofloxacin, lincomycin, oxytetracycline, sulfamethoxazole, and tetracycline, similar for trimethoprim and tylosin, and lower for monensin. The third study investigated bacterial community assembly and ARG profiles in lettuce shoots, roots, rhizosphere soil, and bulk soil upon exposure to antibiotics. Bacterial communities were driven by stochastic processes upon exposure to low level antibiotics, and were more resilient in roots and rhizosphere soil than in bulk soil and shoots. The fourth study explored the importance of demographics, food-relevant habits, and foodborne disease perception to consumers' buy and pay preferences to labeled products by using conventional statistical and novel machine learning methods to analyze survey data. Consumers' willingness to buy or to pay more for certain labeled food products is dependent on certain demographic traits (e.g., urban living) and food-relevant habits (e.g., cooking fresh produce). Machine learning methods achieved sufficient prediction accuracy scores for estimating consumers' willingness to buy or to pay for labeled products, and thus could be useful tools for evaluating survey data and facilitating the development of strategies promoting healthy food production and consumption.

This dissertation is dedicated to my parents, Jun Liu and Wenmin Shen.
Thank you for always supporting and believing in me

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO ABBREVIATIONS

ARB: antibiotic resistant bacteria

ARGs: antibiotic resistance genes

CDC: United States Centers for Disease Control and Prevention

DNA: deoxyribonucleic acid

HPCC: high performance computing center

MDR: multidrug resistance genes

MGEs: mobile genetic elements

MLSB: macrolide-lincosamide-streptogramin B

MSU: Michigan State University

OTU: operational taxonomic unit

PCoA: principle coordinate analysis

qPCR: quantitative polymerase chain reaction

rRNA: ribosomal ribonucleic acid

USDA: United States Department of Agriculture

WHO: World Health Organization

CHAPTER I

Introduction and Objectives

Introduction

New food safety challenges in vegetable production are being brought about by emerging contaminants such as antibiotics, and antibiotic resistance genes (ARGs), as a result of their rapid proliferation in agroecosystems. The imprudent use of antibiotics in human healthcare and animal production has led to trace level of antibiotics in the environment including reclaimed water [1-3]. For example, sulfamethoxazole and trimethoprim were detected at levels up to 22 and 3.1 μg/L in wastewater effluents [4-6]. Vegetables and soils can be exposed to low levels of pharmaceuticals when irrigated with reclaimed waters. Pharmaceuticals (especially antibiotics) may be considered deterministic factors in shaping the microbiome of vegetable production systems as many pharmaceuticals are bioactive to microorganisms. Several studies have shown that exposure to pharmaceuticals and heavy metals from animal manures, wastewaters, or biosolids could change ARG profiles and bacterial communities in soil and water environments [7-12]. Antibiotics and ARGs may also interact with typical microbial pathogens (e.g., *Salmonella*) to collectively impact the safety of vegetables. Since food consumption can result in direct exposure to antibiotics, ARGs, and foodborne pathogens, it is important to understand the changes in bacterial communities and ARG profiles due to their potential influence on food safety and ultimately human health.

Crop irrigation with reclaimed water is increasingly practiced worldwide to meet water demand [13]. However, it was reported that there was higher incidence *Escherichia* coli in lettuce irrigated with overhead sprinklers than with soil-surface irrigation [14]. Thus, it is important to investigate how irrigation methods may influence the microbiome and ARGs in vegetables. Additionally, in soil-plant systems bulk soil, rhizosphere soil, plant roots and shoots may play a critical role in regulating the uptake and accumulation of antibiotics as well as changes in

bacterial microbiome and ARG profiles upon exposure to anthropogenic antibiotics. Thus, it is essential to study the plant uptake of antibiotics, bacterial microbiome, and ARGs in the continuum of bulk soil, rhizosphere soil, roots, and shoots.

The perceived safety of vegetables can drive consumer preferences and demand. Studies have been performed on consumers' willingness to buy and pay more for organic labeled products and other branded products based on demographics and other relevant survey questions [15, 16]. However, few studies have examined the purchasing preferences of consumers to various product labels (e.g., *Raised Without Antibiotics*, *No Medically Important Antibiotics, No Growth Promoting Hormones*, *Cage Free, USDA Organic, Locally Raised, Generic Brand,* and *Major Brand*), as related to food-relevant habits and foodborne disease perceptions of the consumers. Therefore, it is critical that consumers' preferences to labeled products are assessed so that consumer-oriented strategies for mitigating antibiotic resistance can be developed.

The successful launch and progress of the Human Microbiome Project and the Earth Microbiome Project have sparked researchers' enthusiasm to understanding microbial ecology in humans, environment, animals, and plants [17, 18]. Along with the progress in understanding microbiomes, next-generation sequencing technologies have advanced tremendously with increases in speed, read length, efficiency, and a rapid decrease in per-base cost [19]. As a result, the sizes and dimensions of sequencing raw data output have increased exponentially. Data analyses need to be highly efficient and reproducible to meet cutting-edge analytical techniques. The work in this dissertation used high throughput qPCR and 16S rRNA amplicon sequencing in addition to liquid chromatograph mass spectrometry in tandem (LC-MS/MS) and culture-dependent isolation, in combination with computational tools and methods (i.e., R, Python, and machine learning).

Objectives

This dissertation has four main objectives:

1. Characterize bacterial communities and ARGs in a soil-lettuce system upon exposure to antibiotics via overhead and soil-surface irrigations.
2. Investigate the uptake and accumulation of antibiotics in lettuce through the continuum of bulk soil, rhizosphere soil, roots and shoots.
3. Assess the changes in bacterial communities and ARGs profiles in distinct niches of soil-plant systems including bulk soil, rhizosphere soil, roots, and shoots.
4. Determine the consumers' purchasing preferences for various labeled food products as influenced by demographics, food-relevant habits, and foodborne disease perceptions.

The following chapters address the four objectives of my research. Objective 1 is addressed in Chapter II, Objective 2 in Chapter III, Objective 3 in Chapter IV, and Objective 4 in Chapter V. This dissertation ends with Chapter VI that summarizes the findings and identifies future research directions.

CHAPTER II

Pharmaceutical Exposure Changed Antibiotic Resistance Genes and Bacterial Communities in Soil-Surface- and Overhead-Irrigated Greenhouse Lettuce

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*).

Keywords: Pharmaceuticals; Antibiotic resistance genes; Bacterial Community; *intl1*; irrigation; Lettuce

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 [20, 21]. For example, World Health Organization (WHO) recommends daily consumption of > 400 grams of fresh produce to decrease risk of certain noncommunicable diseases and to improve overall health [21]. 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 [22]. 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 [22-24]. 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 [25]. Recently attention is being given to diverse microbiomes in vegetables (specifically opportunistic pathogens) rather than only to obligate pathogens [26-31], as it is believed that plant microbiomes could impact human gut microbiome and thus human health [26, 32].

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 [13]. In fact, globally about 359,000 km^2 of croplands are irrigated with urban wastewater [33]. Reclaimed water often contains trace level of pharmaceuticals

(including antibiotics) [4, 6], due to the extensive and imprudent use of pharmaceuticals in animal production and human healthcare [2, 3]. 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 [4-6]. 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 [29, 34, 35]. Two studies confirmed that antibiotic resistant *E. coli* isolates were more prevalent in vegetables (e.g., lettuce [*Lactuca sativa*]) than in soils and waters used for vegetable production [34, 35]. 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 [7-12]. In soils irrigated with reclaimed water the abundance of ARGs and mobile genetic elements (MGEs) could be increased by 99–8655 folds [10, 36], and are influenced by levels of salinity, pharmaceutical residues, nutrients, and heavy metals, as well as varying wastewater treatment and soil characteristics [12, 37, 38]. 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 [14]. 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 [39]. This stud y may help better utilize reclaimed water while minimizing food safety risks associated with microbial pathogens and ARGs.

Materials and Methods

Lettuce growth experiment and sample collection

Lettuce growth experiment was previously described in detail [40]. 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 cmol(+)/kg cation exchange capacity. The soil did not contain any pharmaceutical tested in this study tested by control samples. The lettuce plants were irrigated daily with fertilizer solution (20-20-20 general purpose fertilizer, 125 mg/L total nitrogen) 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, acid dissociation costant (pKa), water solubility, and hydrophobicity, which were described in detail by Bhalsod et al. [40]. Three non-

antibiotic drugs were selected because pharmaceuticals were often present as mixture in waters. As explained by Bhalsod et al. [40], the concentration of each pharmaceutical (30 μg/L) was at the high end of typical pharmaceutical concentrations in reclaimed water [4, 6], 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. [40]. 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. [40]. 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 (Appendix Table A1). 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.,)[40] 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 (Appendix Table A1). 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 [40]. Thus, the soil samples under overhead irrigation was not selected because no major difference in pharmaceutical concentration was expected from that of surface-irrigated soil samples.

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 (Appendix Table A2). The initial enzyme was activated at 95°C for 10 minutes. 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 (Appendix Table A1). 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 [41]. 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 2×250bp 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 [41]. 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.

Data Analyses

Cycle numbers (C_T) measured by the WaferGen qPCR were used to calculate copy number of genes via Copy Number = $10^{(30-C_T)/(10/3)}$ [42], 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 preprocessed 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 [43, 44]. 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 [45, 46]. 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 greater than 0.6 or less than –0.6 and *p*-value < 0.05) and plotted using Gephi v0.9.1 software. Detailed data analysis procedures are provided in Appendix A.

Results and Discussion

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 Appendix Figure A1. 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 (Appendix Figure A1). Close examination of Figure S1 and Figure 2.1 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 [47, 48]. 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 (Figure 2.1).

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 (Appendix Figure A2). The genes in Figure 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 [49]. The high prevalence of MGEs and MDR genes are also alarming. Class 1 integron (*intl1*) gene was detected in all samples (except for SSC) (Figure 2.1). 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 [11, 12, 50-52]. Its high prevalence may result from its genetic function that integrate exogenous gene sequences into functional genes using integron gene (*intl*), recombination site (*attI*), and an outward-orientated promotor [52]. Our observation supported the proposed strategy to use *intl1* as an indicator gene for the ARG surveillance [33, 50, 53].

As shown in Figure 2.1, 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 (Figure 2.1 and Appendix Figure A3). With pharmaceutical exposure, the relative abundance of some genes increased, whereas other genes decreased (Figure 2.1). Among the most detected genes (Supplemental Figure 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 [10-12]. 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 [40]. 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 (Figure 2.1), which may be due to greater nutrient concentrations and thus bacterial growth and activity in the top soils [11, 54].

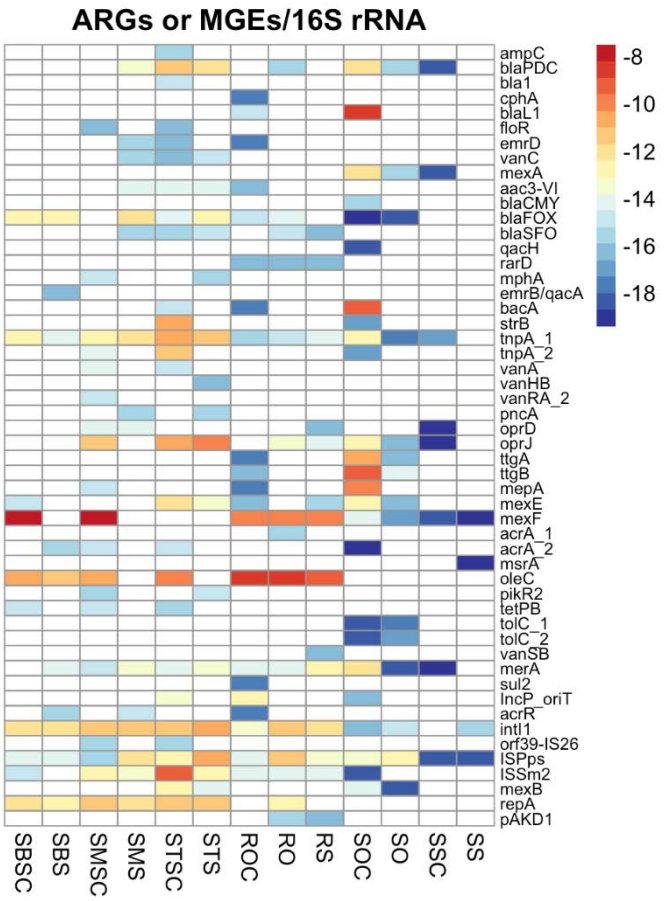

Figure 2.1. Relative abundance (gene copy number/16s rRNA gene copy number) of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs). Data were Log 2 transformed. Blank cells represent genes that were either not detected or below detection limit. Color bar on the right means relative abundance from low (blue) to high (red) levels.

As shown in Figure 2.2, 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.

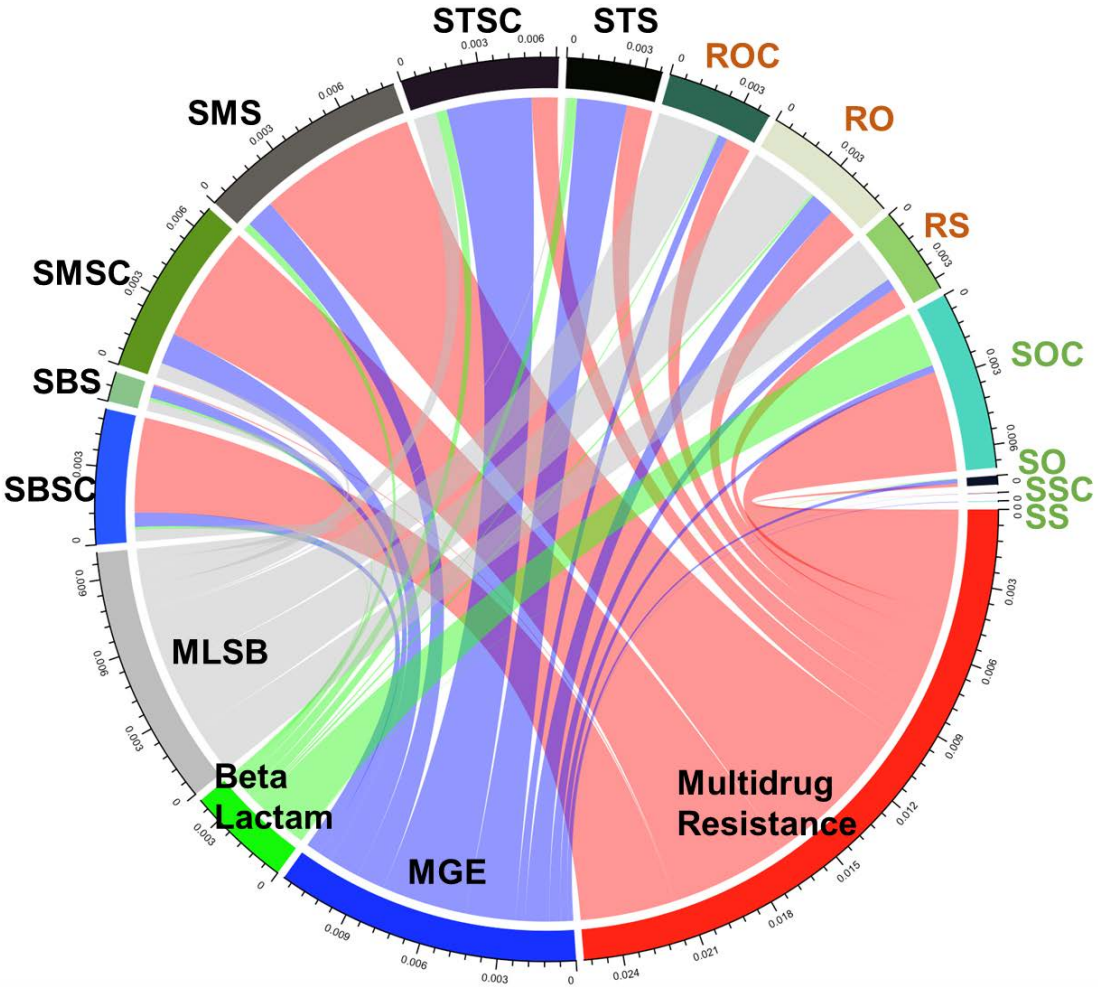

Figure 2.2. Total relative abundance (gene copy number /16s rRNA gene copy number) of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) among various categories. Top four categories include MLSB, Beta Lactam, MGEs, and Multidrug Resistance. The width of the circular bar represents the total abundance of ARGs and MGEs in each sample.

Soil and Lettuce Microbiomes

On both bacterial phylum and family levels pharmaceutical exposure changed bacterial community structures (Figure 2.3). 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 (Figure 2.3A). Proteobacteria were slightly increased in proportion with pharmaceutical exposure (Figure 2.3A). This is interesting as some strains in Proteobacteria can actually grow on various antibiotics and confer various resistance mechanisms [55]. Moreover, Proteobacteria carry many mobile integrons and integron genes even in very ancient times [1, 52, 56]. 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 [57]. Actinobacteria are known for harboring ARGs through their ability to synthesize various secondary metabolites [58]. 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 [59, 60]. It is worth to mention that Bacteroidetes, Firmicutes, and Beta Proteobacteria were found to be highly correlated with some antibiotic resistance strains such as ciprofloxacin resistance heterotrophs, ciprofloxacin resistance enterococci, and sulfamethoxazole resistance enterobacteria [61].

On the family level, *Methylophilaceae* was not observed in the pharmaceuticals-free samples, but became abundant with pharmaceutical exposure (Figure 2.3B). *Methylophilaceae* is a family in the order of *Methylophilales* in Proteobacteria and may contribute to the increase of Proteobacteria with pharmaceutical exposure shown in Figure 2.3A. Species of *Methylophilaceae* have been isolated or sequenced from various ecological niches including aquaculture, wastewater, activated sludge, soil, rhizosphere, and phyllosphere [27, 62-65]. However, no *Methylophilaceae* bacteria were found to cause opportunistic infections in humans and animals [63]. 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* [66]. 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 [57].

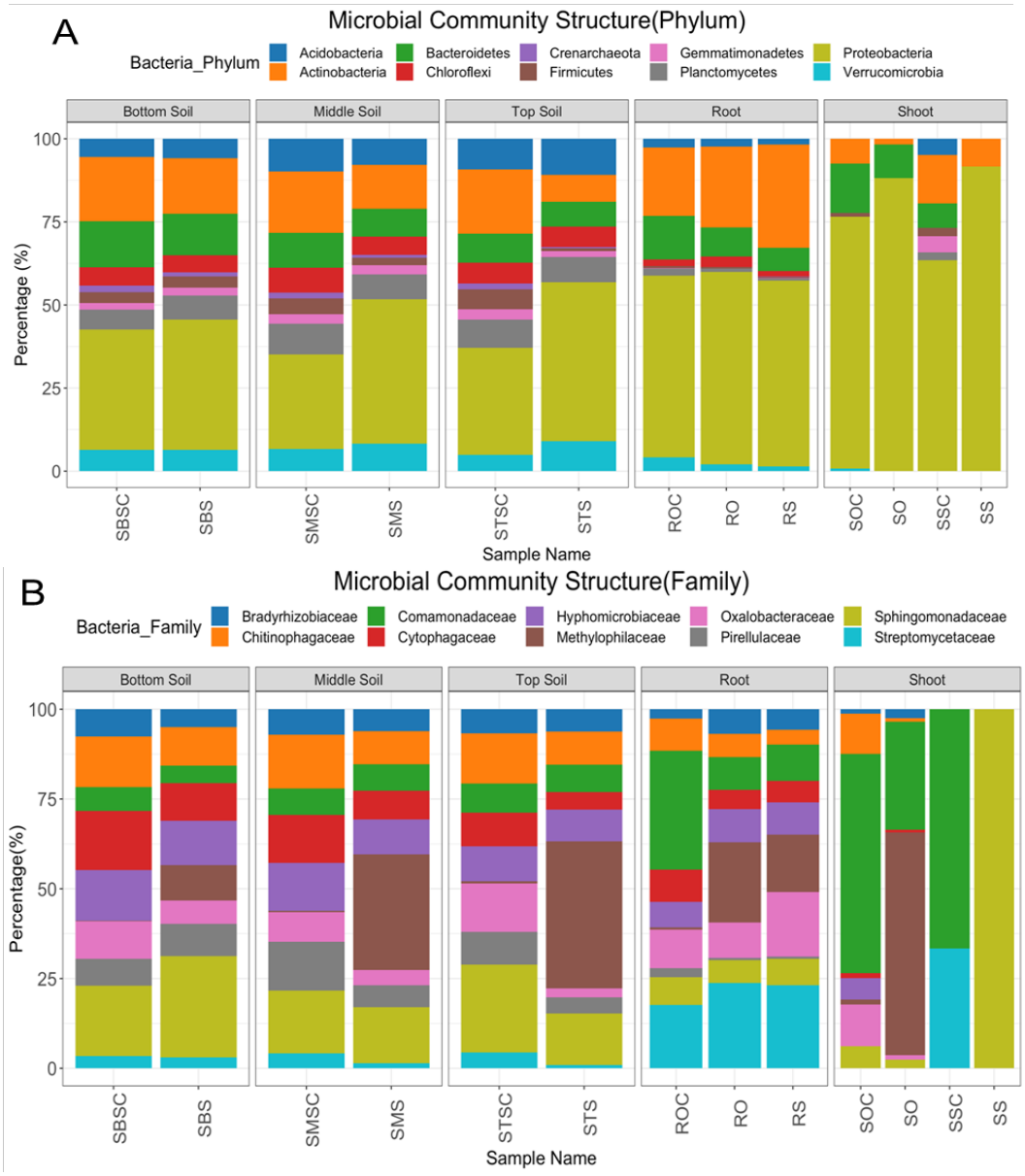

Figure 2.3. Bacterial community composition at the phylum (A) and family (B) levels. Total percentages of the total 10 phyla or families were 100%. Each bar represents the fraction of each bacteria phylum and family.

The bacterial beta diversity analysis showed the ordination position of top ten phyla (Figure 2.4A). Compared with the other methods (Figure 2.4A and Appendix Figure A4), the PCoA analysis is a simpler ordination method that can separate groups with less underlying

assumptions [67]. 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 (Figure 2.4A). 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 [68]. Finally, pharmaceutical exposure decreased bacterial alpha diversity with each sample measured by the Chao 1 diversity index (Figure 2.4B). 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) [45, 46]. 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 [39, 69, 70]. It is interesting to note that bacterial alpha diversity of lettuce shoots was much greater for overhead irrigation than for soil-surface irrigation (Figure 2.4C), again likely due to greater bacterial growth and activity resulted from greater water and nutrient availability in overhead-irrigated lettuce shoots.

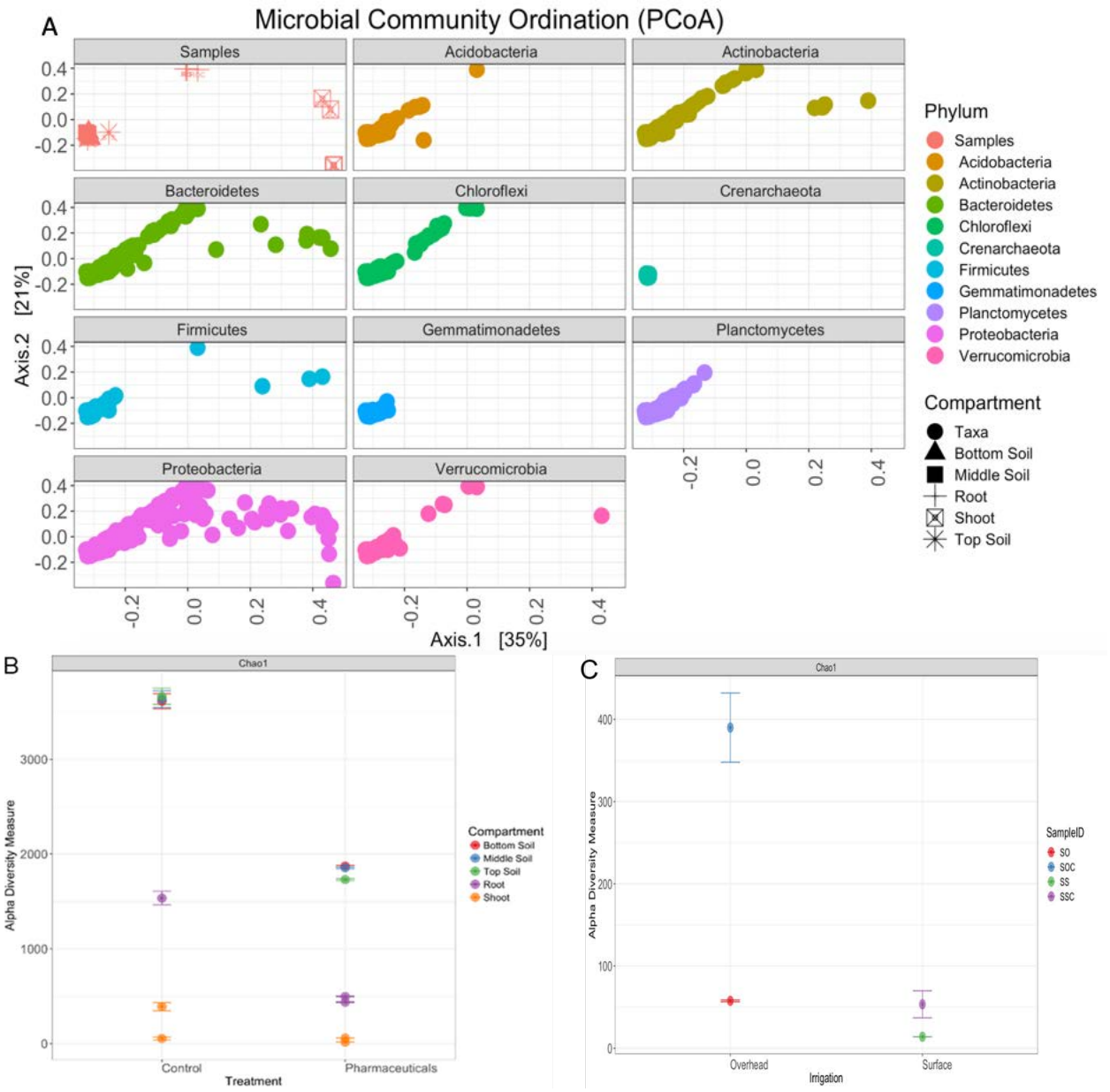

Figure 2.4. Bacterial community alpha diversity based on species level and beta diversity based on phylum level. A. Beta diversity biplot based on principal coordinates analysis (PCoA). Sample panel shows the ordination position of sample type, and the remaining ten panels showed the ordination position 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.

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

The network analysis (Figure 2.5) was performed based on correlation tests as described in Appendix A and the correlation results are summarized in Appendix Table A4. 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 [11, 71]. 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*) (Figure 2.5), 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 [72-74]. One species (*Rhodopirellula baltica* SH1) in Planctomycetes was found to harbor integrons [52]. 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 (Figure 2.5). 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 (t*npA_1*) and bacterial families (Figure 2.5). These two antibiotics classes are commonly used in veterinary medicine and have been detected in wastewater effluents [75-77]. *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 [78]. 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 [57]. MLSB-resistant gene *OleC* was reported to be isolated from *Streptomyces antibioticus* and confers resistance to Oleandomycin because *Streptomyces antibioticus* prodcues natural antibiotics [79]. 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 [80-82]. *OleC* was especially abundant in lettuce roots (Figure 2.1), which may be linked to the abundance of the rhizosphere *Streptomyces* (Figure 2.5), supported by the high fraction of *Streptomycetaceae* in the root samples (Figure 2.3B). This observation is interesting not only from the human heath perspective, but also for the plant protection [26], as *Streptomyces* have been proposed as effective biological control agents against pathogen infection of plant roots [83, 84]. 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.

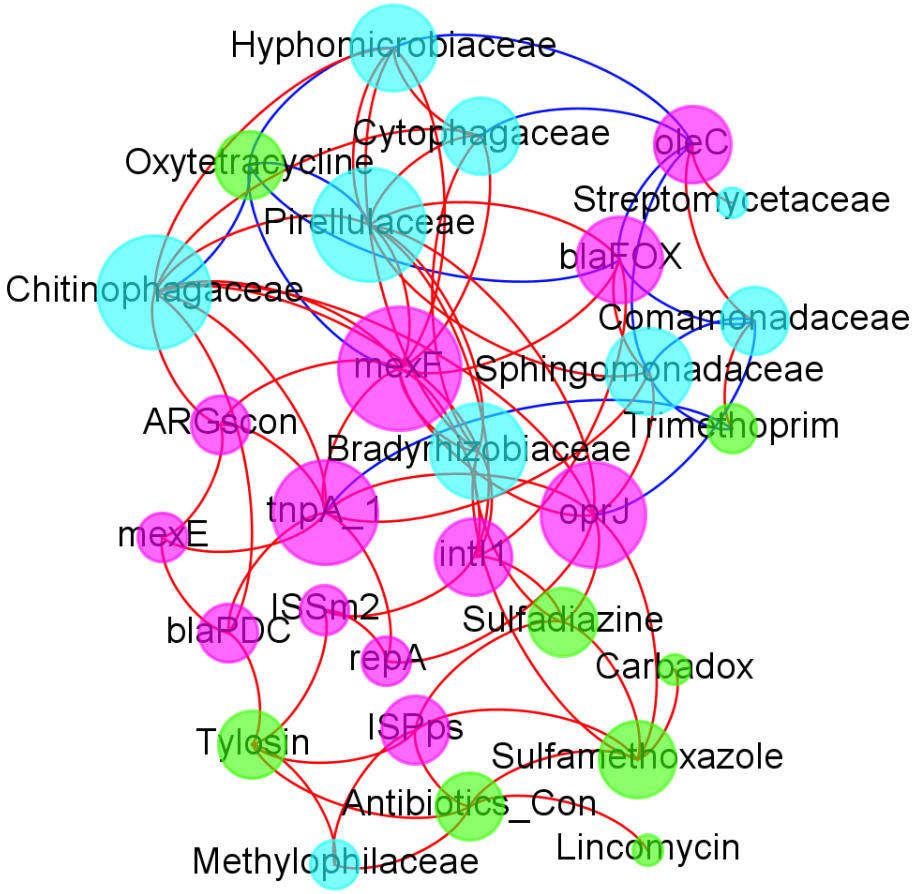

Figure 2.5. Network analysis diagram of the correlations between ARGs, antibiotic concentrations and bacterial families for the lettuce shoot, root and soil samples with pharmaceutical exposure. Green nodes represent the concentrations of antibiotics, blue nodes represent bacterial families, and pink 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, $p < 0.05$).

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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Shen, Y.; Stedtfeld, R. D.; Guo, X.; Bhalsod, G. D.; Jeon, S.; Tiedje, J. M.; Li, H.; Zhang, W. Pharmaceutical exposure changed antibiotic resistance genes and bacterial communities in soil-surface- and overhead-irrigated greenhouse lettuce. Environ. Int. 2019, 131, 105031.

CHAPTER III

Rhizosphere Soil is Key to the Uptake of Antibiotics by Lettuce (*Lactuca Sativa*)

Abstract

Plant uptake of antibiotics raises serious food safety concerns. Measurements and predictions of antibiotic uptake by plants are often based on root concentration factor (RCF) calculated using antibiotic concentrations in bulk soil (RCF_{bs}) rather than in rhizosphere soil (RCF_{rs}) where root uptake actually occurs. This study investigated the fate and transport of nine antibiotics in the continuum of bulk soil, rhizosphere soil, roots and shoots of lettuce (*Lactuca sativa*) under soil-surface irrigation. Antibiotic concentrations in the lettuce shoots remained unchanged during 25–35 days after seedling transplantation. Compared with the RCF_{rs} values, the RCF_{bs} values were significantly greater for ciprofloxacin, lincomycin, oxytetracycline, sulfamethoxazole, and tetracycline ($p < 0.05$), similar for trimethoprim and tylosin, but significantly lower for monensin ($p < 0.05$). Ciprofloxacin, trimethoprim, and tylosin had the lowest translocation factor (TF) values ranging between 0.03–0.05, suggesting limited upward transport to the lettuce shoots. Oxytetracycline, monensin, and sulfamethoxazole had intermediate TF values of 0.36–0.64, whereas lincomycin had the highest TF value of 1.46. This study showed significant differences between the RCF_{bs} and RCF_{rs} values, suggesting the need to reassess the utility of RCF_{bs} in predicting the antibiotic root uptake in diverse soil-plant systems.

Introduction

Food crops can take up trace-level antibiotics from agricultural soils contaminated with antibiotics via applications of animal manure, sewage biosolids, or reclaimed waters [85-89]. Consumption of food crops tainted with low-level antibiotic residues may cause unnecessary human exposure to some clinically important antibiotics [85, 89, 90]. Studies have found that low levels of antibiotics can induce dissemination of antibiotic resistance genes (ARGs) and change bacterial community assembly in soil, plants, and sediments [12, 91]. Increased environmental

abundance of ARGs may increase the likelihood of horizontal ARG transfer to pathogenic bacteria, resulting in human health risks [1, 52]. Thus, the fate and transport of antibiotics in soil-plant systems could have an important impact on food safety and human health and are thus a topic of intensive research.

Many researchers have examined the uptake and accumulation of antibiotics in vegetable crops [40, 85, 87, 89, 90, 92-94]. Most of these studies have investigated the transport of antibiotics from bulk soil or hydroponic solution to plant roots and then to shoots [40, 91-96]. Root concentration factors (RCF) based on antibiotic concentrations in bulk soil are typically calculated [88, 97, 98] and used in plant uptake modeling [93, 99]. However, these studies have overlooked an important fact that antibiotics need to pass through rhizosphere soil before entering plant roots, followed by translocation to phyllosphere [40, 88, 93, 94, 100]. Several studies reported that rhizosphere soil and root exudates may play an essential role in the transport of antibiotics and their bioavailability in soil-vegetable systems [88, 95]. Thus, RCF values based on antibiotic concentrations in rhizosphere soil are needed. Additionally, rhizosphere soil is a hotspot for plant-microbe interaction and plant health, probably modulated by root exudates [101]. Thus, it is important to investigate the distribution and fate of antibiotics in rhizosphere soil and bulk soil in addition to plant roots and shoots.

This study aimed to: i) quantify the transport and distribution of antibiotics in a model soil-plant system; and ii) determine the roles of rhizosphere soil and bulk soil in plant uptake of antibiotics. Lettuce was used as a model vegetable crop because of its popularity and raw consumption. This study could provide further insights into the fate and transport of antibiotics in vegetable production systems.

Materials and Methods

Chemicals and Materials

Nine commonly used antibiotics with varying molecular weight, charge speciation (pKa), water solubility and hydrophobicity were selected (Table 3.1), including sulfamethoxazole, trimethoprim, lincomycin, oxytetracycline, monensin sodium, tylosin, ciprofloxacin, cefalexin, and tetracycline. They were purchased from Sigma-Aldrich (St. Louis, MO, USA), and individually dissolved in HPLC-grade methanol to prepare stock solutions of 500 mg/L, except for ciprofloxacin stock solution at 50 mg/L. Analytical-grade acetonitrile and anhydrous sodium sulfate (Na_2SO_4) were purchased from EMD Chemicals (Gibbstown, NJ, USA), sodium chloride (NaCl), disodium ethylenediaminetetraacetate (Na_2EDTA), and formic acid from J.T. Baker (Phillipsburg, NJ, USA), and ceramic homogenizer, octadecylsilane (C18), and primary secondary amine (PSA) from Agilent Technologies (Santa Clara, CA, USA). Oasis hydrophilic-lipophilic balance (HLB) extraction cartridges (6 cm^2) were purchased from Waters Corporation (Milford, MA, USA).

Table 3.1: Physiochemical properties of antibiotics used in this study. [a] From TOXNET database: http://toxnet.nlm.nih.gov/index.html, [b] Reference [102], [c] From Guidechem database: http://www.guidechem.com/reference/dic-20635.html, and [d] From ChemSpider database: http://www.chemspider.com/Chemical-Structure.10606106.html

Antibiotics	Molecular Weight (g/mol)[a]	Water Solubility (mg/L)[a]	pKa[a]	logKow[a]
Sulfamethoxazole	253.28	610	1.60,5.70	0.89
Trimethoprim	290.32	400	7.12	0.91

Table 3.1 (cont'd)

Lincomycin	406.54	927	7.60	0.20
Oxytetracycline	460.43	313	3.57,7.49,9.44[c]	-0.9
Monensin Sodium	692.87	slightly soluble[b]	4.30[c]	5.43[c]
Tylosin	916.1	5	7.73	3.27[d]
Ciprofloxacin	331.34	30000	6.09,8.74	0.28
Cefalexin	347.39	slightly soluble	5.20,7.30	0.65
Tetracycline	444.43	4	3.30,7.68,9.69	-1.37

Sandy loam soil was collected from a field site of Michigan State University Farms and sieved before the experiment. The field site was planted with poplar trees from 1980s to 2017 and had no previous exposure to pesticides or animal manure. The sandy loam soil had 69.6% of sand, 19.6% of silt, 10.8% of clay, a pH of 6.2, organic matter of 1.7%, 27.5 mg/kg of Bray P1 extractable phosphorus, and 75 mg/kg of ammonium acetate extractable potassium (K), and 79 mg/kg of ammonium acetate extractable magnesium (Mg). The soil had cation exchange capacity of 5.2 cmol(+)/kg with exchangeable bases at 4.9% of K, 16.7% of Mg, and 78.5% of Ca, respectively.

Lettuce growth experiment

A growth chamber experiment was conducted at an average temperature of 24 °C, relative humidity of 37% ± 13%, and 16 hours lighting per day at an artificial light intensity of 565 µmol/m^2-s. Nursery pots (10.0 cm in height and 12.7 cm in diameter) were packed with ~1.28 kg of the sandy soil at a bulk density of 1.1 g/cm^3 to a height of ~9.5 cm. The soil had a field capacity of 0.18 as measured by the gravimetric method. A total of 30 lettuce plants (15

plants each in the control and antibiotics treatment chambers) were first seeded in compost mixture, then transplanted and grown in the nursery pots (1 plant per pot). Nursery pots in each chamber were shifted daily to ensure the similar illumination from the Roleadro grow light (1000W) (Roleadro, Shenzhen, China). All plants were irrigated at the soil surface to avoid direct contact with the lettuce shoots. The control plants received antibiotics-free water, whereas the treated plants were irrigated with antibiotics-containing water. In the antibiotics treatment, antibiotics-spiked irrigation water (4 L) was made every week at the concentration of 30 μg/L for each antibiotic, and was used for the first five days in any given week. Two liters of antibiotics-spiked fertilizer water containing 125 mg/L total nitrogen (20-20-20 general purpose fertilizer) was prepared and applied to each lettuce plant for the remaining two days of the week. Control plants received deionized (DI) water for the first five days in any given week, followed by the two days of antibiotics-free fertilizer solution. This irrigation sequence of five-day irrigation without fertilizer followed by two-day irrigation with fertilizer was repeated for five weeks. Lettuce growth pots were weighed every day, and replenished with irrigation water to maintain 80% of field capacity.

Sample collection

A sequential sampling scheme was used to collect the lettuce root, shoot, rhizosphere soil, and bulk soil samples. Lettuce plants grown with or without antibiotics exposure were harvested in triplicate at Day 25, 27, 30, 35, respectively. Three additional plants from both the control and antibiotics treatment chambers were harvested at Day 35, and only the extra control samples were later used for testing extraction efficiencies of all antibiotics. On the day of harvest, the fresh lettuce shoots were separated from lettuce roots and then weighed. The bulk roots were carefully taken out from the soil, with the remaining fine roots separated from the soil

by crushing small aggregates and passing through a 2-mm sieve. Lettuce roots were then placed in a 50-mL centrifuge tube containing 30-mL of DI water and shaken for 1 hour. Afterward, the lettuce roots were carefully removed from the centrifuge tube, washed with DI water to remove any visible soil particles, dried with Kimwipes, weighed, and placed in a −20 °C freezer. These loosely attached soil particles that detached from the roots during shaking were considered to comprise the rhizosphere soil [103, 104], in contrast to all the soil particles in a rhizobag that may include both soil particles loosely associated with the roots and those further away from the roots [105, 106]. This sampling method allows us to better assess the effect of plant roots on the bioavailability and root uptake of antibiotics in the rhizosphere soil. Wash water was kept in the centrifuge tube to prevent any loss of antibiotics in the wash water. The 50-mL centrifuge tube containing the rhizosphere soil and the wash water were placed in a −20 °C freezer. Bulk soil samples were thoroughly mixed by passing through the sieve, collected in sterilized bags, and vigorously shaken for 2 minutes to homogenize the soil samples. All lettuce shoot, root, rhizosphere soil, and bulk soil samples were stored at −20 °C in a freezer and subsequently freeze-dried, ground, and mixed for antibiotic analyses. Water samples were collected once or twice for every batch of antibiotic solution with and without fertilizer.

Extraction and measurements of antibiotics

Briefly, 0.25 g of the lettuce shoot and root samples and 2.5 g of the rhizosphere soil and bulk soil samples were individually placed in 50 mL centrifuge tubes. Afterward, 2 mL of 150 mg/L Na_2EDTA was added and vortexed for 1 minute. After revortexing with an added ceramic homogenizer for an additional minute, 1.75 mL of methanol and 3.25 mL of acetonitrile were added to the centrifuge tube, followed by vortexing for 2 minutes. Next, 0.5 g NaCl and 2.0 g Na_2SO_4 were added to the centrifuge tube and vortexed for 1.5 minutes to absorb excess water.

The centrifuge tube was centrifuged at 6500 rpm for 10 minutes. Then 1.3 mL supernatant was transferred to a prefilled d-SPE tube for sample cleanup (including 0.0125 g C18, 0.0125 g PSA, and 0.2250 g Na_2SO_4), and further vortexed for 1 minute. The d-SPE tubes were centrifuged for 5 minutes at 10,000 rpm, and then 900 µL of the supernatant was transferred to a sampling vial containing 100 µL of methanol. Standard curves were constructed on the same day with the matrix match extraction solutions from the lettuce shoot, root, rhizosphere soil, and bulk soil samples. Extraction efficiency was measured by spiking 200 µL of a mixed antibiotic standard solution (500 µg/L for each antibiotic) to 0.25 g of the lettuce shoot and root samples or 2.5 g of the soil samples (Appendix Table B2). These spiked samples were then evaporated for 10 minutes in a fume hood and extracted using the same modified QuEChERS method. The water samples were passed through Oasis hydrophilic-lipophilic balance (HLB) solid-phase extraction (SPE) columns and then eluted with analytical grade methanol following the manufacturer's instructions (Waters, USA). The methanol solutions containing eluted antibiotics were stored at 4 °C for future analyses. All the samples were analyzed by Shimadzu Prominence high performance liquid chromatograph (Colombia, MD,USA) coupled with an Applied Biosystems Sciex QTrap 4500 triple quadrapole mass spectrometer (Foster City, CA, USA) (LC-MS/MS) on the same day of extraction (except for the water samples) using an Agilent Eclipse Plus C18 column (2.1 mm × 50 mm, particle size of 5µm). Sample injection volume was 10 µL with mobile phase A (0.3% formic acid in DI water) and phase B (acetonitrile/methanol (1/1) with 0.3% formic acid) at the flow rate of 0.35 mL/min. Appendix Table B1 provided the LC-MS/MS parameters for the nine antibiotics. Antibiotic concentrations in the lettuce root, shoot, rhizosphere soil and bulk soil samples were then calculated on dry weight basis using the LC/MS-MS measurements. As shown in Appendix Table B2, for the lettuce shoot samples, the

extraction efficiencies were low for sulfamethoxazole (4.3%) and tetracycline (42.9%), and ranged from 52.7% to 89.1% for other antibiotics. For the lettuce root samples, the extraction efficiencies of sulfamethoxazole (18.8%), oxytetracycline (28.7%), and tetracycline (29.0%) were relatively low, whereas the other antibiotics had extraction efficiencies between 53.5%–73.9%. For the soil samples, the extraction efficiency was low for oxytetracycline (0.8%), ciprofloxacin (6.9%), and tetracycline (2.7%), and was between 57.2%–99.2% for other antibiotics. The method detection limits of most antibiotics for the lettuce samples were reported by Chuang et al. [96], ranging between 0.7–4.7 µg/kg.

Data analyses

The roots concentration factor (RCF) for rhizosphere soil (RCF_{rs}) and bulk soil (RCF_{bs}) were calculated as follows:

$$\mathrm{RCF_{rs}} = \frac{\mathrm{C_{rt}}}{\mathrm{C_{rs}}}$$

$$\mathrm{RCF_{bs}} = \frac{\mathrm{C_{rt}}}{\mathrm{C_{bs}}}$$

The root-to-shoot translocation factor (TF) was calculated as:

$$\mathrm{TF} = \frac{\mathrm{C_{st}}}{\mathrm{C_{rt}}}$$

where C_{rt}, C_{st}, C_{rs}, and C_{bs} (µg/kg) are the concentrations of antibiotics by dry weight in the lettuce root, shoot, rhizosphere soil, and bulk soil, respectively. Because there were no significant differences between the RCF values at different harvest days (Day 27, 30, 35), the RCF values were averaged for each antibiotic in the rhizosphere soil and bulk soil, respectively. The differences between the RCF_{rs} and RCF_{bs} values were compared using the student's T-test.

We removed the RCF and TF values for Day 25 since they were significant outliers compared to the remaining data. Kruskal-Wallis non-parametric multivariable statistical tests were used to calculate statistical significance in all other comparisons.

The cumulative input mass of each antibiotic at Day 25, 27, 30 and 35 was calculated by multiplying the average antibiotics concentration by the volumes of non-fertilized or fertilized irrigation water during each irrigation sequence, followed by the sum of antibiotics mass for all sequences until the day of sampling. For each sampling day, the concentrations of each antibiotics in the lettuce shoot, root, bulk soil and rhizosphere soil samples were multiplied by their individual dry mass to obtain the recovered mass, followed by division with the cumulative input mass to determine the recovered mass percentage in each sample type. All calculations and statistical tests were coded and performed using a custom written Python workflow, and all figures were plotted in R version 3.6.1. Python and R scripts are available at Yike Shen's Github page (https://github.com/YikeShen).

Results and Discussion

Antibiotics residues in the lettuce and soil samples

The concentrations of antibiotics ranged from non-detection for cephalexin to 7.02 ± 3.22 µg/kg for trimethoprim in the lettuce shoots (Figure 3.1), which were very low and close to their method detection limits [96]. No significant changes in the concentrations of each antibiotic were observed during the four harvest days, indicating the concentrations of antibiotics in the lettuce shoots had become constant from Day 25 until Day 35 after the seedling transplantation. It is plausible that the uptake of the tested antibiotics to the lettuce shoots were balanced off by in-plant metabolism and degradation at the late growth stage. For example, in one study a

substantial percentages of tylosin (22%), monensin (24%), lincomycin (26%), trimethoprim (41%), oxytetracycline (48%) and sulfamethoxazole (97%) were lost in radishes after 7 days of exposure [107]. The shoot concentrations were comparable to those of tylosin, monensin and trimethoprim (0.5–9.1 μg/kg) with low translocation to the shoots in our previous greenhouse study [40], but much lower than those of lincomycin and oxytetracycline with intermediate translocation (21–48 μg/kg). This discrepancy was probably due to differences in transpiration and growing conditions between this study and the greenhouse study. Specifically, tylosin is a relatively large molecule with a molecular weight of 916 g/mol, which showed limited uptake into lettuce shoots, in agreement with previous studies [40, 94, 108]. The stable concentrations of lincomycin (6.46 ± 1.86 μg/kg) in lettuce shoots could be due to its relative high stability and continuous uptake [40, 107]. The relatively low sulfamethoxazole concentrations at the end of the experiment were probably due to its high degradation in the soil-plant system [40], and low extraction efficiency (Appendix Table B2). In addition, sulfamethoxazole was previously reported to reach the maximum concentration in cabbage within 10 days of uptake[109], with this antibiotic experiencing more pronounced dissipation in the late growth stage. Compared to other antibiotics, ciprofloxacin concentrations were relatively low in the shoots. Its uptake into plant shoots depends on both the spiked concentration and growth duration of plant [110], with low concentrations of ciprofloxacin in manured soils not taken up by plant shoots [111]. The ciprofloxacin residues found in the lettuce shoots may have important health implications, as many bacteria have been found resistant to ciprofloxacin (an important antibiotic drug in fluoroquinolones), especially *Salmonella* species [112, 113]. Thus, despite low ciprofloxacin concentrations detected in the lettuce shoots (< 2.5 μg/kg), exposure of plant-associated bacteria to ciprofloxacin may promote the selection of antibiotic resistance, resulting in greater health

risks. Finally, cephalexin was not detected in any lettuce shoot, root, rhizosphere soil, or bulk soil samples.

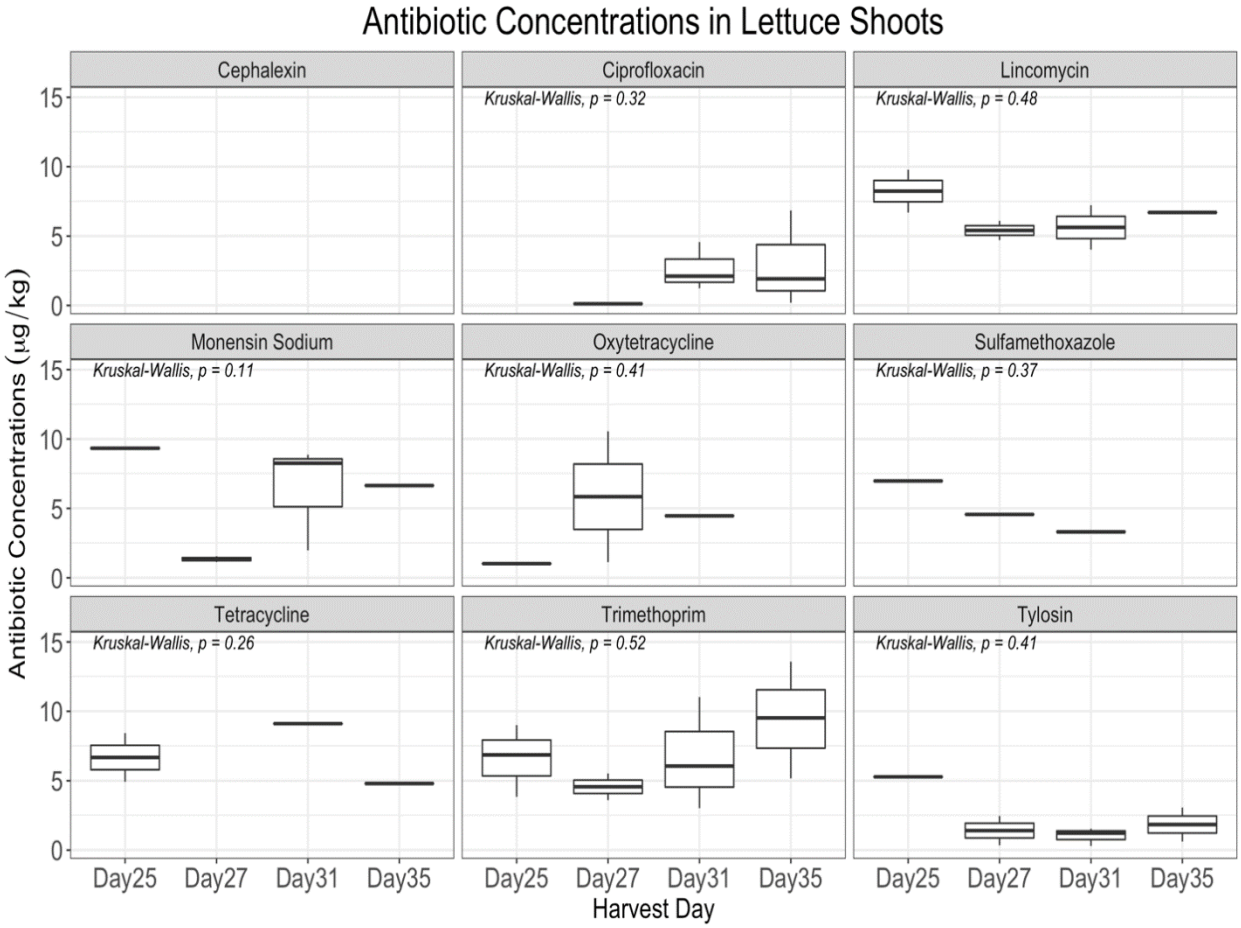

Figure 3.1: Antibiotic concentrations in the lettuce shoots by dry weight. Antibiotic concentrations in lettuce shoots by dry weight. Kruskal-Wallis statistics were performed for the samples at all four harvest days. No symbol for a data point indicates no detection of antibiotics. Boxplots without quantiles indicate that the concentration of triplicates were not detected.

Antibiotic concentrations in the lettuce roots varied substantially (Figure 3.2), reflecting varying root uptake potential for the tested antibiotics. The concentrations of ciprofloxacin and trimethoprim were in the higher range (125–230 µg/kg), and appeared to increase during Day 25 to 35, suggesting strong root uptake. The root concentrations of tylosin and monensin were lower than those of other antibiotics, likely due to their larger molecular weights resulting in limited entry through root membrane [40, 94]. The concentrations of oxytetracycline and tetracycline were intermediate, similar to those observed by Bhalsod et al., whereas the lower concentration of sulfamethoxazole likely resulted from its higher degradation [40, 107], and low extraction efficiency (Appendix Table B2). The low concentrations of lincomcyin in the lettuce roots were unexpected and cannot at this point be explained.

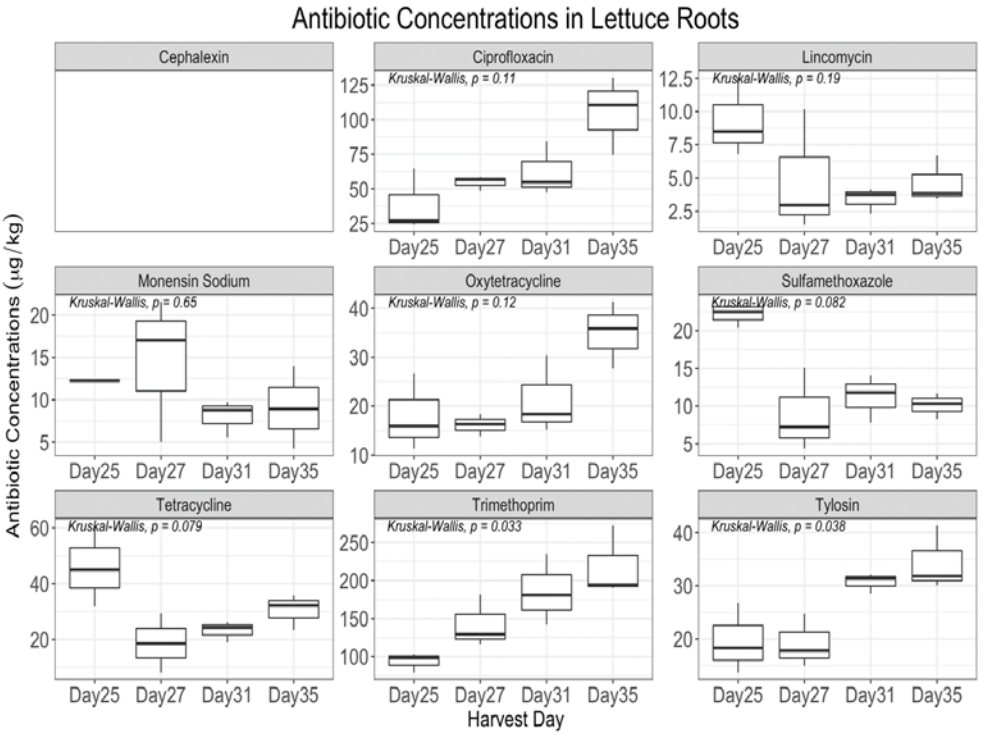

Figure 3.2: Antibiotic concentrations in the lettuce roots by dry weight. Kruskal-Wallis statistics were performed for the samples at all four harvest days. No symbol for a data point

Figure 3.2 (cont'd) indicates no detection of antibiotics. Boxplots without quantiles indicate that the concentration of triplicates were not detected.

As shown in Figure 3.3, the very low concentration of oxytetracycline and tetracycline in the bulk soil were likely due to their strong sorption to soil resulting in lower extractability (Appendix Table B2) [114] and their fast degradation [120]. Tylosin and trimethoprim had the higher concentrations in the bulk soil than other compounds, likely due to their relatively high stability (Figure 3.3, Appendix Figure B1) [115, 116]. The remaining antibiotics were present at low concentrations in the bulk soil. In the rhizosphere soil, the concentrations of ciprofloxacin and sulfamethoxazole increased with time ($p < 0.05$), whereas the other antibiotics were not statistically different across various sampling days (Figure 3.4). Antibiotic concentrations in rhizosphere soil collectively depend on the transport of antibiotics from the bulk soil to the rhizosphere, their sorption and degradation in soil, and their uptake by plant roots. Thus, ciprofloxacin and sulfamethoxazole could over-supply for the root uptake, and the other antibiotics had reached equilibrium. Next we will discuss the difference between the RCF_{bs} and RCF_{rs} values.

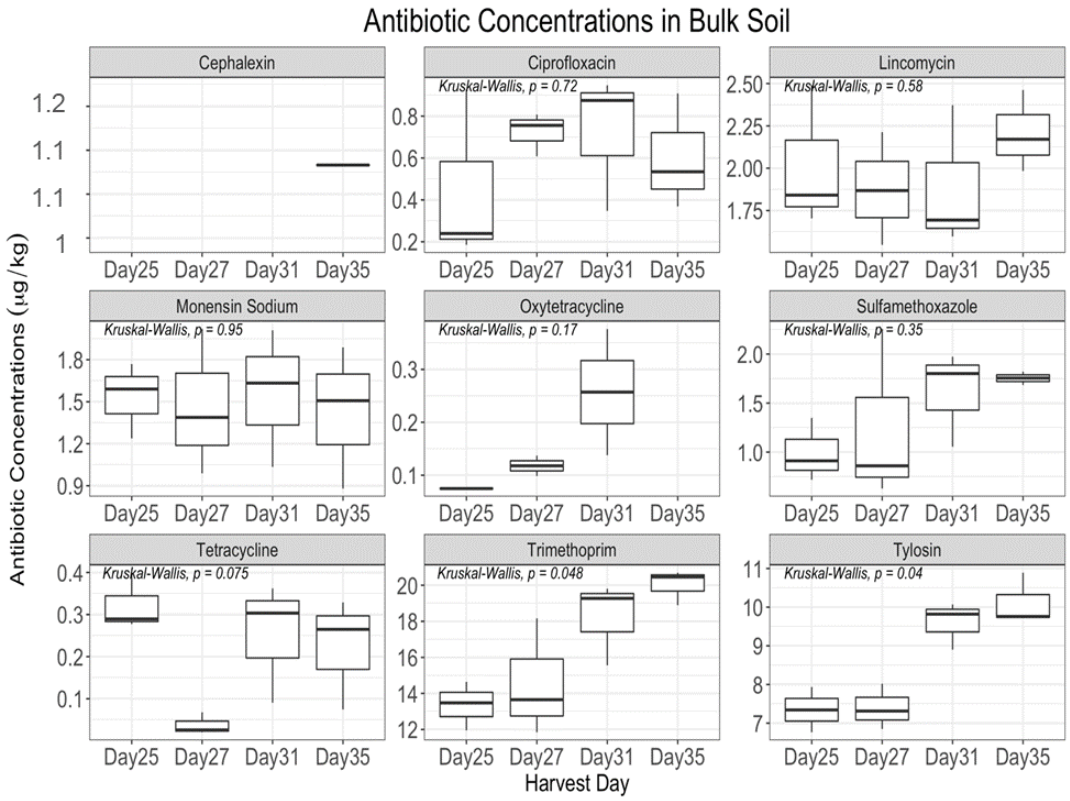

Figure 3.3: Antibiotic concentrations in the bulk soils by dry weight. Kruskal-Wallis statistics were performed for the samples at all four harvest days. No symbol for a data point indicates no detection of antibiotics. Boxplots without quantiles indicate that the concentration of triplicates were not detected.

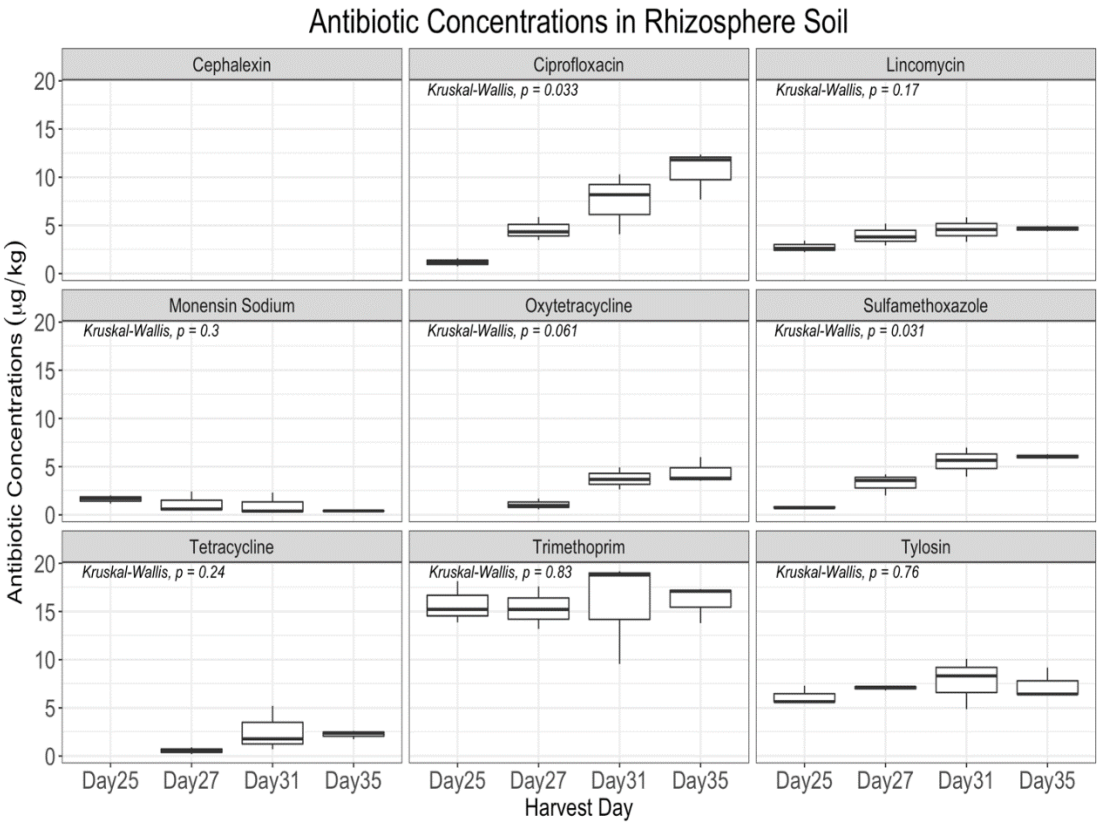

Figure 3.4: Antibiotic concentrations in the lettuce rhizosphere soil by dry weight. Kruskal-Wallis statistics were performed for the samples at all four harvest days. No symbol for a data point indicates no detection of antibiotics. Boxplots without quantiles indicate that the concentration of triplicates were not detected.

Root concentration factors and translocation factors

Compared with the RCF_{rs} values, the RCF_{bs} values were significantly greater ($p < 0.05$) for ciprofloxacin (126 ± 85), lincomycin (2 ± 1), oxytetracycline (115 ± 43), sulfamethoxazole (7 ± 2.5), and tetracycline (300 ± 258), similar for tylosin and trimethoprim, and significantly lower for monensin sodium ($p < 0.05$) (Figure 3.5, Appendix Table B3). Comparing with Bhalsod et al.[40], the dry-weight based RCF_{bs} values were comparable for monensin and tylosin, substantially

greater for oxytetracycline, and lower for lincomycin, sulfamethoxazole and trimethoprim. Additionally, Eggen et al. reported a RCF_{bs} value of 0.3 for ciprofloxacin in barley, which is 400 times lower than the measured RCF_{bs} in lettuce [97]. This suggests that RCF_{bs} measurements could be subject to very large variations, probably due to crop species, growing condition, and more importantly uneven sampling of the bulk soil. This large discrepancy also could be partly due to the varying sorption, transport, and degradation of antibiotics in different soils under different setting. Therefore, the RCF_{bs} values are determined by not only root uptake, but also sorption and transport of antibiotics in the bulk soil, whereas the RCF_{rs} are not affected by interactions of antibiotics with the bulk soil. Assuming that plant roots can better control the bioavailability of antibiotics in the rhizosphere via root exudates, the RCF_{rs} values may be more agreeable and applicable in different soil settings. Furthermore, the RCF values based on pharmaceutical concentrations in soil pore water have been proposed to better represent the root uptake of bioavailable pharmaceuticals in soils and can correlate with RCF_{bs} well if sorption of pharmaceuticals to soils is known [93]. This approach is better than merely relying on the RCF_{bs}, but still cannot account for the effect of plant root exudates in the rhizosphere. Thus, the RCF_{rs} values may be of value due to inclusion of the plant root effect.

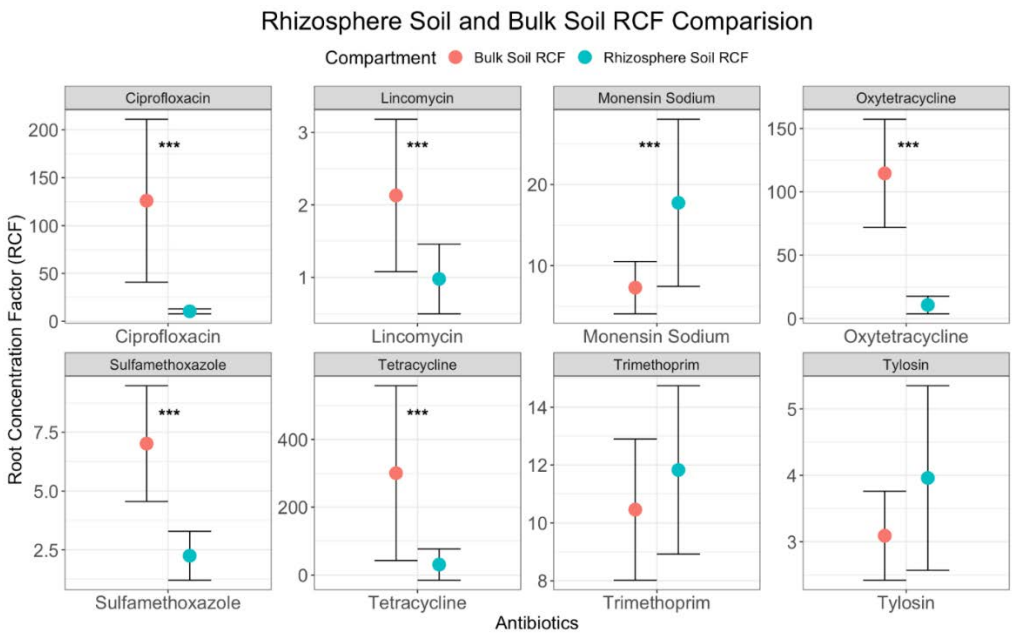

Figure 3.5: Root concentration factors (RCF) based on antibiotic concentrations in either rhizosphere soil or bulk soil. Solid red circles represent the bulk soil-based RCF_{bs}, and solid green circles represent the rhizosphere soil-based RCF_{rs}. Error bars are standard deviation. *** indicates the significant differences in means tested by the student T-test ($p < 0.05$).

Ciprofloxacin, trimethoprim, and tylosin had average TF values of 0.03, 0.04, and 0.05, respectively, indicating relatively strong affinity to the lettuce roots (Figure 3.6). Lincomycin had a TF value of 1.46 ± 0.89, and oxytetracycline, monensin, and sulfamethoxazole had TF values between 0.36–0.64 (Appendix Table B3). These TF values were in agreement with those of Bhalsod et al. [40]. The better agreements in the TF values than in the RCF_{bs} values between this study and Bhalsod et al. [40] were expected as translocation from roots to shoots was less impacted by distinct soil conditions in different experimental settings. In fact, trimethoprim was previously shown to have a strong affinity to lettuce roots [94], leading to its minimal translocation to the lettuce shoots. Additionally, cell membranes may exclude large-sized monensin and tylosin,

resulting in their accumulation in the lettuce roots [94]. When present in biosolids at environmentally-relevant concentrations, ciprofloxacin had minimal uptake potential in crops (including lettuce), with a leaf concentration factor < 0.01 [117]. Also, ciprofloxacin had a relatively low TF of 0.2–0.3 for vetiver grass grown hydroponically [118]. Based on its TF value, greater accumulation of lincomycin would be expected in the shoots, which may disproportionally influence the shoot endophiles. In contrast, other drugs would influence the bacteria in the roots more. Sulfamethoxazole, oxytetracycline, and tetracycline were intermediate-sized molecules, which had a higher potential to be taken up by the lettuce plants. However, the large variation might be the result of fluctuating low-level concentrations in the lettuce shoots, due to in-plant dissipation and low extraction efficiencies (Appendix Table B2).

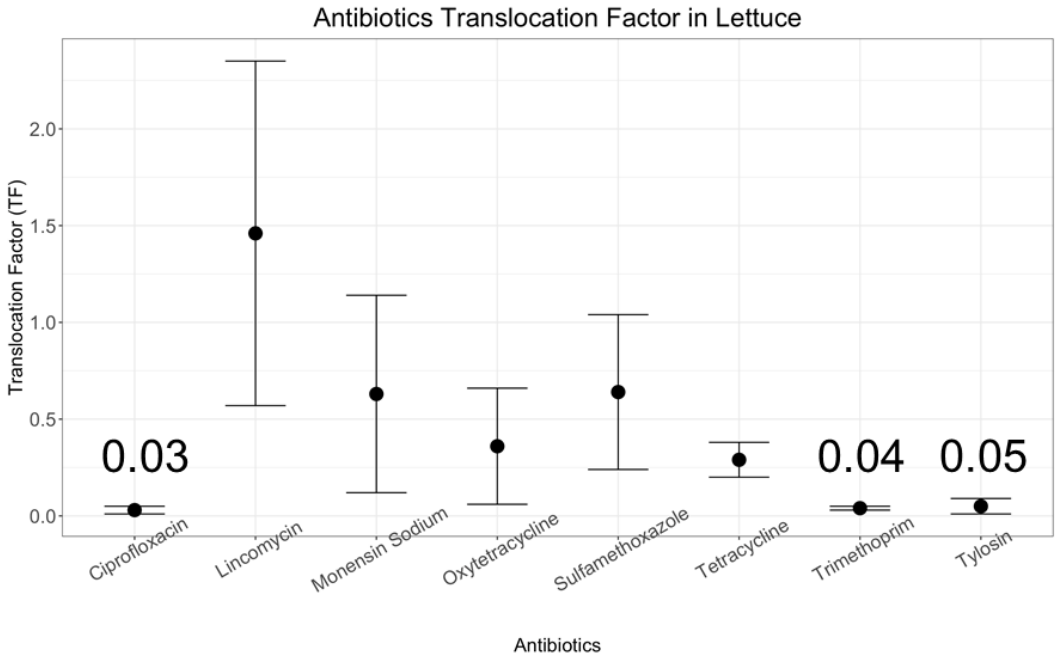

Figure 3.6: Translocation factor in lettuce shoots. Errors bar indicates standard deviation.

The majority of the recovered antibiotics were distributed in the bulk soil, partly due to the large mass of the bulk soil (Figure B1). Trimethoprim had the highest total mass recovery of approximately 75%, whereas tylosin ranked second at about 60% (Figure B1). Low recovery for some antibiotics (e.g., oxytetracycline and tetracycline) may due to their quick dissipation and strong sorption to soil matrices, which may lower their extractability [119, 120]. In addition, degradation, transformation and metabolisms in soils and lettuce plants could also contribute to the lower recovery of most antibiotics (i.e., ciprofloxacin, lincomycin, monensin, and sulfamethoxazole [121-123].

Conclusion

This study investigated the fate and transport of antibiotics in the soil-lettuce system. Compared with the RCF_{rs} values, the RCF_{bs} values were significantly greater for ciprofloxacin, lincomycin, oxytetracycline, sulfamethoxazole, and tetracycline, similar for trimethoprim and tylosin, and significantly lower for monensin. The large discrepancy between the RCF_{bs} and RCF_{rs} values suggests the need of reassessing the accuracy using the RCF_{bs} values in predicting antibiotic root uptake in diverse soil-plant systems. Our study highlights the importance of measuring the concentrations of antibiotics in rhizosphere soil to improve our knowledge regarding the fate and transport of antibiotics in soil-plant systems. This study was limited to only one crop type, one soil type, and 9 antibiotics, and its applicability to diverse soil types, crop species, and antibiotics should be examined in future studies. It will be important to further assess if there is greater variations among the RCF_{bs} values than the RCF_{rs} values, and confirm if the RCF_{rs} values will be more suitable for predicting plant uptake of antibiotics from soils.

CHAPTER IV

Bacterial Community Assembly and Antibiotic Resistance Genes in the Lettuce-Soil System upon Exposure to Anthropogenic Antibiotics

Abstract

Vegetables are important to a healthy and nutritious human diet. Microbiomes and antibiotic resistomes in vegetables may influence the human gut microbiome and ultimately human health. However, little is known about how vegetable microbiomes and antibiotic resistomes respond to anthropogenic antibiotics in crop irrigation water containing trace-level antibiotics. This study investigated bacterial community assembly and profiles of antibiotic resistance genes (ARGs) in lettuce (*Lactuca sativa*) shoots and roots, rhizosphere soil, and bulk soil irrigated with antibiotic-containing water, using 16S rRNA amplicon sequencing and high throughput real time qPCR, respectively. Using normalized stochastic ratio (NST) calculations, bacterial communities were more of stochastic assembly in the rhizosphere soil (83%–86%) and bulk soil (81%–84%), and less of stochastic assembly in the lettuce roots (45%–48%), suggesting a stronger deterministic control of plant roots in bacterial community assembly. Antibiotic exposure did not substantially change the stochasticity of the bacterial communities, despite the NST values were significantly increased by ~3% ($p < 0.05$) for the rhizosphere soil and lettuce roots and significantly decreased by ~3% ($p < 0.05$) for the bulk soil, when exposed to antibiotics. Alpha diversity values for the rhizosphere soil and lettuce roots exposed to antibiotics remained unchanged, but were significantly decreased for the bulk soil and lettuce shoots ($p < 0.05$). The levels of *Methylophilaceae* and *Beijerinckiaceae* were significantly different between the antibiotic and antibiotics-free control treatments. Finally, the rhizosphere soil is a hotspot for interactions between ARGs, mobile genetic elements, bacterial communities, and antibiotic residues.

Introduction

Vegetables are important to a healthy and nutritious human diet [124, 125]. In addition to nutrients in vegetables such as vitamins, calcium, iron, and antioxidants, recent studies highlighted the importance of the vegetable microbiomes on the human gut microbiome and ultimately human health [26, 27, 34, 39]. In water-stressed regions, crop irrigation with reclaimed water (e.g., treated wastewater effluents) or other water sources contaminated with trace contaminants is increasingly necessary [126]. For example, in California 37% of reclaimed water has been used in agricultural irrigation [127].

Due to their extensive use in human medicine and livestock production, antibiotics have become one group of trace contaminants widely present in irrigation water sources. Concentrations of tetracycline, sulfamethoxazole, ciprofloxacin, and trimethoprim ranged from 2.5 to 23.6 µg/L in some wastewater effluents [5, 128]. Plant contact with or absorption of irrigation water containing trace levels of antibiotics may change both commensal and pathogenic bacterial populations in vegetables [91, 129]. However, most bacteria naturally carry background antibiotic resistance genes (ARGs) and are capable of acquiring ARGs via horizontal gene transfer [1, 52, 55, 130], with antibiotic resistant bacteria (ARB) having been isolated from vegetables produced in home gardens, commercial fields, and greenhouses [131, 132]. In fact, the frequency of horizontal gene transfer is thought to increase upon exposure to low dose of antibiotics [12, 33]. Thus, ARB in the vegetable microbiome can be an important food safety issue, considering that antibiotic resistant pathogens cause about 35,000 deaths each year in the US [133]. In particular, some *Salmonella* phenotypes have shown increased multidrug-resistance and decreased susceptibility to antibiotics [131], and may contaminate vegetables through contact with animal manure, wild life or soil particles during growth, harvesting, or postharvest processing. Drug-resistant non-

typhoidal *Salmonella* may result in 212,500 infections and 70 deaths each year in the US [133]. Therefore, it is important to assess the drivers of change in bacterial communities (including invading pathogens such as *Salmonella*) and ARG profiles associated with vegetables upon exposure to trace-level antibiotics via irrigation.

In principle microbial community assembly is driven by deterministic and stochastic processes [134, 135]. The deterministic processes are non-random, niche-based, and may include interspecies interactions (e.g., competition, mutualism, and predation), and environmental factors (e.g., pH, temperature, salts, etc.) [135]. The stochastic processes include ecological drift, dispersal, and diversification [135]. Microbial community assembly is highly dependent on niches [135]. Previous studies have most often determined the changes in alpha diversity for within-sample species diversity and beta diversity for inter-sample species diversity, as well as shifts in ARG profiles, under environmental stresses such as antibiotics, heavy metals, and other contaminants [7, 11, 12, 91]. These studies showed that environmental stresses altered bacterial community and ARG profiles. However, the relative contributions to bacterial community changes by stochastic or deterministic processes were not assessed. Elucidating the deterministic or stochastic control of bacterial communities is important to developing effective mitigation strategies.

When soil-plant systems (e.g., roots, shoots, rhizosphere soil or bulk soil) are exposed to anthropogenic antibiotics, bacterial community may first experience an initial shock and then either stabilize via three stabilization scenarios (dying of less competitive strains; co-existence of all strains by occupying different metabolic niches; or separation of strains into different spatial niches) or develop an unstable continued aggression [136]. Thus, it is important to first determine the bacterial taxonomic differences with or without antibiotic exposure, which can be performed using the linear discriminant analysis (LDA) effect size (LEfSe) method [137]. The contribution of

stochastic and deterministic processes on bacterial community assembly can then be assessed by determining normalized stochastic ratio (NST) recently proposed in 2019 [134]. The NST method has been used to assess microbial community assembly in various ecosystems such as groundwater, river, and rhizosphere [134, 138, 139]. Stochastic processes were found to drive the community assembly of desert soils from four continents and were responsible for the spatial and temporal patterns of river microbial communities during the wet and dry seasons [138, 140]. In one study, injecting organic carbon in groundwater shifted the groundwater community from deterministic to more stochastic assembly, with a later reversion back to the deterministic assembly [134]. Thus, the use of the NST method may provide insights into bacterial community assembly in soil-lettuce systems after antibiotics exposure.

Therefore, this study aimed to: i) assess bacterial community assembly with or without antibiotic exposure, using bacterial diversity indexes, taxonomic profiles, and NST values; and ii) quantify ARGs in a soil-lettuce system using high throughput real-time qPCR. Specifically, we evaluated the bacterial communities and ARGs in lettuce shoots, roots, rhizosphere soil and bulk soil after antibiotic exposure. Lettuce was used as a model vegetable crop since it is both highly popular and consumed raw.

Materials and Methods

Growth of lettuce

A growth chamber experiment was performed at an average temperature of 24 °C, artificial light intensity of 565 μmol/m^2-s, 16 hours of lighting per day, and a relative humidity of 37%±13% in a Biosafety Level 2 Lab. Sandy loam was collected from a field site planted with poplar trees from 1980s to 2017 and had no previous exposure to pesticides or animal manure.

Characteristics of the soil after sieving were measured as follows: 69.6% sand, 19.6% silt,10.8% clay, pH of 6.2, 1.7% organic matter, 27.5 mg/kg Bray P1 extractable phosphorus, and ammonium acetate extractable potassium (K) and magnesium (Mg) of 75 and 79 mg/kg, respectively. The soil had a cation exchange capacity of 5.2 cmol(+)/kg with the exchangeable bases of 4.9% K, 16.7% Mg, and 78.5% Ca, respectively. Nursery pots (10.0 cm in height and 12.7 cm in diameter) were packed with ~1.28 kg of the sandy soil at a bulk density of 1.1 g/cm^3 to a height of ~9.5 cm.

Nine commonly used antibiotics, including sulfamethoxazole, trimethoprim, lincomycin, oxytetracycline, monensin sodium, tylosin, ciprofloxacin, cefalexin, and tetracycline , were selected based on varying molecular weight, acid dissociation constant (pKa), hydrophobicity, and water solubility (Appendix Table C1). A total of 30 lettuce plants, including 15 plants in the control chamber and 15 plants in the antibiotics treatment chamber, were first seeded in a compost mixture, and then transplanted and grown in nursery pots. Nursery pots were shifted daily to ensure similar light coverage within the growth chambers. The control plants were irrigated with antibiotics-free water at the soil surface, whereas the plants in the antibiotics treatment received antibiotics-containing water. The detailed experimental procedure and irrigation schedule were described in CHAPTER III.

To mimic co-occurrence of commensal and pathogenic bacteria, the leaves of the lettuce plants were inoculated with two nonpathogenic *Salmonella* strains (*Salmonella enterica* serovar Typhimurium LT2 and MHM (ATCC2828)). The strains were first grown in tryptic soy broth-yeast (TSB-YE) at 35°C for 48 h and then combined in equal volumes to obtain a 2-strain cocktail. The leaves of each lettuce plant were inoculated by gently smearing 1 mL of the

Salmonella cocktail at Day 25 and then irrigated as described above. The lettuce plants were grown for 35 days after seedling transplantation.

Sample collection

A sequential sampling scheme was used to collect lettuce root, shoot, rhizosphere soil, and bulk soil samples. Three triplicates lettuce plants grown with or without antibiotic exposure were harvested on Days 25, 27, 30, and 35. Lettuce shoot samples were cut at the stem base using a 70% ethanol-sterilized scissor and then separated into two subsamples. One subsample was immediately assessed for the numbers of *Salmonella* and the other weighed and placed in a −20 °C freezer. The frozen samples were later freeze-dried, ground, and mixed for analyses of bacterial microbiome, ARGs, and antibiotic concentrations. To enumerate the *Salmonella* concentration in the lettuce shoot samples, the lettuce shoot subsample from each plant was placed in a sterile disposable whirl-pak bag, diluted in phosphate-buffered saline (PBS) at the ratio of 1:18, and homogenized in a Stomacher®400 circulator (Fermion X Ltd, UK) at 300 rpm for 1 minute. The homogenate was appropriately diluted in PBS and surface-plated on XLT4 agar (Neogen, USA), with the plates examined for *Salmonella* colonies after 24 h of incubation at 35 °C. The *Salmonella* concentration was calculated follows:

$$\mathrm{Ps} = \frac{\mathrm{CFU\ ave}}{(1 \times 2 + 0.1 \times \mathrm{n}) \times \mathrm{D} \times \frac{\mathrm{W_L}}{\mathrm{W_L} + \mathrm{W_{PBS}}} \times 10}$$

where P_s is the *Salmonella* concentration on fresh lettuce shoot (CFU/g), CFU_{ave} is the average CFU for two serial dilutions, D is the highest dilution, W_L is the lettuce fresh weight, W_{PBS} is the PBS weight, and n is the number of agar plates with detected CFUs at the lower dilution. This result was then multiplied by 10 to account for the initial serial dilution in PBS. The *Salmonella* concentrations were then logarithmically transformed.

Immediately after harvest and sample collection as described in CHAPTER III, the lettuce shoot, root, rhizosphere soil, and bulk soil samples were stored in a −20 °C freezer.

DNA extraction for microbiome and ARGs

Triplicate samples of lettuce shoot, root, rhizosphere soil, and bulk soil samples collected with or without antibiotic exposure on Day 25, 27, 30, and 35 were individually placed in PowerBead tubes, weighed, and extracted for DNA using the DNeasy Powersoil kit (Qiagen, USA) as per the manufacturer's instruction (Qiagen, USA). Total DNA concentration was determined by the Qubit® dsDNA BR Assay Kit (Life Technologies, Eugene, OR). All DNA samples were either concentrated or diluted to 15 ng/μL. There were 96 samples in this study including triplicates for the lettuce shoot, root, rhizosphere soil, and bulk soil samples at the four harvest days with or without antibiotic exposure. The bacterial microbiome was measured using the 16S rRNA amplicon sequencing technique. All DNA samples were amplified with the cycling conditions (95 °C for 2 min, 95 °C for 20 s for 30 cycles, 55 °C for 15 s, 72 °C for 1 min, and 72 °C for 10 min). The PCR products were purified and normalized with a SequalPrep Normalization Plate Kit (Invitrogen, Carlsbad, CA). A sample library was prepared by combining 5 μL of each sample. The V4 hypervariable region of the 16S rRNA gene was amplified with Illumina dual-indexed compatible primers 515f/806r to minimize the cost of long customized primers and produce more high-quality sequences [41]. Batch normalization of the amplicon libraries was conducted with Invitrogen SequalPrep DNA Normalization Plates. Products eluted from the plates were then combined and subject to quality control. After quantification with the Qubit dsDNA HS, Caliper LabChipGX HS DNA, and Kapa Illumina Library Quantification qPCR assay, the amplicon pool was loaded into an Illumina MiSeq v2 standard flow cell and sequenced in a 2×250 bp paired-end format with a v2 500 cycle MiSeq

reagent cartridge. Primers complementary to the sequences of 515f/806r primers were added to appropriate wells in the reagent cartridge to serve as sequencing and index primers [41]. Raw data were output as fastq files.

The WaferGen SmartChip Real-time qPCR System (WaferGen Bio-systems, Fremont, CA) was used to quantify ARGs and mobile genetic elements (MGEs) in the lettuce shoot, root, rhizosphere soil, and bulk soil samples. The system has 5184 individual SmartChip nanowells that provide high throughput reactors for multiple primers. The 54 primer sets of ARGs and MGEs were selected from 384 primer sets based on their detected concentrations in our previous study [91]. A total of 72 samples from three harvest days (Day 25, 30, 35) were measured for ARGs and MGEs.

Data analyses

Bacterial microbiome

The raw 16s rRNA fastq.gz data were preprocessed using Trimmomatic to cut adaptors [141]. The trimmed data were analyzed through the QIIME 2 pipeline [142]. Briefly, the dataset was demultiplexed, and ran through DADA2 to denoise and remove chimeric sequences. The feature table was calculated and mapped to match the feature ID to sequences in order to obtain the representative sequence. The representative sequence was then trained using an unsupervised machine learning to cluster through the Silva reference database [143] for OTU picking with a 99% similarity threshold determination to obtain the taxonomy composition. Afterwards, chloroplast and mitochondria contamination were removed using the Qiime2 filter table function. The dataset was then computed for all alpha and beta diversity statistics in the Qiime2 pipeline. Finally, the OTU table, alpha and beta diversity data, and related statistics were output for

downstream analyses and graphics. The entire process was written in a batch file and analyzed in the High Performance Computing Center (HPCC) at Michigan State University. To obtain adequate average sequencing depth and minimize the loss of low read samples, the lettuce shoot, root, rhizosphere soil, and bulk soil samples were separated and ran using the aforementioned batch script to obtain the alpha and beta diversity statistics. An average sequencing depth of 30,000 reads was selected for the rhizosphere soil and bulk soil samples because of greater bacterial community diversity. Meanwhile, an average sequencing depth of 10,000 was selected for the lettuce root samples. Since the lettuce shoots samples had low reads after removing choloroplast and mitochondria, an average sequencing depth of 500 was selected. The Shannon diversity matrix was selected to measure alpha diversity incorporating the Kruskal-Wallis nonparametric statistical testing. The pairwise permanova test with 999 permutation based on weighted unifraq distance was selected to calculate the statistical significance of beta diversity.

Bacterial taxonomies for the top 10 phyla and families were computed using the phyloseq package in R [144]. The linear discriminant analysis (LDA) effect size (LEfSe) method was used to determine the taxon that can most likely explain the differences between the control and antibiotics treatment groups [137]. An LDA score was first calculated using the online user interface [137] by setting the alpha value for the factorial Kruskal-Wallis and Wilcoxon tests between subclasses to 0.005 and the LDA cutoff score to 3.0. The LDA score table was output as a lefse.lefse_internal_res file and later using Unix to remove non-significant taxa. The LDA score table was then cleaned up and plotted in R using a further LDA cutoff score 3.5.

The NST values were calculated to estimate average stochasticity within a group of samples for bacterial community assembly [134] using the NST R package. Briefly, the OTU table and eight treatment groups (4 sampling compartments with or without antibiotics) were used as

community and group input files. Next, the tNST function calculated the stochasticity (the NST values) based on the Bray-Curtis distance matrix subsample for 1000 iterations. Then, tNST data were boot-strapped by subsample 1000 iterations and analyzed using the Wilcoxon test. Finally, the median, quantile, and statistical significance were input into R for graphics representation.

Antibiotic resistance genes

Cycle numbers (C_T) measured by the WaferGen qPCR were used to calculate the gene copy number via Copy Number = $10^{(30-C_T)/(10/3)}$ [42], using a cutoff threshold of $C_T < 30$. If the targeted gene were absent in two of three replicates, the ARGs detected in only one replicate were removed. The C_T values for the triplicate samples were averaged before downstream analysis. The relative abundance of detected ARGs was computed by dividing the estimated ARG copy number by the copy number of 16S rRNA. The patterns and characteristic profiles of ARGs and MGEs were then plotted as a heatmap. Finally, network analysis of four sample compartments among the percentages of family-level bacterial communities, antibiotic concentrations, and ARGs/MGEs relative abundance were conducted based on correlation tests (correlation coefficient > 0.6 or < −0.6 and *p*-value < 0.05) and plotted using Gephi v0.9.2 software. The script for ARGs analysis was performed using a custom written workflow in R. All python, R, and batch scripts are available at Yike Shen's Github page (https://github.com/YikeShen).

Results and Discussion

Antibiotic exposure on bacterial community assembly

Antibiotic exposure shifted the bacterial community composition in bulk soil, rhizosphere soil, lettuce roots, and lettuce shoots samples. Bacterial alpha diversity (Shannon diversity index)

was significantly higher for the bulk soil and lettuce shoot samples in the control treatment than in the antibiotic treatments, as shown by the Wilcoxon test ($p < 0.005$, Figure 4.1). Interestingly, antibiotic exposure did not significantly affect alpha diversity of the rhizosphere soil and lettuce root samples (Figure 4.1). Additionally, based on the Kruskal-Wallis test, the day of harvest did not significantly affect alpha diversity (Appendix Figure C2). The different trends of alpha diversity between the rhizosphere soil and root samples, relative to those of the bulk soil and shoot samples, were intriguing. When exposed to sub-inhibitory levels of antibiotics in the environment, bacterial communities may experience continued aggression or counteracting competition [136]. Thus, the bulk soil and lettuce shoots may have experienced more continued aggression, resulting in decreased alpha diversity. Additionally, antibiotic exposure may also inhibit some less abundant susceptible bacteria to undetectable levels, thus decreasing species richness [26, 69]. The counteracting competition maybe one reason why alpha diversity of the rhizosphere soil and lettuce root samples remained unchanged regardless of antibiotic exposure [136]. When species compete with one another during exposure to a stressor (antibiotic exposure), other community members in the rhizosphere may be able to neutralize the added antibiotics to stabilize the bacterial community [136]. Indeed, soil microbiome in the rhizosphere remains crucial for plant health in terms of nutrient uptake, pathogen colonization prevention, modulation of host immunity [101], and microbiome density and diversity and can therefore be considered as the "second genome" of a plant [101]. Additionally, the rhizosphere microbiome can be shaped by plant mucilage and root exudates [145]. Schlatter and Kinkel [146] found that *Streptomyces* associated with plant roots were highly antibiotic resistant, suggesting that plant have more deterministic control than soils in shaping bacterial microbiome. Another study found that actinobacterial communities in the rhizosphere of strawberry plants grown in different soils remained similar

regardless of different soil properties [147]. Soil bacterial communities can also reportedly tolerate increased levels of antibiotics when amended with root exudates [148]. Therefore, it is not surprising that bacterial communities of the rhizosphere soil and lettuce roots were not affected by antibiotic exposure in this study. However, whether changes in bacterial community diversity lead to any functional changes remains unanswered [149].

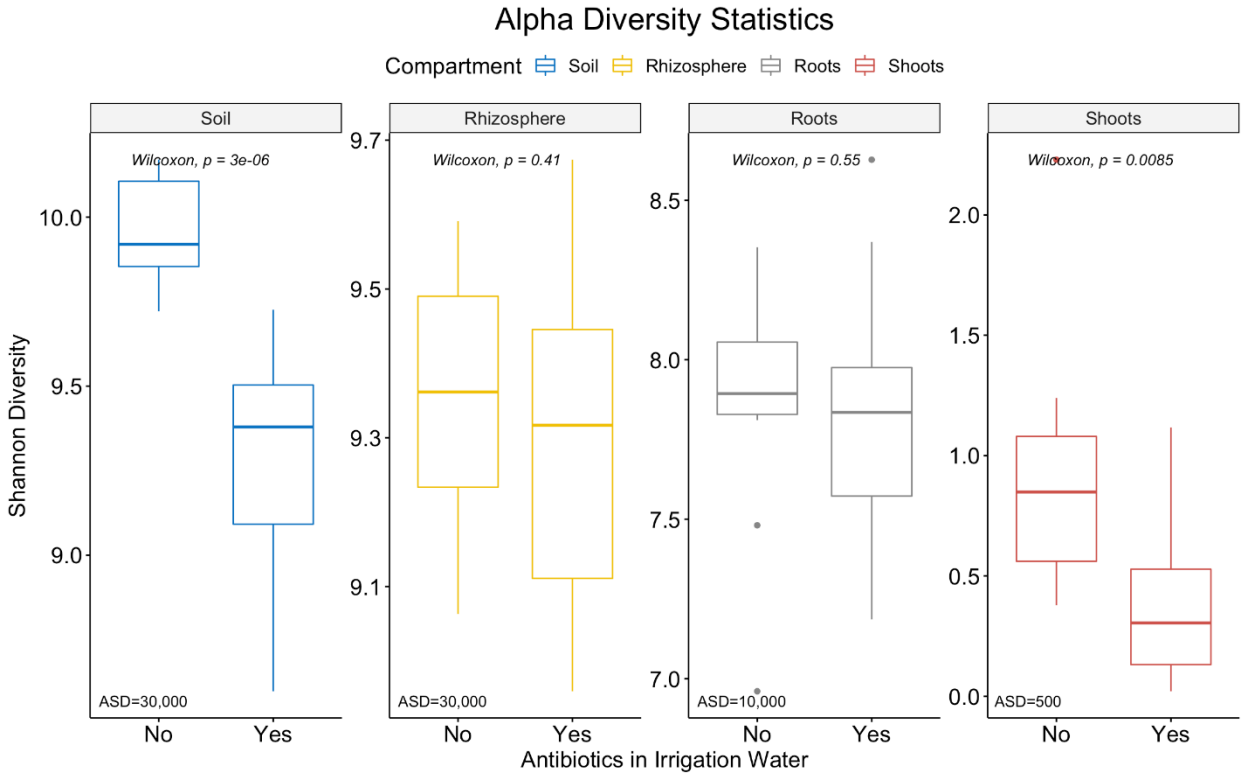

Figure 4.1: Alpha diversity statistics based on antibiotic exposure. ASD meant average sequencing depth (the number of reads).

It is important to understand which community members may contribute to bacterial community changes. Proteobacteria was the most abundant phylum in every compartment (Appendix Figure C1). The LDA score can be interpreted as the degree of consistent difference

in relative abundance between features in two classes of analyzed microbial communities [137]. The LDA score for *Methylophilaceae* was greater than 4 for the bulk soil, rhizosphere soil, and lettuce root samples. As a member of proteobacteria, *Methylophilaceae* was the significantly different taxa that was increased by antibiotic exposure (Figure 4.2). Our previous study also found that *Methylophilaceae* increased with exposure to a mixture of antibiotics and other common drugs in the soil-lettuce system, likely due to its potential to degrade exogenous chemicals [91]. *Beijerinckiaceace,* a member of phylum Proteobacteria and order Rhizobiales, was also the significant taxa with antibiotic exposure in the bulk soil, rhizosphere soil, and lettuce root samples (Figure 4.1). *Burkholderiaceae,* a member of phylum proteobacteria, was the significant taxa in both the bulk soil and rhizosphere soil samples from the control treatment (Figure 4.1). Although the changes were not statistically significant in the lettuce shoot and root samples, *Burkholderiaceae* had high abundance and ranked as the top 10 families in every compartment (Appendix Figure C1). *Micrococcaceae*, a member of Actinobacteria, was significantly different in the bulk soil with antibiotic exposure, whereas they were significantly different in the lettuce root samples from the control treatment. It is of interest to pinpoint bacteria that are either benefited from or inhibited by exposure to anthropogenic antibiotics in different niches. Our taxonomic profiles and the LDA score on significant taxa provided some insights. One well-known function of methylotrophs *Methylophilaceae* and *Beijerinckiaceae* is their ability to fix nitrogen, which is irrelevant to our study [150]. The significance of both taxa with antibiotic exposure requires further investigation. Interestingly, in taxon – *Burkholderiaceae* – that is significant in our control treatment, the same LDA pattern was also present for both bulk soil and rhizosphere soil. *Burkholderiaceae* was commonly found in diverse ecological niches, including soil, water, plants, animals, and fungi [151]. The significant pattern in the control

treatment might be because they are native to soil niches. One study found that sulfonamide and fluoroquinolone significantly decreased the relative abundance of *Micrococcaceae* in swine manure [152]. *Micrococcaceae* was also detected in the phyllosphere [153]. However, the reason for the opposite findings in the bulk soil and lettuce roots remain unknown. Also significant difference patterns may be still unable to reveal the competition mechanisms in community assembly - continued aggression or counteracting competition. Therefore, we further investigated the correlation patterns between significantly different taxa and antibiotic concentrations (Figure 4.5). The family *Methylophilaceae* likely benefited from total antibiotics and sulfamethoxazole, showing a significant positive correlation in the bulk soil. Interestingly, in the lettuce roots where we observed an increase and significant differences in *Methylophilaceae* (Figure 4.2, Appendix Figure C1) with antibiotic exposure, a significant negative correlation was seen for tylosin and trimethoprim. *Beijerinckiaceae*, a member of the methylotrophs, had a significant positive correlation with trimethoprim (Figure 4.3). Although both taxa had significant differences (Figure 4.3) with the overall relative abundance of both taxa increasing with antibiotic exposure (Appendix Figure C1), ongoing competition in the roots may undermine the small microbial community changes from antibiotic exposure, resulting in non-significant changes in alpha diversity. In addition, no significant correlation was observed between *Methylophilaceae* and any of the antibiotics in the rhizosphere soil (Figure 4.3). Thus, *Methylophilaceae* may have benefited from the inhibition of other bacteria by added antibiotics, and thus outcompeted to achieve increased relative abundance. The taxa *Beijerinckiaceae* may directly benefit from ciprofloxacin and trimethoprim in the rhizosphere soil and roots, resulting in significantly increased relative abundance with antibiotic exposure (Figure 4.2, Figure 4.3, and Appendix

Figure C1). Correlation results based on the Spearman coefficient values and *p* values can be found in Appendix Table C4.

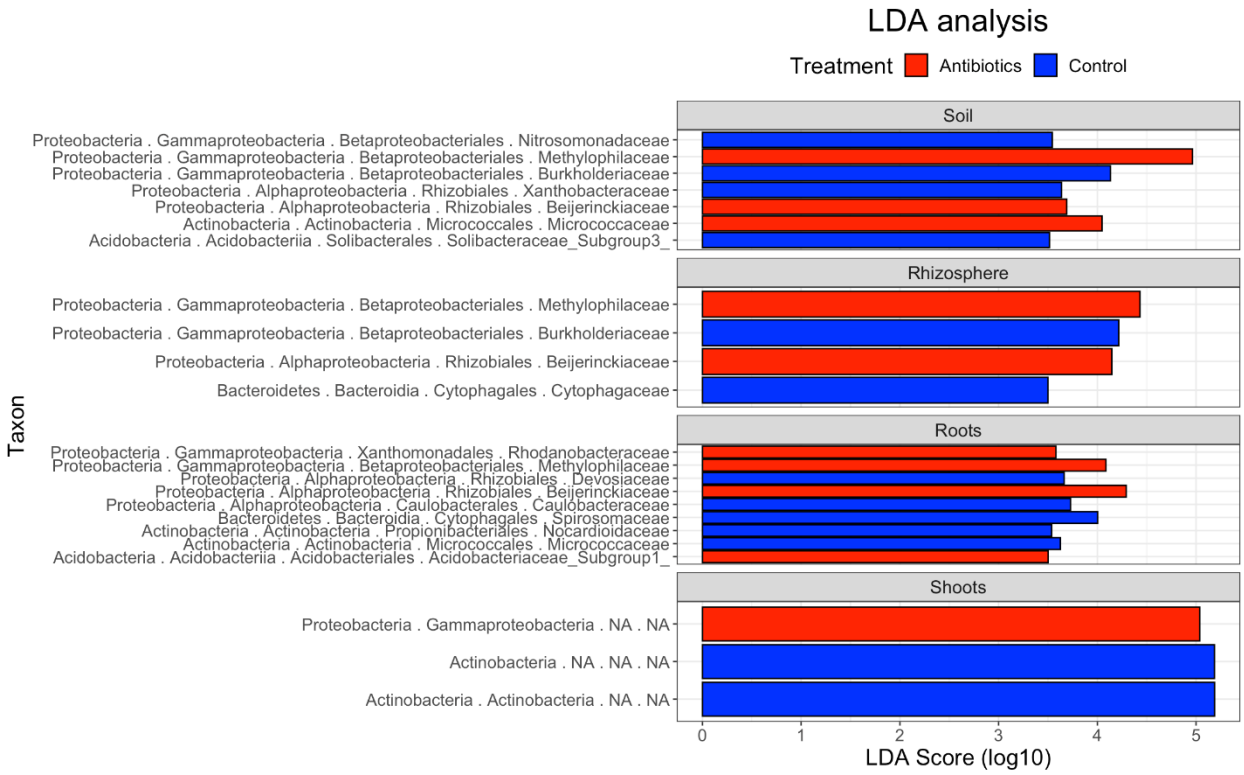

Figure 4.2: LDA analysis of the bacterial community. X-axis showed log10 transformed LDA score. Cutoff LDA>3.5 was used.

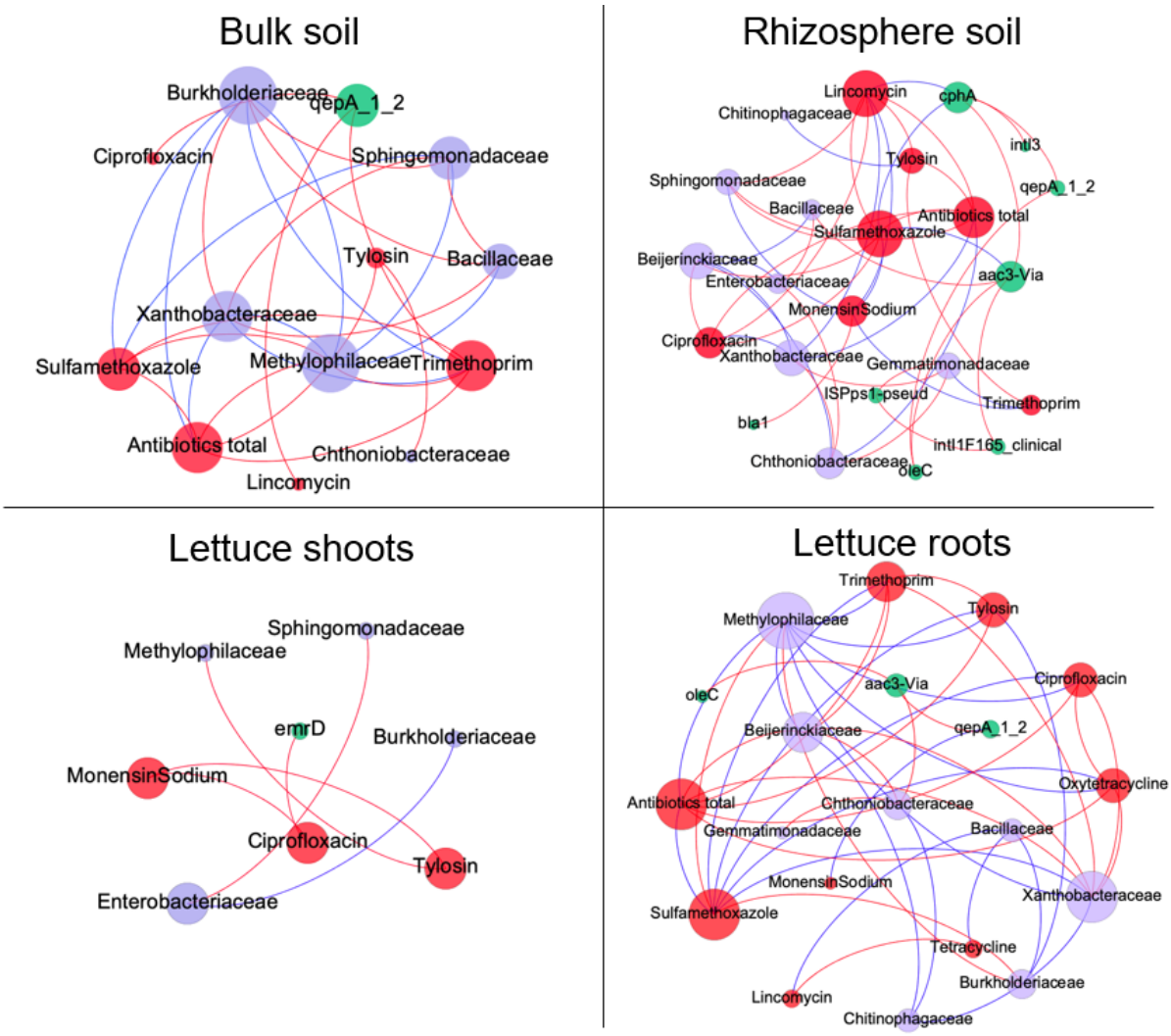

Figure 4.3: Network of the Spearman's correlations between ARGs, antibiotic concentrations and bacterial families for the lettuce shoot, root and soil samples exposed to antibiotics. Red nodes are the antibiotic concentrations, purple nodes are bacterial families, and green nodes are ARGs and MGEs. Red lines indicate positive correlations (Correlation coefficient > 0.6, $p < 0.05$), and blue lines indicate negative correlations (Correlation coefficient < −0.6, $p < 0.05$).

The beta diversity was statistically significant with or without antibiotic exposure in each compartment (Figure 4.4). The weighted unifrac distance matrix considered both structure and membership of the community, while PCoA ordination gave the simplest and best separation between groups. The pairwise permanova test with 999 permutation increased the confidence of the ordination results. Beta diversity was used to measure ecological distances [154]. Exposure to antibiotics significantly increased the distance of beta diversity in each compartment. Meanwhile, there were no statistical differences in beta diversity for the samples collected on four harvest days (Appendix Figure C3). The lettuce and soil bacterial communities were stable within the span of 10 days. Another study found that beta diversity was stable over a period of seven months, regardless of the field seasonal patterns in Michigan [155]. However, it is unknown if antibiotics were the deterministic factor. The mechanisms of community assembly need to further studied to comprehend community changes.

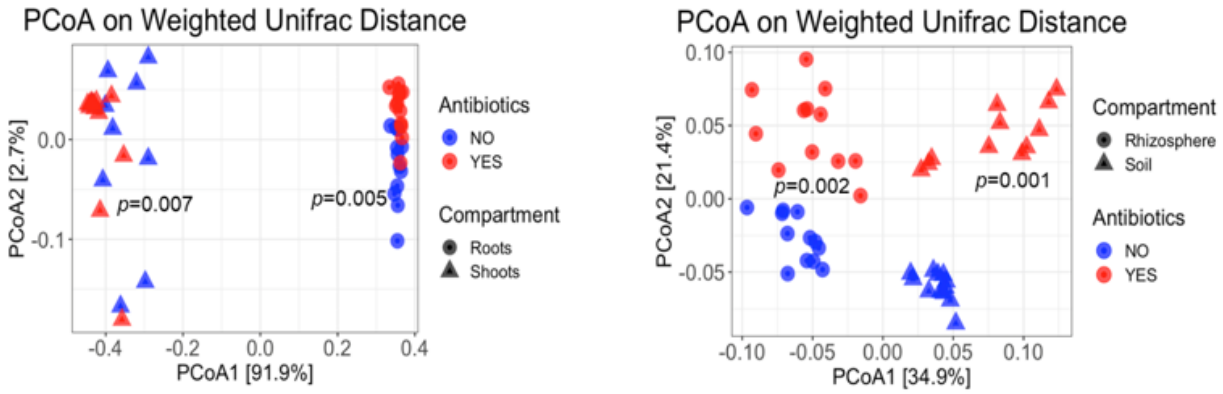

Figure 4.4: Beta diversity ordination and statistics for the bulk soil, rhizosphere soil, lettuce roots, and lettuce shoots with or without antibiotic exposure.

The NST values are used to determine if community assembly changes were more stochastic or deterministic [134]. Arbitrarily a NST value above 50% indicate a more stochastic assembly, whereas a NST value below 50% suggests a more deterministic assembly [134]. Moreover, it is often more revealing to examine the changes of NST values among treatments (e.g., antibiotic exposure). In this study we hypothesize that antibiotic exposure can be considered as a deterministic factor, and other deterministic and stochastic factors remained the same for both antibiotic exposure and control treatments. The soil samples with or without antibiotic exposure had the NST value above 50% (i.e., 83%–86% in the rhizosphere soil and 81%–84% in the bulk soil) (Figure 4.5), favoring the stochastic processes. Adding antibiotics slightly increased the NST value for the rhizosphere soil and decreased the value for bulk soil (Figure 4.5). Root samples had a NST values below 50% (i.e., 45%–48%), favoring a deterministic assembly. Antibiotics were not the deterministic force in lettuce roots since the NST value increased slightly with antibiotic exposure. Lettuce shoots had too few reads to compute the NST values. Furthermore, samples from different harvest days showed no apparent trend in NST changes (Appendix Figure C4), likely due to the short sampling period. Generally, the changes of the NST values were small with antibiotic exposure (Figure 4.5). When exposed to antibiotics, the NST values were significantly increased by ~3% ($p < 0.05$) for the rhizosphere soil and lettuce roots and significantly decreased by ~3% ($p < 0.05$) for the bulk soil. Thus, antibiotic exposure did not solely control the bacterial community assembly. In fact, bacterial community in each niche always had interspecies interaction – competition – as the deterministic force. It is interesting to observe the consistent trend of changes for the rhizosphere soil and lettuce roots with increased stochasticity and constant alpha diversity. Lettuce roots had a major impact on the stochasticity of both roots and the rhizosphere. In one study the microbial

communities of the plant roots exhibited complex interactions that influenced bacterial assemblage [156]. Liu et al. [139] found that rhizosphere bacterial assembly was more of a deterministic process when nitrogen was added. The rhizosphere and plant root microbiome are incredibly resistant to stresses. One study found that the core membership of the microbiome in the bean rhizosphere was persistent across different continents [157]. In fact, the bacterial communities in the rhizosphere soil and lettuce roots may have master species that govern the community assembly, increase the selectivity, and diminish the effect of environmental stressors. The absolute value of stochasticity also depends on different environmental niches. Previous study found that bacterial community in soil niches were much more diverse than those in plant root and shoot niches [91]. The diversity of soil microbiome was also demonstrated by the higher sequencing reads than the lettuce root microbiome. When the community was more diverse, stochastic processes may be more common. For example, when the community had a higher number of species, the effect of ecological drift, and random death and birth could be more pronounced. More species may also enable horizontal gene transfer between different bacteria species, enabling diversification (a key stochastic process). The major stochastic processes in soil niches are more dominant than added trace-level antibiotics, resulting in the overall more stochastic assembly. The quantification of the NST values with antibiotic exposure, for the first time, provides new insights into how antibiotic stress drove the community assembly in the rhizosphere soils and roots. The results helped us better understand the dynamics of bacterial community assembly.

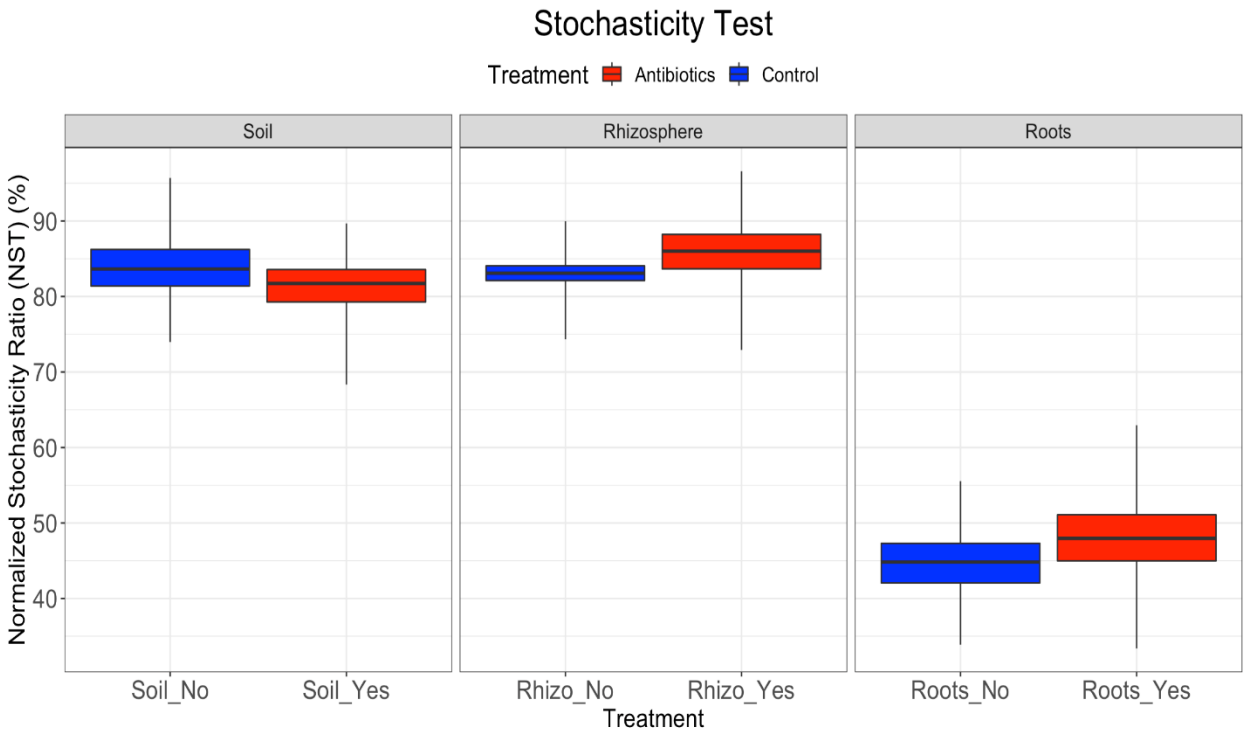

Figure 4.5: Normalized stochastic ratio based on antibiotics exposure.

We further investigated the survival of an opportunistic pathogen (*Salmonella*) on lettuce shoots to assess if changes in the lettuce microbiome reduced *Salmonella* invasion. Overall, *Salmonella* was unaffected by antibiotics exposure (Figure 4.6). Since the antibiotics were taken up into the lettuce shoots, direct contact between the antibiotics and *Salmonella* on the shoot surface was likely minimal. Given the non-ideal growth conditions on lettuce shoots, low nutrient levels and humidity on the lettuce shoots may play a more prominent role in the *Salmonella* survival. Additionally, the spiked *Salmonella* concentrations might be too high to detect small changes in their survival, if any.

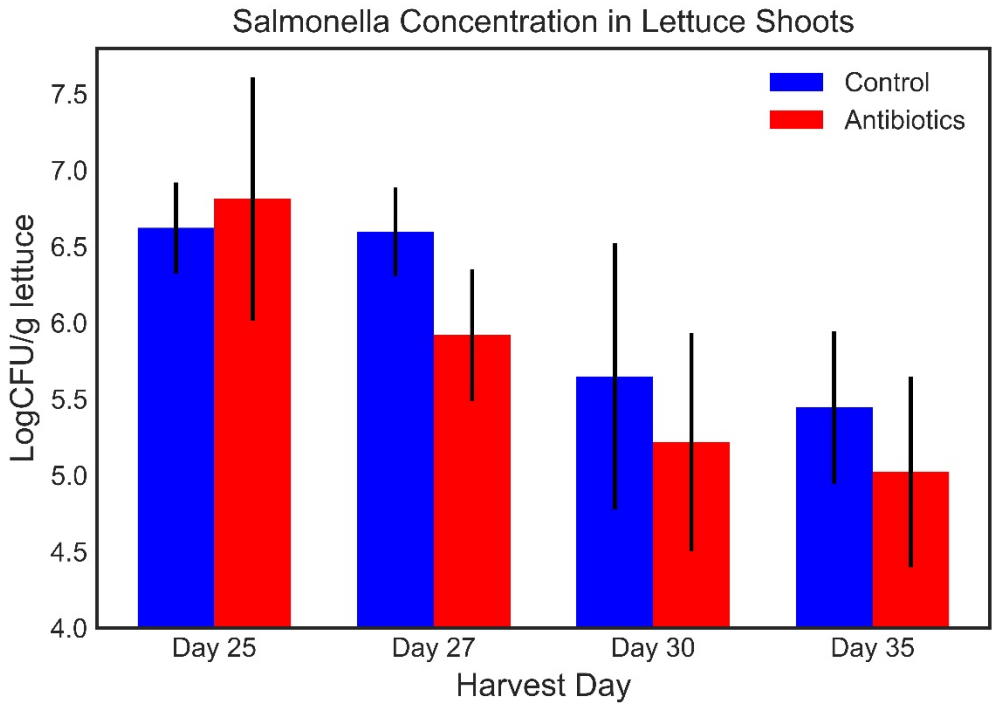

Figure 4.6: *Salmonella* concentrations in lettuce shoots over time. Compared by the student T-test. No significant changes with or without antibiotic exposure.

Antibiotic resistance genes

A total of 18 ARGs and MGEs were detected out of the 52 ARGs targeted and their primer sets are listed in Appendix Table C2. The sum of log 2 transferred relative abundance of ARGs or MGEs to 16S rRNA refers to each resistance classes which is summarized in Appendix Table C3. Figure 4.7 shows the relative abundance profiles of ARGs and MGEs. The rhizosphere soil samples in the control treatment yielded the highest number of ARGs and MGEs. With antibiotic exposure, multidrug resistant genes (MDR) (*tnpA* and *mepA*) decreased to undetectable levels. However, a consistent increase in MGEs was observed with antibiotic exposure

(*incP_oriT*, *intl1F165_clinical*, *ISPps1-pseud*, *ISSm2-Xanthob*, and *intl3*). Horizontal transfer of ARGs is often associated with MGEs such as integrons when suitable recipient bacteria are present [52, 55, 130]. Increased dissemination of MGEs may increase the risk of horizontal gene transfer of ARGs. Interestingly, no consistent pattern of change in ARGs and MGEs were observed in the bulk soil exposed to antibiotics. The *intl3* and clinical integrase (*intl1F165_clinical*) gene both increased in the bulk soil by the last day of harvest. Lettuce roots exhibited an increase in MGEs (*incP_oriT*, *ISSm2-Xanthob*, *intl3*) similar to the rhizosphere soil. It visually appeared that except for MGEs the remaining ARGs resistance classes did not have a consistent pattern of change with antibiotic exposure. Overall, ARGs or MGEs detected in the lettuce shoots were minimal compared with those in the lettuce root, rhizosphere soil, and bulk soil samples. Antibiotic exposure did increase the relative abundance of MDR (*emrD* and *mepA*) in the lettuce shoots, or the beta-lactam resistant gene (*blaTEM*) in the lettuce shoots and roots.

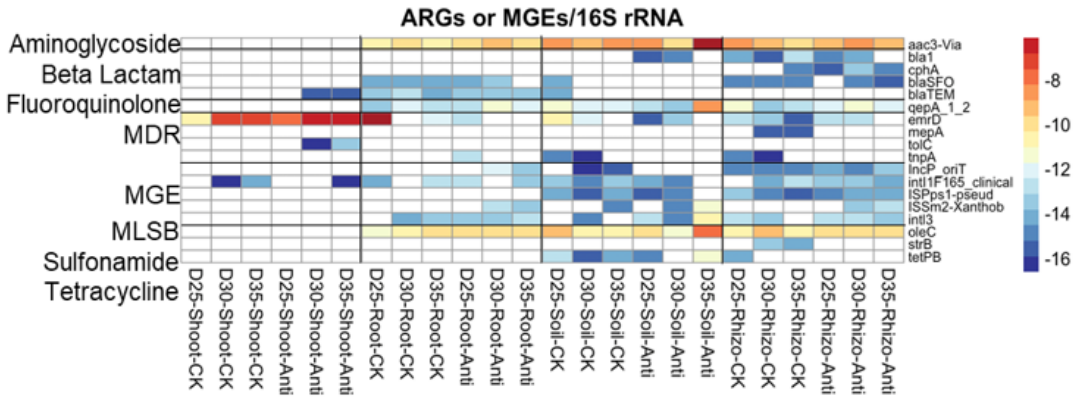

Figure 4.7: Relative abundance (gene copy number/16S rRNA gene copy number) of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs). Data were Log 2 transformed. Blank cells indicate that genes were either not detected or below the detection limit. Colored bar on the right indicates relative abundance from low (blue) to high (red).

The rhizosphere soil and lettuce roots had higher bacteria community interactions and ARGs exchanges than the bulk soil and lettuce shoots (Figure 4.3). Interestingly, the only MGEs (*intl3*, *intl1F165-clinical*, and *ISPps1-pseud*) having significant correlation with bacterial community and antibiotic concentrations were found in the rhizosphere soil (Figure 4.3). The MGEs could be horizontally transfer from one bacteria to another bacteria host, increase the chance of bacteria resistance to antibiotics [1]. The rhizosphere soil and lettuce roots as hotspots for bacterial interactions and gene exchanges further supported the resilience of the bacterial community in the rhizosphere soil and lettuce roots to antibiotic stress.

Conclusion

This study provides several new insights into better understanding bacterial community assembly and ARG profiles when soil-plant systems are exposed to anthropogenic antibiotics. With antibiotic exposure, alpha diversity of bacterial community did not change in the rhizosphere soil and lettuce roots, but decreased in the bulk soil and lettuce shoots. The differences may because of the resilience of root and rhizosphere microbiome to external stressors. Significant taxa may either benefited from antibiotic exposure, showing significant positive correlations, or inhibited by antibiotic exposure, showing significant negative correlations. The ongoing competition between antibiotics and bacterial community may offset small community changes in the rhizosphere soil and lettuce roots, resulting in non-significant changes in alpha diversity. Beta diversity was statistically significant with antibiotic treatments. Using the NST calculations, bacterial communities were more of stochastic assembly in the rhizosphere soil (83%–86%) and bulk soil (81%–84%), and less of stochastic assembly in the lettuce roots (45%–48%), suggesting a strong deterministic control of plant roots in bacterial community assembly. Additionally, antibiotic exposure did not substantially change the

stochasticity of the bacterial communities, despite the NST values were significantly increased by ~3% ($p < 0.05$) for the rhizosphere soil and lettuce roots (3%) and significantly decreased by ~3% ($p < 0.05$) for the bulk soil, when exposed to antibiotics. The MGEs in the rhizosphere soil generally increased with antibiotic exposure, whereas the lettuce root, shoot and bulk soil had non-uniform changes. Furthermore, the hotspots of ARGs, bacterial community, and antibiotic interactions were found in the rhizosphere soil and lettuce roots. This study provides knowledge of bacterial community assembly in the model vegetable production system using antibiotics as the only environmental stressor.

CHAPTER V

Predicting Customers' Buy and Pay Preferences for Labeled Products with Machine Learning

Abstract

Food labeling is being promoted as an effective approach for encouraging safe and healthy practices of food production and consumption. It is important to understand how consumers' buy and pay preferences for labeled products vary as related to their demographics, food-relevant habits, and foodborne disease perceptions. This study used both conventional statistical and novel machine learning models on survey data to predict consumers' buy and pay preferences regarding eight labels related to food production and consumption. Results showed that increases in the frequency of cooking fresh produce corresponded with significant increases in the probability of participants' willingness to buy andwillingness to pay more for *USDA Organic* products. Living in urban areas significantly increased the probability of participants' willingness to buy and willingness to pay more for products labeled with *Raised without Antibiotics* and *Does Not Contain Medically Important Antibiotics*. Our machine learning models provided a new means for evaluating food safety and labeling survey data and produced adequate average prediction accuracy scores for all eight labels. The label, *Raised Without Antibiotics*, had a high average prediction accuracy for both participants' willingness to buy and willingness to pay more. Thus, the machine learning models may be used to analyze food survey data and help develop strategies for promoting healthy food consumption.

Introduction

Food labeling is being promoted as an effective approach for encouraging safe and healthy practices of food production and consumption [159-161], and the number of food items with healthy labels (e.g., organic, antibiotics-free, and cagefree) is increasing in the market [161-163]. Consumers' purchasing preferences are thought to relate to information directly available on the food package [160, 162], including brand, ingredients, product origin, and production method. Each

food label in the market has different price premiums compared with other products with no specific labels [162]. In fact, food producers are increasingly responding to consumers' preference for "green" or "healthy" products by certifying their food products with sustainable and/or healthy labels. One common example in the US is *USDA Organic* certified foods, which are produced without the use of certain synthetic chemicals such as antibiotics, growth hormones, synthetic pesticides, and chemical fertilizers [164]. Food producers follow a set of protocols to produce organic-certified foods: (*i*) before harvest, the land must be free of prohibited chemicals for 3 years; (*ii*) animals must be raised on 100% organic feed or on 80% organic feed for 9 months followed by 100% organic feed [164]. Organic foods are generally perceived as beneficial to both human and environmental health. However, some *USDA Organic* certification criteria are difficult to achieve. Therefore, some producers seek for alternative practices to meet consumers' buy and pay preferences [165]. There is growing consumer interest in decreasing the use of antibiotics in livestock production to reduce the risk of proliferating antimicrobial resistance [165, 166]. As a result, an increasing number of food products with labels such as *Raised Without Antibiotics*, *No Medically Important Antibiotics*, and *Cage Free* are now on the market. Previous studies showed that consumers prefer to buy and pay more for a familiar brand [167, 168], thus consolidating most market shares into major brands. However, local and community-supported farms are rapidly gaining popularity along with the label *Locally Raised* [169].

Various factors can shape consumers' perception of labeled food products, including demographics (e.g., gender, age, ethnicity, household size, education, employment, marital status, and residence type), food-relevant habits (e.g., fresh produce washing and cooking frequencies, and grocery shopping destinations), and foodborne disease perception (pathogen risk perceptions) [170-173]. Thus, it is important to understand how consumers' demographics, food-

relevant habits, and foodborne disease perception are linked to their behaviors in buying and paying more for specially labeled products.

Traditional approaches to understanding consumer preferences utilize conventional statistical methods to find the fewest critical independent variables that contribute to changes in dependent variables [174-178]. Statistical regression methods usually combine similar categorical independent variables to one or two categories, transform ranked categorical independent variables to numerical variables, and identify numerical variables to achieve the best fit. One challenge for this approach to analyzing survey data is to incorporate all variables from multiple questions to determining the conditional probability of participants' buy and pay preferences. This challenge can be overcome by novel machine learning methods. Machine learning methods can integrate specific questions and all survey responses into models without transforming or combining survey data. They can also provide a prediction accuracy score to evaluate the accuracy of individual customers' buy and pay preferences using their survey answers. Thus, machine learning methods enable computers to learn and predict patterns of behaviors through established statistical models. Nonetheless, the interpretation of machine learning models is a long-standing issue [179-181].

Previous work suggests that consumers' willingness to buy and pay more for some food labels are significantly predicted by demographics and other relevant questions [162, 166, 172, 182]. For example, woman, elderly, and higher income household are willing to pay price premiums for products with All–Natural labels [162]. One study found eco-label placed in the middle of conventional and organic labelled apples for consumers' preferences [172]. Additionally, convenient behaviors were found to increase the preferences of consumers buying organic products [182]. However, these previous studies are limited in two critical ways. First, few studies

have been conducted to assess multiple questions and multiple labeled products. Additionally, no studies have incorporated both machine learning and statistical methods to understand food-related survey data. Thus, this study fills this gap by synthesizing and interpreting survey responses to a group of questions for informing consumers' preferences to labeled products, using both statistical and machine learning methods. We hypothesize that demographics, food-relevant habits, and foodborne disease perceptions will shape the customers' willingness to buy and pay more for labeled products.

Materials and Methods

Survey Instrument

The annual survey of the Assessment of Sustainability Knowledge and the Sustainability Attitudes Scale by the Environmental Science and Policy Program at Michigan State University was implemented through the Qualtrics platform. Each iteration of this survey included special topics related to humans and the environment. This study used the de-identified secondary survey data from an 11-question module designed specifically on food safety and food labels. Survey recruitment began on February 21, 2019 and ended on March 13, 2019. A total of 1080 participants started the survey, but 340 participants were screened out by Qualtrics for failing to complete the survey or providing low quality data. The remaining 740 participants were used in the current study. Figure 5.1 provides a spatial distribution map of survey participants based on their ZIP Codes using the ggmap package in R.

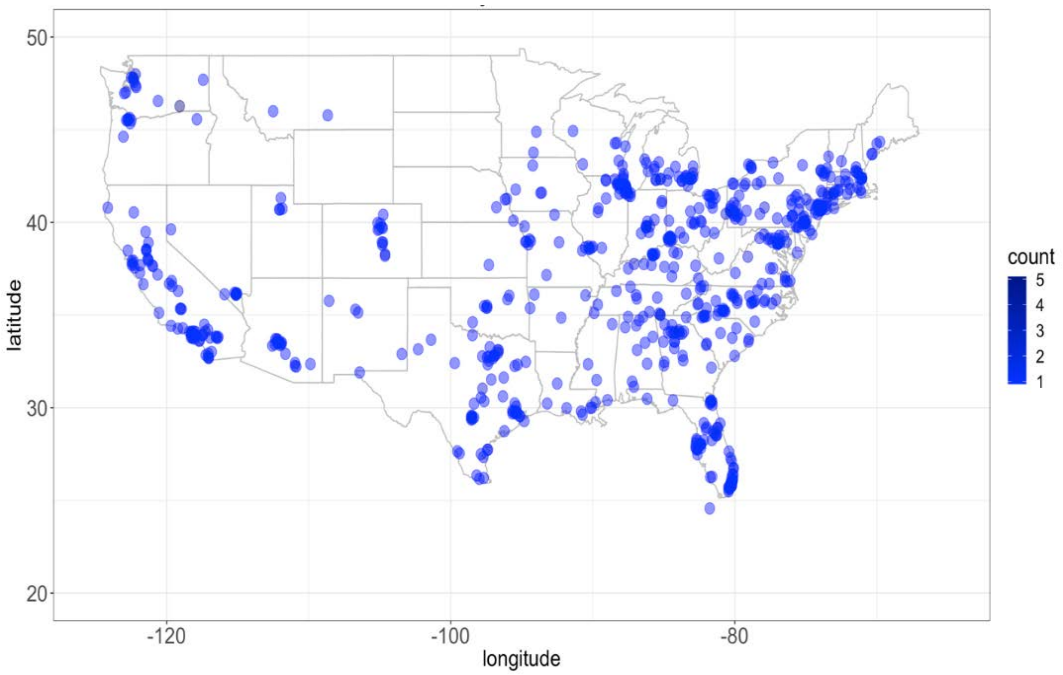

Figure 5.1: Spatial Distribution of Survey Participants by ZIP Codes.

The food safety and food label survey module was comprised of three part, with a total of 11 questions. Part one assessed participants' food relevant habits and their daily behavior regarding food safety. Briefly those questions included: (*i*) the frequency of participants eating meat or leafy greens, (*ii*) places where participants shop for groceries and have meals, (*iii*) their eating and food preparation habits regarding fresh produce (wash and cook frequencies), and (*iv*) the reasons for their preparation of fresh produce. Part two measured participants' foodborne disease perceptions, specifically focusing on their knowledge regarding the severity of common foodborne bacterial/viral infections including nontyphoidal *Salmonella*, *Escherichia coli* O157 H7, *Listeria monocytogenes*, *Staphylococcus aureus*, and Norovirus. They were asked to report their perception on the severity of the above pathogens on a 5-point scale ranging from the low (1) to high (5) severity. They were also given the option indicating "do not know" (0) in this

question. Participants' past experiences with foodborne disease and their awareness of *Salmonella* in raw chicken were also asked. Part three gathered information about participants' willingness to buy and willingness to pay more for labeled products. Eight labels were specifically included (*Without Antibiotics*, *No Medically Important Antibiotics*, *No Growth Promoting Hormones*, *Cage Free*, *USDA Organic*, *Locally Raised*, *Generic Brand*, and *Major Brand*) and participants provided binary (yes/no) responses to whether they would buy a product with this label and whether they would be willing to pay more for it, respectively. Participants were also given the option to state that "they do not care", which were classified as "no" because we assume that participants who did not care would not buy or pay more for the product. The percentage of "they do not care" for any label was below 31%. Thus, this treatment would skew our analysis toward to the unwillingness to buy and pay more for labeled products. Appendix Table D1 and D2 list all of the questions and response options.

Data analyses

Raw data were downloaded from Qualtrics as an SPSS (.sav) file. The file was uploaded into R version 3.5.2 and R studio for data tidying and preprocessing to select demographics and food safety and food label block questions and the 740 participants pool. Descriptive statistics for each question were summarized using the frequency function in R. All codes are available at github page: https://github.com/YikeShen

Conventional statistical model

A probit regression model was used to find significant independent variables shaping the probability of participants' buy and pay preferences to labeled products. The independent variables were the answers for the questions on demographics, food-relevant habits, and

foodborne disease perception. For all independent variables, categorical variables were transformed and summarized into dummy variables based on each question. Representative groups for each categorical question was inputted as reference groups. Below is a summary of how categorical questions with more than two answers were transformed. For ethnicity, groups were classified as White and non-White, using White as the reference group. Residence type included “own” or “rent”, using “own” as the reference group. The marital status of “never married”, “divorced”, “widowed”, and “separated” were combined to a non-married group with the married group used as the reference group. Grocery store choices were classified as conventional (e.g. Meijer, Walgreens, Walmart) or non-conventional (organic grocery chains, local grocery stores, etc.), using the conventional group as the reference group. The reasons people cook their fresh produce were transformed into “taste better”, “food safety”, and other reasons, using “taste better” as the reference group. For the remaining binary questions, reference groups were “male” (gender), “rural” (residence type), “yes” (had the foodborne disease), and “yes” (aware raw chicken contains *Salmonella*). Questions with ranked answers were transformed into numeric values. Education was coded from 1 to 5, starting from “did not finish high school” (1) to “graduate degree” (5). Employment was coded from 1 to 3, i.e., “not employed” (1), “half-time” (2), and “full-time” (3). The number of males, females, children, and infants was summed to obtain the household size. Household sizes greater than 4 were counted as four to prevent excessive outliers in constructing the probit regression model. The frequencies of people eating meat, leafy greens, washing fresh produce, and cooking fresh produce were transformed into numeric values, increasing with frequencies from low (1) to high (3). Eating locations were ranked 1–5 starting with people who always eat meals from restaurants (1) to always eat home-prepared meals (5), respectively. The answers to foodborne disease perception

for non-typhoidal *Salmonella*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Staphylococcus aureus,* and Norovirus were ranked from 0 to 5 according to the degree of severity, from "I don't know" (0), not at all severe (1) to extremely severe (5).

Our dependent variables included two questions for each of eight food labels, asking whether consumers are willing to buy or willing to pay more for those labels. Participants were able to respond "yes", "no", and "I do not care". In our analysis we combined "no" and "I do not care" as explained before in Survey instrument section. Therefore, there were 16 individual dependent binary variables.

Each dependent variable was regressed to the independent variables using R packages "MASS", "stats", "fastDummies", and "sandwich". A probit regression model was used since binary dependent variables were included, and using the probability to buy or pay more is more iterative than the logit model. Multicollinearity diagnosis screening was performed in R using "performance" package. The multicollinearity diagnoses showed low multicollinearity with variance inflation factor (VIF) less than 2.5. Statistical results tables were summarized using R "stargazer" package.

Machine learning

Machine learning workflow was separately performed from the statistical model, and the machine learning analyses started from raw input datasheet. Raw independent variables from demographics, food-relevant habits, and foodborne disease perceptions were transformed to numbered (e.g., 0, 1, 2, 3, 4, 5) categories in R and subsequently transformed into one hot encoded feature matrix (binary vectors) in Python. Continuous variable age was transferred into six categorical groups, > 18, 19–24, 25–34, 35–49, 40–64, and > 65, respectively. Predictors

were used as machine learning independent variables hereafter. Predictors served as the input feature matrix X_j for machine learning models. There were 96 categories ($X_j = 96$) combined from all predictors in the machine learning feature matrix. Raw dependent variables, participants' willingness to buy or pay more for labeled products, were transferred into binary answers (Yes/No). The results collected from the survey were considered ground truth. The same sample pool of 740 participants was used in the machine learning method.

Four machine learning predictive models were used in this study to train, validate, and test the predicted willingness of participants' to buy or pay more for labeled products, including logistic regression, support vector machine, random forest, and neural network. All four models were supervised learning designed specifically for categorical values. Except for tuned parameters in hyperparameter tuning, default parameters in Python scikit learn package version 0.20.1 were used to run those models [183]. The four methods corresponded with four classifiers, i.e., logistic regression classifier, support vector classifier, random forest classifier, and neural network classifier, respectively. All four machine learning models followed ylabel (i)~feature matrix (X_j) where i = 1–8 representing participants' willingness to buy the labeled product and i = 9–16 representing participants' willingness to pay more for labeled products. Prediction accuracy was calculated as the number of correct prediction from machine learning models divided by the yes or no response results collected from the survey (i.e., the ground truth).

Ten-fold cross-validation was used for the machine method. The dataset was divided into three parts, 80% of it for training, 10% of it for validation, and 10% of it for test. First, the dataset was divided into 10 subsections. The first 8 sections were used for training, the 9^{th} section was used for validation, and the 10^{th} section was used for the test. Next, the dataset assigned the 2^{nd} to 9^{th} sections to training, the 10^{th} section to validation, and the 1^{st} section for

test. The dataset was looped and run ten times to cover each section in training, validation, and test. Hyperparameter tuning was performed during the training and validation process. The best parameters were calculated and selected according to a prediction accuracy score to be used in the test dataset to get the best prediction performance. Tuned parameters were selected from the description from the scikit learn package [183]. Only a few parameters listed in Appendix Table D3 needed to be tuned, and most of the parameters performed well under default conditions [183]. The parameters were initially pooled and tested to see the best performance on prediction accuracy results. For the logistic regression model, the parameter C, representing the inverse of regularization strength, was tuned. For the supporting vector machine model, the parameter C for misclassifying data points and gamma for rbf kernel coefficient (the decision region) were tuned. For the neural network model, the hidden layer sizes representing the number of neurons in each layer and the number of layers were tuned. In addition, the solver for weight optimization was tuned. For the random forest model, n_estimator representing the number of trees in the forest and max_depth representing the maximum depth of the tree were tuned. A detailed number of selections for parameters and models were listed in Appendix Table D3. The prediction accuracy results for the 10-fold cross-validation were averaged to obtain the average prediction accuracy. Standard deviations were also calculated. The flowchart for the machine learning method is represented in Figure 5.2.

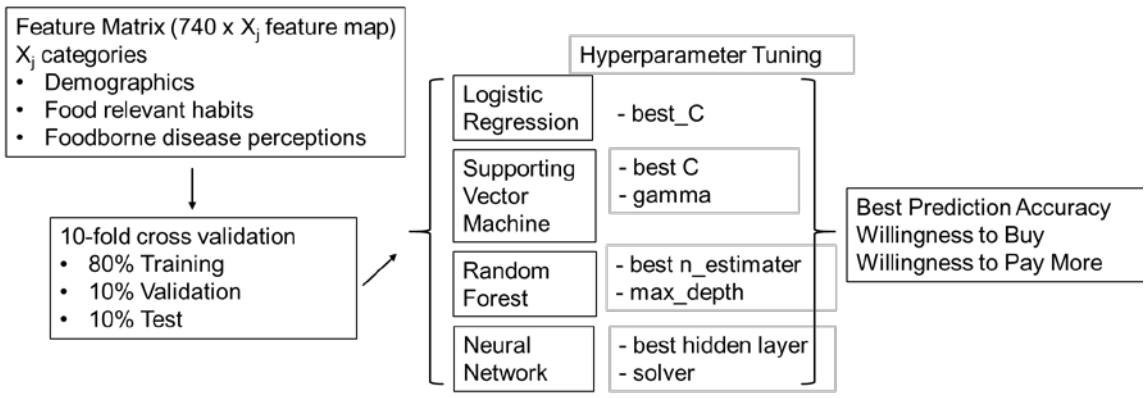

Figure 5.2: Flow of the machine learning models. Xj represented the input feature matrix.

Results and Discussions

Statistical model

Three food labels were significantly affected by one of two independent variables for both willingness to buy and willingness to pay more ($p < 0.05$) (Figure 5.3). The detailed statistical table can be found in Appendix Table D4 and D5. For *USDA Organic* labeled products, increasing cooking frequencies of fresh produce significantly increased both the probability of participants' willingness to buy and pay more. The cooking frequency of fresh produce was the significant factor for both the participants' willingness to buy and to pay more for *USDA Organic* products (Figure 5.3). The statistical model showed non-white participants, increasing the fresh produce washing frequencies, and increasing the risk perception of *Salmonella* significantly increased the probability of participants' willingness to buy. Higher education levels and more knowledge of Norovirus may play a role in increasing the probability of participants' willingness to pay more. Norovirus is the most common transmissible and self-limiting foodborne disease [184, 185]. It counted for 58% of the community gastroenteritis cases

reported in 2011 [186]. Increased participants' risk perception may shape their attitudes to high-quality organic produce. However, increases in household size, age, and fresh produce consumption frequencies decreased the probability of participants' willingness to pay more for *USDA Organic* products. Thus, it appears that the consumers' willingness to buy may be associated with their knowledge towards specific issues, whereas their willingness to pay more may be more associated with demographics. Indeed, consumers with higher education levels, which may relate to higher social affluence, may have greater purchasing power for organic products. Dimitri and Dettmann [16] found that well-educated and relatively high-income populations have a higher tendency to buy organic vegetables. People who live in a larger household may have a larger demand for foods, decreasing their probability of paying more for organic products. Seniors may have less incentive to pay the price premium compared to what they usually pay. People who eat fresh produce less often may care less about organic products. Generally, people who had a healthier lifestyle balance their diets with more fresh produce. People who have a healthy lifestyle may pay more attention to food quality, thus willing to pay the price premium for organic products. Similarly, Zhang et al. [15] pointed out that well-educated urban households were willing to buy more organic produce.

In our results, living in urban areas significantly increased the probability of "buy" and "pay" preferences to products labeled with *Raised without Antibiotics* and *Does not Contain Medically Important Antibiotics*. Generally, people living in urban areas are exposed to more products with new labels as a result of a wider selection of grocery stores. Indeed, Hjelmar [182] found that people who have visually been exposed to organic labels have a higher chance of buying them. Urban areas may also have more high-end jobs available, creating a higher demand for foods produced with higher standards. However, the demographic distribution in this survey

may bias our results since approximately 80% of participants were from urban settings. Interestingly, to the best of our knowledge, there was no survey attempted to find relationships between these two labels and residential location. Indeed, having non-conventional grocery stores significantly increased participants' willingness to buy *Raised without Antibiotics* products (Figure 5.3). Although increased frequency of washing fresh produce cannot eliminate the risk of antibiotic resistance, it still significantly increased the probability of participants' buy and pay more for these two labeled products. Age negatively correlated with their buy and pay preferences to restricted antibiotics use labels. The perceptions on foodborne pathogen and antibiotic resistance were not significantly related to the consumers' "buy" and "pay more" preferences for labels related to antibiotics. This might result from a disconnect between antibiotic resistant pathogens and the use of antibiotics in production for consumers.

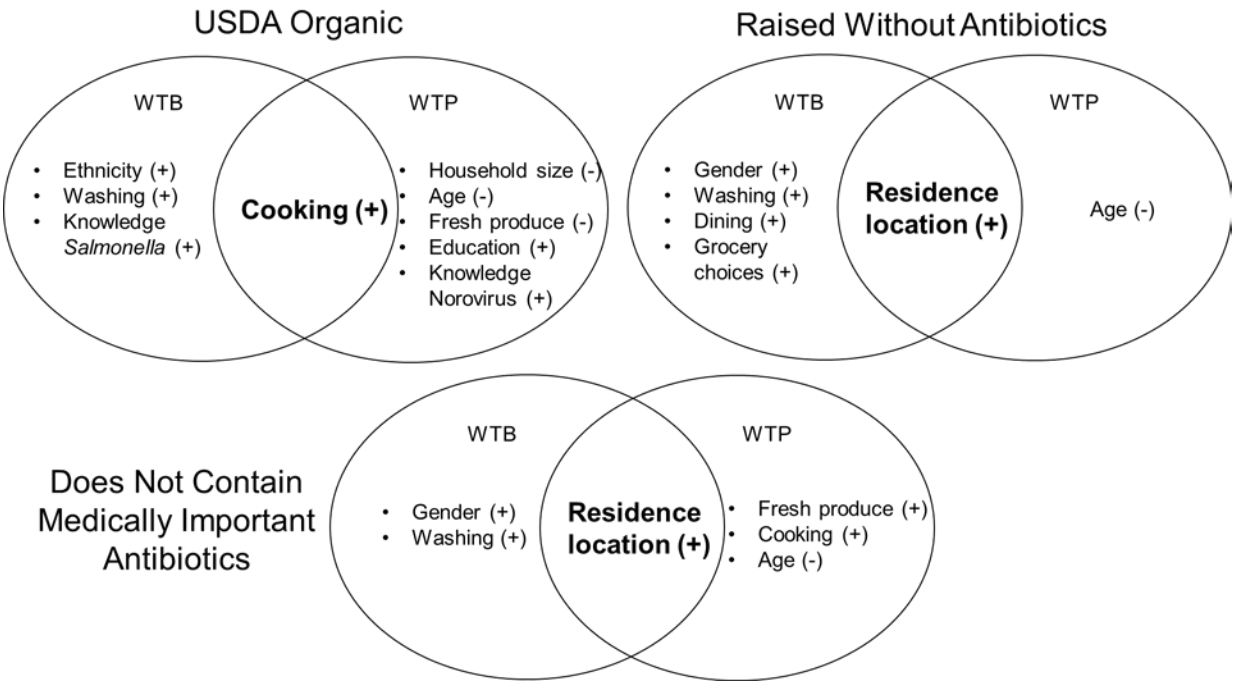

Figure 5.3: Important independent variables found by the probit regression model. Refer Appendix Table D4 and D5 for detailed statistical results. Full name of independent variables in

Figure 5.3 (cont'd) the venn graph. Ethnicity – non-white; washing – how often do you wash your fresh produce, low to high; knowledge *Salmonella* - how severe do you believe the following food-borne diseases are, low to high; Household size – how many people live in your household, low to high; age – young to old; fresh produce – how many times per day do you eat leafy greens (lettuce, spinach etc), low to high; education level – low to high; knowledge Norovirus – how severe do you believe the following food-borne diseases are, low to high; gender – female; dining – do you eat more home-prepared meals or meals from restaurants, low to high from more home prepared to more restaurants; grocery choices – non-conventional grocery stores; residence location – urban; cooking - how often do you cook your fresh produce (vegetables), low to high.

Machine learning models

Overall, the four machine learning models had the average performance higher than random guess (0.5 prediction accuracy) (Appendix Table D6), meaning that the survey questionnaire was useful in predicting consumers' willingness to buy and pay more for labeled products. The highest average prediction accuracy across four machine learning models was selected for each label for both willingness to buy and willingness to pay more (Figure 5.3). The *Raised Without Antibiotics* label had a high average prediction accuracy for both participants' willingness to buy and pay more using logistic regression and support vector machine models, respectively. *USDA Organic* label products yielded an average prediction accuracy for willingness to buy of 0.619, and a relatively high willingness to pay more of 0.657. The neural network model for willingness to pay more for the generic brand label had the highest average prediction accuracy of 0.691. Previous studies found that household attributes such as house ownership, the number of residents in the house, places of dinning, food preparation practices,

grocery shopping destinations, and the consumption of meat and fresh produce played an important role in buying organics [162]. With the machine learning method, we cannot specify which independent variables significantly change the prediction accuracy. Instead, the average prediction accuracy provided an overall idea if a customer will buy or pay more for specific products. The machine learning models could have a large input feature matrix containing the original answers to all questions, while statistical methods can only fit important independent variables. By using the machine learning models, some underrepresented questions can be considered in the analysis, which would have been ignored in the statistical regression models. For example, ethnicity was defined as White or non-white in our statistical model. In the machine learning models, White, Black, Hispanic, Asian, and other races were all predictors and input into our model. Similarly, conventional grocery chains, local grocery stores, organic grocery chains, and other stores were inputted as four predictors. In contrast, only conventional and non-conventional grocery stores were used in our statistical regression. Therefore, having machine learning results can help tailor the prediction to individual customers, including as many features as possible. To the best of our knowledge, no previous studies have been conducted using machine learning to predict participants' willingness to buy and pay more for the specific eight labels. Our results suggest that machine learning models can be used to analyze food survey data.

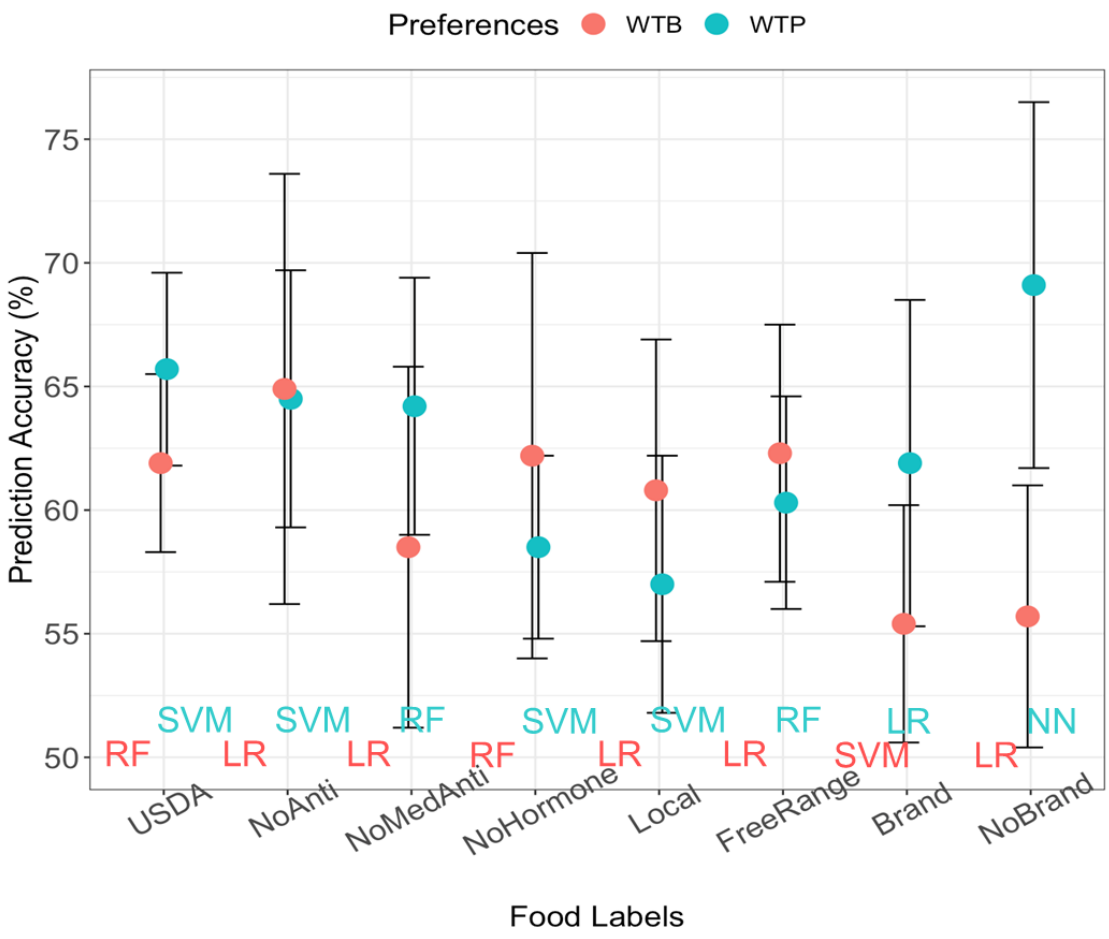

Figure 5.4: Prediction accuracy results of consumers' willingness to buy (WTB) or willininngnss to pay more (WTP) for the machine learning models. Figure name abbreviations: Logistic regression, LR; supporting vector machine, SVM; neural network, NN; random forest, RF. USDA Organic, USDA; Raised Without Antibiotics, NoAnti; No Medically Important Antibiotics, NoMedAnti; No Growth Promoting Hormones, NoHormone; Locally Raised, Local; Cage Free, FreeRange; Major Brand, Brand; Generic Brand, NoBrand. Refer to Appendix Table D6 for detailed machine learning results.

Conclusion

This study shed unique insights into the importance of demographics, food relevant habits, and foodborne disease perception to consumers' buy and pay preferences for labeled products using both conventional statistical and novel machine learning methods. It was also the first study to use machine learning models to analyze food survey data. We found that increased fresh produce cooking frequencies significantly increased the probability of participants' willingness to buy and pay more for *USDA Organic* labeled products. It was also found that urban residents are more likely to buy and pay more for products labeled *Raised without Antibiotics* and *Does not Contain Medically Important Antibiotics*. Elderly customers are less likely to buy or pay more for products produced with a restrained antibiotic use. The *Raised without Antibiotics* label had a high average prediction accuracy for both willingness to buy and willingness to pay more with the machine learning methods. Finally, our machine learning models achieved an adequate average prediction accuracy score for eight labels, thus providing a new tool for evaluating food safety and food labeling survey data.

CHAPTER VI

Conclusions and Future Recommendations

Conclusions

The results of this dissertation improved our understandings on potential contamination of vegetables by antibiotics in recycled resources (reclaimed water) and the consequent changes in bacterial community and ARGs profiles. The use of reclaimed water is beneficial to farmers, especially in water-stressed regions. This work provided insights into potential risks of antibiotics, ARGs and bacterial pathogens in vegetables and offered suggestions on how to safely use reclaimed water in vegetable production. This work also connected scientific research to social science through a survey study on food safety and living habits related questions to determine consumers' purchasing preferences to different food labels. The information is critically needed to evaluate the quality and safety of agricultural food products and understanding consumers' preferences to food products. Major conclusions were summarized as follows:

I: 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.

II: Root concentration factors based on antibiotic concentrations in bulk soil (RCF_{bs}) were significantly greater than those based on antibiotic concentrations in rhizosphere soil (RCF_{rs}) for ciprofloxacin, lincomycin, oxytetracycline, sulfamethoxazole, and tetracycline, similar for trimethoprim and tylosin, and lower for monensin. The difference between the RCF_{bs} and RCF_{rs} values indicates that the RCF_{bs} values may not accurately predict the root uptake of antibiotics in diverse soil-plant systems.

III. Bacterial communities are driven by stochastic processes upon exposure to low level of anthropogenic antibiotics, and were more resilient in the roots and rhizosphere soil than in the bulk soil and shoots.

IV. Consumers' willingness to buy or to pay for certain labeled food products is dependent on certain demographic traits (e.g., age or urban living) and food-relevant habits. Machine learning methods achieved sufficient prediction accuracy scores for estimating consumers' willingness to buy or to pay, and thus could be useful tools for evaluating food safety and food labeling survey data.

Future Work

Building upon this work, future research will be directed to understanding the mechanisms of ARGs and microbiome changes using multi-omics technologies. The 16S rRNA sequencing could not assess functional changes; neither can it determine the metabolic pathways of bacterial communities. Shotgun metagenomics and metabolomics can help address these challenges. At the gene level, WaferGen real-time qPCR can only quantify the abundance of genes based on their copy number, and the analyses must rely on relative abundance to 16S rRNA gene, which may have multiple copies in some bacteria. The transcriptomics technology and long-read sequencing can be used to identify more detailed functions of specific genes.

In the One Health framework connecting animal health, environmental health, and human health, this dissertation work mainly focused on agricultural and environmental sciences. It is critically needed to connect environmental health to human health to understand human health implications of changing environmental microbiomes and antibiotic resistomes.

APPENDICES

Appendix A: CHAPTER II Supplementary Materials

Pharmaceutical Exposure Changed Antibiotic Resistance Genes and Bacterial Communities in Soil-Surface- and Overhead-Irrigated Greenhouse Lettuce

Supplementary Methods

Raw results of ARGs and MGEs were extracted from the WaferGen qPCR software (Version 2.8.1.23). Raw data spreadsheet with cycle numbers (Ct) were imported into R Studio (version 1.1.383) interface in R (Version 3.4.2). Genes were removed if they were only detected once in technical triplicate measurements of a sample. The cutoff threshold of $C_T < 30$ was selected and genes that had no detection or a $C_T > 30$ were then removed. Next, the C_T values from at least two measurements of each sample were averaged. Afterwards if a gene was only detected once in the triplicate pharmaceutical treated samples of the same treatment, the gene was then removed from further analyses. Average C_T values for the genes detected in at least two treatment replicates were computed, which could eliminate potential false positive gene detection. Copy number of genes was calculated via Gene Copy Number = $10^{(30-C_T)/(10/3)}$ [42]. Relative abundance of detected genes was computed by dividing the estimated gene copy number with the gene copy number of 16S rRNA. R packages 'tidyr', 'tidyverse', 'dplyr' and a R workflow were used to perform the above data preprocessing. Relative abundance heatmap of genes was plotted using "pheatmap" and "RColorBrewer" packages. Relative abundance heatmap can visualize the abundance and distribution of genes in samples, including ARGs and MGEs. Chord diagram was plotted using "circlize" package and can be used to visualize the most abundant ARGs and MGEs in each sample. UpSet plot of gene intersections of shoots, roots, and soils samples was plotted using "UpSetR" package, which shows the sharing of ARGs

and MGEs among all samples. Ordination of ARGs and MGEs was plotted using “Vegan” package based on Bray-Curties distance, indicating the degree of similarity in ARGs and MGEs between different samples.

Bacteria community analysis was first preprocessed using the MacQIIME pipelines v. 1.9. following online tutorial for operational taxonomic unit (OTU) picking [43]. A total of 14,884 bacteria genus were picked up using close reference OTU picking based on a 97% similarity threshold with default uclust to cluster to Greengenes reference database [43, 44]. Bacteria detected at least twice in the triplicates measurements with OTU greater than 0 for total 27 samples in the Illumina MiSeq among triplicates were averaged. Next, bacteria belonging to mitochondria and chloroplast were removed because small subunit ribosomal RNA genes of plant organelles (mitochondria and chloroplast) are easily amplified by PCR and thus contaminate bacterial gene pool. This is because those genes are originated from endosymbiotic bacteria [187, 188]. A total 6519 taxa were picked for downstream analysis. The OTU table, taxa table, sample composition table, and tree table were placed into a “phyloseq” dataframe, followed by the downstream analysis using “phyloseq”, “vegan”, “ggplot2”, and “ape” packages (Paradis et al., 2004; Paul and Susan, 2013). Top 10 phyla and families were selected to plot the composition of bacterial communities. Top ten phyla were selected for ordination analysis with singleton (OTU = 1) and doubleton (OTU = 2) removed based on Bray-Curtis distance. We first use multiple constrained and unconstrained ordination methods to analyze the beta diversity of our samples, including detrended correspondence analysis, canonical correspondence analysis, redundancy analysis, detrended principle coordinates analysis, non-metric multidimensional scaling, multidimensional scaling, and principal coordinates analysis (PCoA) (Supplementary Figure S5). PCoA was then selected because it revealed the best position among our samples and made less assumptions in

calculating distance. Alpha diversity was calculated by the Chao1 estimator [45, 46] and plotted using "phyloseq" package [144].

Network analysis among antibiotics concentrations, ARGs/MGEs relative abundance, and percentages of family-level bacterial communities were conducted based on correlation test. First, correlations between antibiotics concentrations and the relative abundance of ARGs/MGEs or bacterial families were performed for the lettuce and soil samples with pharmaceutical exposure (7 averaged measurements respectively). Then, correlations between the relative abundance of ARGs/MGEs and bacterial communities were performed for all samples (13 averaged measurements respectively). We selected ARGs and MGEs detected in more than half samples in the correlation tests to eliminate false positive correlations. Correlation coefficient greater than 0.6 and less than −0.6 with p-value < 0.05 were selected (Supplementary Table S4). The network was plotted using Gephi v0.9.1 software. The 8 antibiotics were included in the network analysis. As acetaminophen and caffeine only inhibited certain bacteria at non-environmentally relevant high concentrations of 756–1516 mg/L and 300–10,000 mg/L, respectively [189-191], they were not considered to be related to microbiomes and ARGs. It was recently reported that at an environmentally relevant concentration (50 μg/L) carbamazepine enhanced horizontal transfer of several plasmid-borne ARGs [192]. However, our preliminary test found minimal interactions of carbamazepine with ARGs in this study (i.e., negative correlation with only one *mexF* gene). To simplify the network analysis, we only included 8 antibiotics.

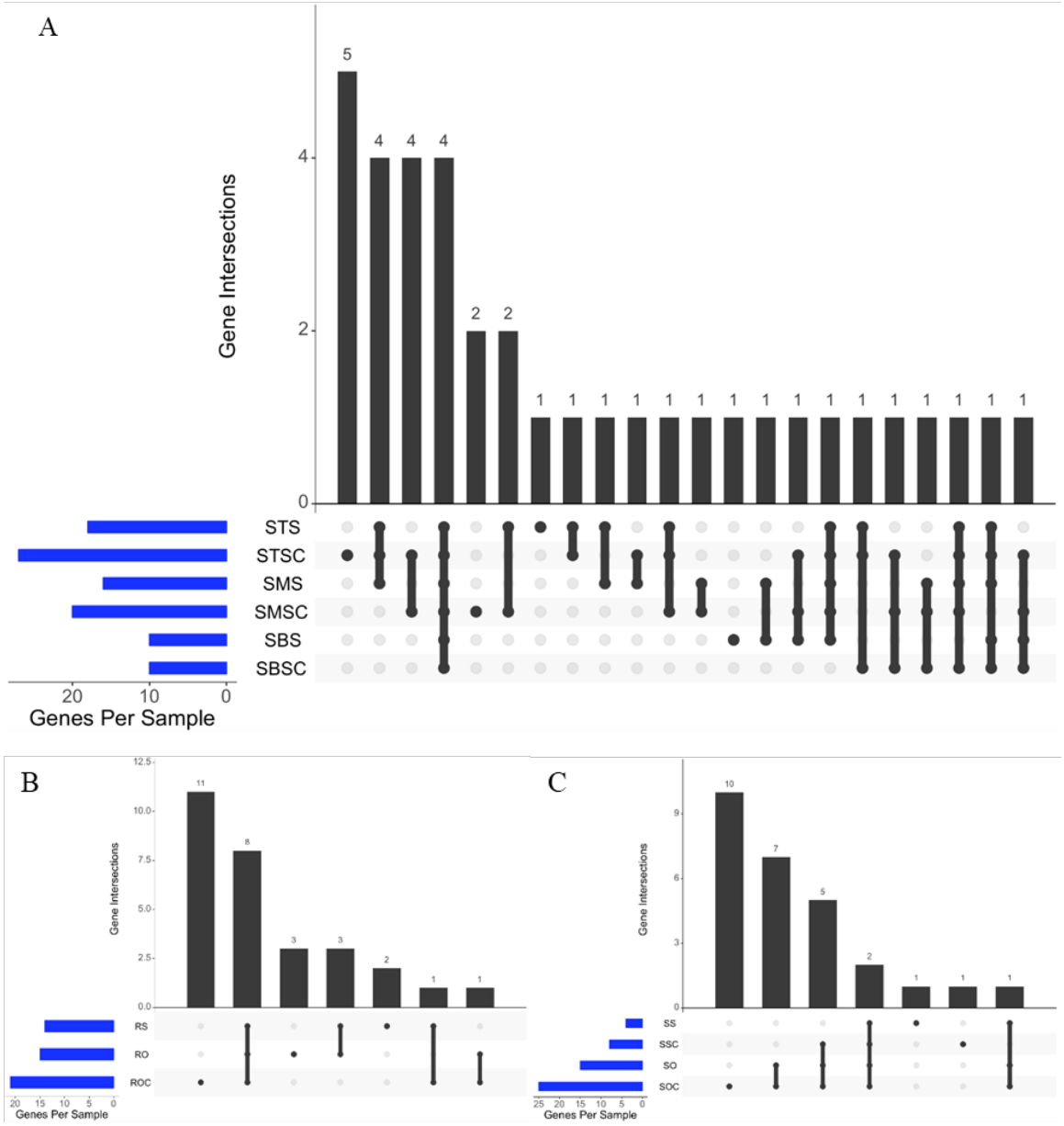

Figure A1. Intersections of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in soil, lettuce root and lettuce shoot samples. Left blue bar charts represent the total count of ARGs in each sample. The right black bar charts represent gene intersection (1-11 genes). The dark black dot highlights the samples of soils, lettuce shoots, and lettuce roots that share certain genes. For example, the three black dots connected in the second column in Figure S1A indicate that there were four commonly shared genes among STS, STSC, and SMS (See Table S1 for sample naming convention).

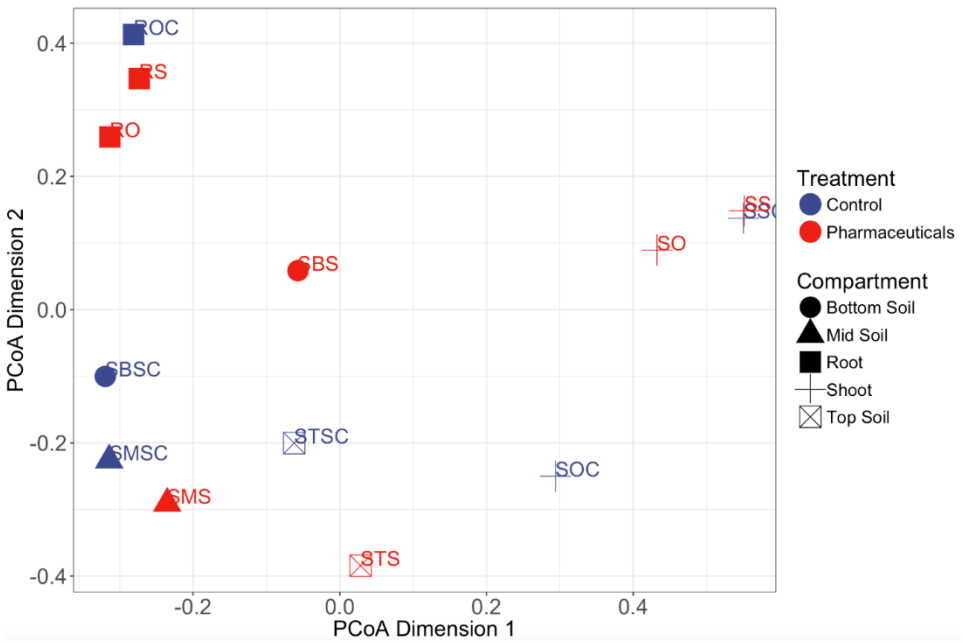

Figure A2. Principal coordinates analysis of antibiotic resistance genes (ARGs) and mobile genetic elements MGEs in the soil, lettuce root and shoot samples based on Bray-Curtis distance.

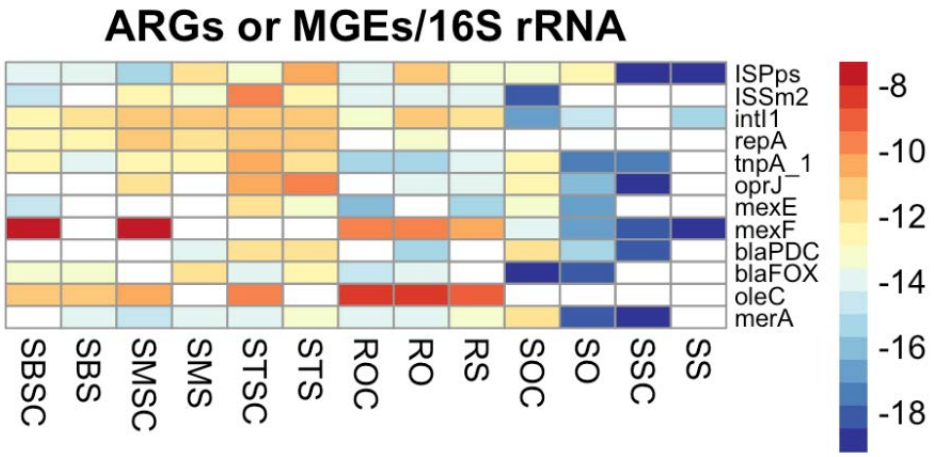

Figure A3. Relative abundance (gene copy number/16s rRNA gene copy number) of antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs) in the soil, lettuce root and shoot samples. Sample naming convention is provided in Table S1. Data were Log 2 transformed. Blank cells represent genes that were either not detected or below detection limit. Color bar on the right means relative abundance from low (blue) to high (red) levels. Top 12 genes were selected based on more than half detection in all samples (> 7/13).

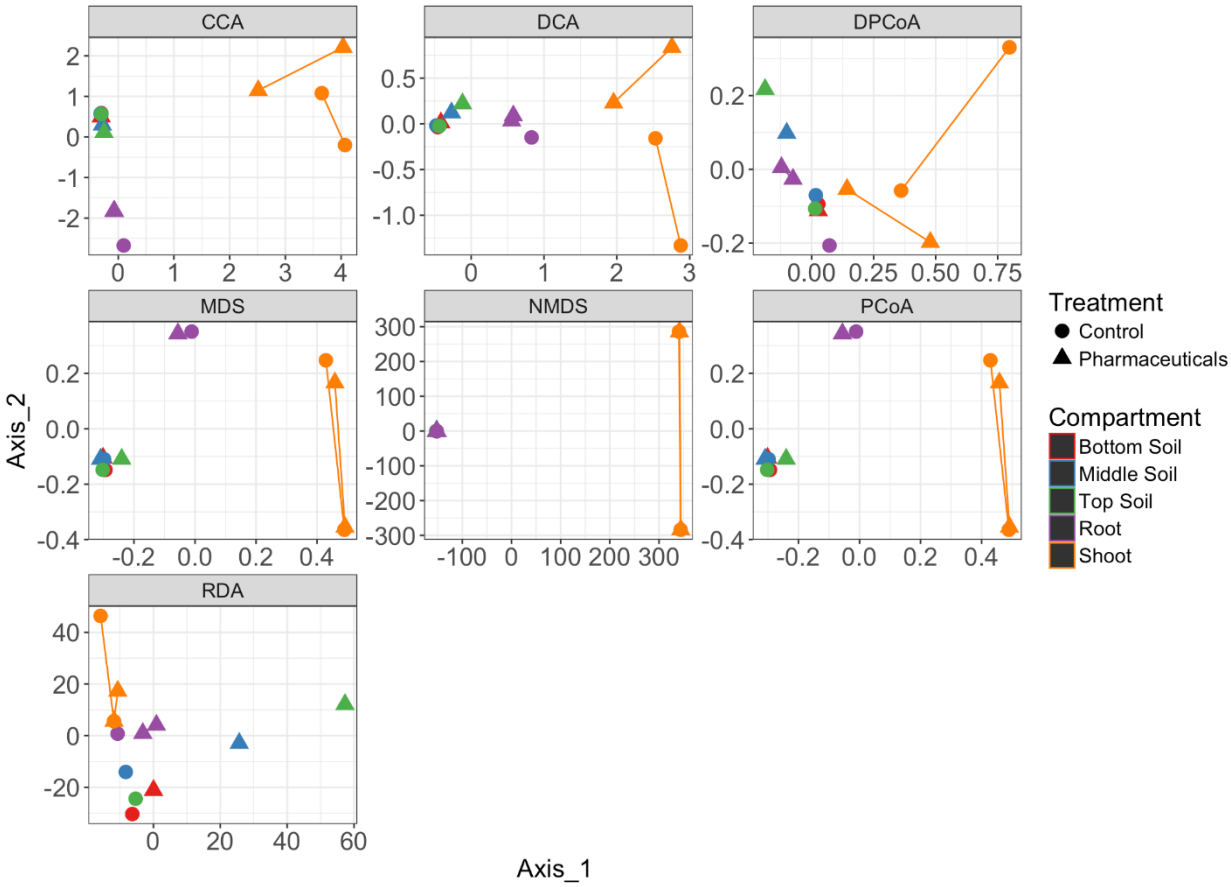

Figure A4. Multivariate analysis of bacterial community based on Bray-Curties distance.

Table A1: Experimental treatment and sample name abbreviation

Sample name	Sample Triplicates	Experimental treatment
STSC	No replicate	Top soil layer for soil-surface irrigation without pharmaceuticals (control treatment)
SMSC	No replicate	Middle soil layer for soil-surface irrigation without pharmaceuticals (control treatment)
SBSC	No replicate	Bottom soil layer for soil-surface irrigation without pharmaceuticals (control treatment)
STS	STS1	Top soil layer for soil-surface irrigation with pharmaceuticals
	STS2	
	STS3	
SMS	SMS1	Middle soil layer for soil-surface irrigation with pharmaceuticals
	SMS2	
	SMS3	
SBS	SBS1	Bottom soil layer for soil-surface irrigation with pharmaceuticals
	SBS2	
	SBS3	
ROC	No replicate	Lettuce root receiving overhead irrigation without pharmaceuticals (control treatment)
RO	RO1	Lettuce root receiving overhead irrigation with pharmaceuticals
	RO2	
	RO3	
RS	RS1	Lettuce shoot receiving soil-surface irrigation with pharmaceuticals
	RS2	
	RS3	
SOC	No replicate	Lettuce shoot receiving overhead irrigation without pharmaceuticals (control treatment)
SSC	No replicate	Lettuce shoot receiving soil-surface irrigation without pharmaceuticals (control treatment)

Table A1 (cont'd)

SO	SO1	Lettuce shoot receiving overhead irrigation with pharmaceuticals
	SO2	
	SO3	
SS	SS1	Lettuce shoot receiving soil-surface irrigation with pharmaceuticals
	SS2	
	SS3	

Table A2. Primer set for 16S rRNA, antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs).

Assay	Name	Forward Primer	Reverse Primer	Target
1	16S rRNA	GGGTTGCGCTCGTTGC	ATGGYTGTCGTCAGCTCGTG	16S rRNA
33	ampC	TCCGGTGACGCGACAGA	CAGCACGCCGGTGAAAGT	Beta Lactam
36	blaPDC	CGCCGTACAACCGGTGAT	GAAGTAATGCGGTTCTCCTTTCA	Beta Lactam
39	bla1	GCAAGTTGAAGCGAAAGAAAAGA	TACCAGTATCAATCGCATATACACCTAA	Beta Lactam
46	cphA	GCGAGCTGCACAAGCTGAT	CGGCCCAGTCGCTCTTC	Beta Lactam
48	blaL1	CACCGGGTTACCAGCTGAAG	GCGAAGCTGCGCTTGTAGTC	Beta Lactam
56	floR	ATTGTCTTCACGGTGTCCGTTA	CCGCGATGTCGTCGAACT	Amphenicol
64	emrD	CTCAGCAGTATGGTGGTAAGCATT	ACCAGGCGCCGAAGAAC	MDR[a]
72	vanC	AAATCAATACTATGCCGGGCTTT	CCGACCGCTGCCATCA	Vancomycin
89	mexA	AGGACAACGCTATGCAACGAA	CCGGAAAGGGCCGAAAT	MDR[a]
93	aac3-VI	CGTCACTTATTCGATGCCCTTAC	GTCGGGCGCGGCATA	Aminoglycoside
111	blaCMY	GCGAGCAGCCTGAAGCA	CGGATGGGCTTGTCCTCTT	Beta Lactam
113	blaFOX	GGTTTGCCGCTGCAGTTC	GCGGCCAGGTGACCAA	Beta Lactam
121	blaSFO	CCGCCGCCATCCAGTA	GGGCCGCCAAGATGCT	Beta Lactam
125	qacH	CATCGTGCTTGTGGCAGCTA	TGAACGCCCAGAAGTCTAGTTTT	MDR[a]
132	rarD	TGACGCATCGCGTGATCT	AAATTTTCTGTGGCGTCTGAATC	Amphenicol
140	mphA	CTGACGCGCTCCGTGTT	GGTGGTGCATGGCGATCT	MLSB
156	emrB/qacA	CTTTTCTCTAACCGTACATTATCTACGATAAA	AGAACGTAGCGACTGATAAAATGCT	MDR[a]
157	bacA	CGGCTTCGTGACCTCGTT	ACAATGCGATACCAGGCAAAT	other
177	strB	GCTCGGTCGTGAGAACAATCT	CAATTTCGGTCGCCTGGTAGT	Aminoglycoside
202	tnpA_1	CCGATCACGGAAAGCTCAAG	GGCTCGCATGACTTCGAATC	Transposase
203	tnpA_2	GGGCGGGTCGATTGAAA	GTGGGCGGGATCTGCTT	Transposase
210	vanA	AAAAGGCTCTGAAAACGCAGTTAT	CGGCCGTTATCTTGTAAAAACAT	Vancomycin
215	vanHB	GAGGTTTCCGAGGCGACAA	CTCTCGGCGGCAGTCGTAT	Vancomycin
217	vanRA_2	CCACTCCGGCCTTGTCATT	GCTAACCACATTCCCCTTGTTTT	Vancomycin
229	pncA	GCAATCGAGGCGGTGTTC	TTGCCGCAGCCAATTCA	MLSB

Table A2 (cont'd)

234	oprD	ATGAAGTGGAGCGCCATTG	GGCCACGGCGAACTGA	MDR[a]
235	oprJ	ACGAGAGTGGCGTCGACAA	AAGGCGATCTCGTTGAGGAA	MDR[a]
243	ttgA	ACGCCAATGCCAAACGATT	GTCACGGCGCAGCTTGA	MDR[a]
244	ttgB	TCGCCCTGGATGTACACCTT	ACCATTGCCGACATCAACAAC	MDR[a]
245	mepA	ATCGGTCGCTCTTCGTTCAC	ATAAATAGGATCGAGCTGCTGGAT	MDR[a]
246	mexE	GGTCAGCACCGACAAGGTCTAC	AGCTCGACGTACTTGAGGAACAC	MDR[a]
247	mexF	CCGCGAGAAGGCCAAGA	TTGAGTTCGGCGGTGATGA	MDR[a]
256	acrA_1	TACTTTGCGCGCCATCTTC	CGTGCGCGAACGAACAT	MDR[a]
257	acrA_2	CGTGCGCGAACGAACA	ACTTTGCGCGCCATCTTC	MDR[a]
276	msrA	AACGAAATCAAGCGCAACAA	CAACCGTGCCTTTTTCTTTTG	MLSB
285	oleC	CCCGGAGTCGATGTTCGA	GCCGAAGACGTACACGAACAG	MLSB
290	pikR2	TCGTGGGCCAGGTGAAGA	TTCCCCTTGCCGGTGAA	MLSB
292	tetPB	ACACCTGGACACGCTGATTTT	ACCGTCTAGAACGCGGAATG	Tetracycline
299	tolC_1	CAGGCAGAGAACCTGATGCA	CGCAATTCCGGGTTGCT	MDR[a]
300	tolC_2	GCCAGGCAGAGAACCTGATG	CGCAATTCCGGGTTGCT	MDR[a]
310	vanSB	GCGCGGCAAATGACAAC	TTTGCCATTTTATTCGCACTGT	Vancomycin
331	merA	GTGCCGTCCAAGATCATG	GGTGGAAGTCCAGTAGGGTGA	Murcury
332	sul2	TCCGATGGAGGCCGGTATCTGG	CGGGAATGCCATCTGCCTTGAG	Sulfonamide
342	IncP_oriT	CAGCCTCGCAGAGCAGGAT	CAGCCGGGCAGGATAGGTGAAGT	plasmid incompatibility
350	acrR	TGCAACACGCGCTTTCTC	ACGATTGCGGGCAGGTT	MDR
359	intI1	CGAACGAGTGGCGGAGGGTG	TACCCGAGAGCTTGGCACCCA	Integrase
366	orf39-IS26	GCGCGTCGAGCATCAATAG	CAGTTGTGCTGCTGGTGGTC	Insertional sequence
369	ISPps	CACACTGCAAAAACGCATCCT	TGTCTTTGGCGTCACAGTTCTC	Insertional sequence
370	ISSm2	TGGATCGACCGGTTCCAT	GCTGACCGAGCTGTCCATGT	Insertional sequence
374	mexB	CTGGAGATCGACGACGAGAAG	GAAATCGTTGACGTAGCTGGAA	MDR[a]
378	repA	CCCCCAGGACTTGCGAGCG	GAGGCATGCACGCCGACCA	plasmid replication
380	pAKD1	GGTAAGATTACCGATAAACT	GTTCGTGAAGAAGATGTA	plasmid replication

[a] MDR is multidrug resistance.

Table A3: Bacterial community composition for top 20 families. Number represents percentage (%) of each bacteria in each sample.

	SBSC	SBS	SMSC	SMS	STSC	STS	ROC	RO	RS	SOC	SO	SSC	SS
Methylophilaceae	0.1	6.6	0.2	23.5	0.4	28.1	0.5	17.5	12.5	0.3	16.5	0.0	0.0
Chthoniobacteraceae	3.6	3.8	4.8	5.6	4.4	7.1	0.7	0.5	0.3	0.2	0.0	0.0	0.0
Chitinophagaceae	10.0	7.2	9.9	6.7	8.7	6.3	7.4	5.2	3.2	2.4	0.3	0.0	0.0
Sphingomonadaceae	13.9	18.9	11.6	11.4	15.4	9.9	6.3	5.0	5.8	1.3	0.6	0.0	15.3
Pirellulaceae	5.3	6.1	9.0	4.5	5.8	3.1	2.1	0.6	0.5	0.0	0.0	0.0	0.0
Xanthomonadaceae	3.0	2.9	2.0	1.3	3.0	1.6	2.9	3.4	2.1	15.6	14.2	33.3	0.0
Pseudomonadaceae	0.3	0.2	0.0	0.2	0.1	0.1	0.9	0.3	0.3	50.6	37.9	8.3	6.1
Ellin6075	1.5	2.2	3.0	3.4	4.9	9.1	0.3	0.8	0.7	0.0	0.0	0.0	0.0
Bradyrhizobiaceae	5.4	3.3	4.7	4.5	4.2	4.3	2.2	5.3	4.5	0.3	0.7	0.0	0.0
Micromonosporaceae	2.8	3.5	5.4	2.8	4.2	1.4	6.2	3.8	5.2	0.0	0.0	8.3	0.0
Bacillaceae	4.5	3.8	5.7	2.6	6.3	0.9	0.5	0.5	0.4	0.0	0.0	0.0	0.0
Micrococcaceae	3.8	2.2	4.2	2.4	5.6	1.5	0.5	0.4	0.5	8.8	1.8	16.7	0.0
Nocardioidaceae	4.4	4.2	4.6	3.2	5.1	1.9	2.2	2.6	4.0	0.9	0.0	8.3	0.0
Methylobacteriaceae	0.4	1.3	0.4	4.3	0.5	7.5	0.1	7.6	7.0	0.1	14.6	0.0	78.6
Cytophagaceae	11.7	7.0	8.9	5.9	5.9	3.3	7.5	4.3	4.6	0.3	0.2	0.0	0.0
Comamonadaceae	4.7	3.3	4.8	5.4	5.1	5.3	27.4	7.1	8.0	12.9	8.0	16.7	0.0
Hyphomicrobiaceae	10.1	8.3	8.9	7.1	6.1	6.1	5.9	7.2	7.1	1.3	0.0	0.0	0.0
Sphingobacteriaceae	5.0	8.9	3.7	1.4	3.3	0.6	2.5	1.8	1.4	2.6	4.9	0.0	0.0
Streptomycetaceae	2.4	2.0	2.8	1.0	2.7	0.6	14.7	18.6	18.1	0.0	0.0	8.3	0.0
Oxalobacteraceae	7.4	4.3	5.5	3.2	8.5	1.7	9.0	7.7	14.1	2.5	0.3	0.0	0.0

Table A4: Correlation tests in the network analysis.

	Variable 1	Variable 2	ρ	p value
1	Antibiotics_Con	ISPps	0.79	0.04
2	Antibiotics_Con	Lincomycin	0.82	0.02
3	blaFOX	Oxytetracycline	-0.90	0.04
4	mexF	Oxytetracycline	-0.90	0.04
5	oprJ	Sulfadiazine	1.00	0.00
6	intI1	Sulfadiazine	0.82	0.02
7	ISPps	Sulfadiazine	0.89	0.01
8	Antibiotics_Con	Sulfamethoxazole	0.79	0.04
9	oprJ	Sulfamethoxazole	1.00	0.00
10	ISPps	Sulfamethoxazole	0.86	0.01
11	Carbadox	Sulfamethoxazole	0.86	0.01
12	Sulfadiazine	Sulfamethoxazole	0.96	0.00
13	tnpA_1	Trimethoprim	-0.81	0.05
14	Antibiotics_Con	Tylosin	0.79	0.04
15	blaPDC	Tylosin	1.00	0.00
16	ISPps	Tylosin	0.86	0.01
17	ISSm2	Tylosin	1.00	0.00
18	Antibiotics_Con	Methylophilaceae	0.82	0.02
19	Tylosin	Methylophilaceae	0.86	0.01
20	Oxytetracycline	Chitinophagaceae	-0.93	0.00
21	Trimethoprim	Sphingomonadaceae	-0.88	0.01
22	Oxytetracycline	Pirellulaceae	-0.90	0.01
23	Sulfadiazine	Bradyrhizobiaceae	0.89	0.01
24	Sulfamethoxazole	Bradyrhizobiaceae	0.82	0.02
25	Trimethoprim	Comamonadaceae	0.85	0.02
26	Oxytetracycline	Hyphomicrobiaceae	-0.76	0.05
27	ARGscon	Chitinophagaceae	0.75	0.00
28	Chitinophagaceae	Pirellulaceae	0.80	0.00
29	Sphingomonadaceae	Pirellulaceae	0.62	0.03
30	Chitinophagaceae	Bradyrhizobiaceae	0.69	0.01

Table A4 (cont'd)

31	Pirellulaceae	Bradyrhizobiaceae	0.78	0.00
32	Chitinophagaceae	Cytophagaceae	0.82	0.00
33	Pirellulaceae	Cytophagaceae	0.90	0.00
34	Bradyrhizobiaceae	Cytophagaceae	0.77	0.00
35	Sphingomonadaceae	Comamonadaceae	-0.90	0.00
36	Chitinophagaceae	Hyphomicrobiaceae	0.82	0.00
37	Pirellulaceae	Hyphomicrobiaceae	0.91	0.00
38	Bradyrhizobiaceae	Hyphomicrobiaceae	0.86	0.00
39	Cytophagaceae	Hyphomicrobiaceae	0.94	0.00
40	Chitinophagaceae	blaPDC	0.93	0.00
41	Sphingomonadaceae	blaFOX	0.70	0.04
42	Pirellulaceae	blaFOX	0.68	0.04
43	Comamonadaceae	blaFOX	-0.85	0.00
44	ARGscon	tnpA_1	0.66	0.02
45	Chitinophagaceae	tnpA_1	0.71	0.01
46	Sphingomonadaceae	tnpA_1	0.60	0.04
47	blaPDC	tnpA_1	0.79	0.04
48	Chitinophagaceae	oprJ	0.83	0.01
49	Sphingomonadaceae	oprJ	0.83	0.01
50	Pirellulaceae	oprJ	0.76	0.03
51	Comamonadaceae	oprJ	-0.79	0.02
52	tnpA_1	oprJ	0.90	0.00
53	ARGscon	mexE	0.79	0.04
54	blaPDC	mexE	1.00	0.00
55	tnpA_1	mexE	0.96	0.00
56	ARGscon	mexF	0.72	0.02
57	Chitinophagaceae	mexF	0.80	0.01
58	Pirellulaceae	mexF	0.92	0.00
59	Bradyrhizobiaceae	mexF	0.89	0.00
60	Cytophagaceae	mexF	0.86	0.00
61	Hyphomicrobiaceae	mexF	0.93	0.00
62	blaFOX	mexF	0.94	0.00

Table A4 (cont'd)

63	tnpA_1	mexF	0.68	0.04
64	oprJ	mexF	0.83	0.04
65	Sphingomonadaceae	oleC	-0.86	0.01
66	Cytophagaceae	oleC	-0.86	0.01
67	Comamonadaceae	oleC	0.89	0.01
68	Hyphomicrobiaceae	oleC	-0.79	0.04
69	Streptomycetaceae	oleC	0.96	0.00
70	blaFOX	oleC	-0.90	0.04
71	Pirellulaceae	intI1	0.66	0.02
72	Bradyrhizobiaceae	intI1	0.73	0.01
73	blaFOX	intI1	0.75	0.02
74	mexF	intI1	0.80	0.01
75	Methylophilaceae	ISPps	0.86	0.00
76	intI1	ISSm2	0.77	0.02
77	tnpA_1	repA	0.93	0.00
78	oprJ	repA	1.00	0.00
79	ISSm2	repA	0.89	0.02

Appendix B: CHAPTER III Supplementary Materials

Rhizosphere Soil is Key to the Uptake of Antibiotics by Lettuce (*Lactuca sativa*)

Table B1: LC-MS/MS properties for nine antibiotics.

Antibiotics	Precursor ion (m/z)	Product ion (m/z)	Declustering potential (volts)	Collision energy (volts)	Collision cell exit potential (volts)
Sulfamethoxazole	254	156	60	20	8
Trimethoprim	291	261	80	30	12
Lincomycin	407	126	60	30	8
Oxytetracycline	461	426	60	30	8
Monensin Sodium	694	676	120	50	8
Tylosin	916	173	100	40	10
Ciprofloxacin	332	231	76	45	14
Cefalexin	332	231	76	45	14
Tetracycline	445	410	80	30	6

Table B2: Extraction efficiencies (%) of antibiotics from the spiked lettuce shoots, lettuce roots, and soil samples.

	Shoots (%)	Roots (%)	Soil (%)[a]
Sulfamethoxazole	4.3 ± 0.7)	18.8 ± 2.9	85.0 ± 7.1
Trimethoprim	85.2 ± 0.9	73.9 ± 0.8	88.6 ± 5.3
Lincomycin	83.2 ± 4.3	71.6 ± 7.8	88.6 ± 4.8
Oxytetracycline	52.7 ± 5.5	28.7 ± 3.6	0.8 ± 0.2
Monensin Sodium	87.6 ± 2.0	65.2 ± 3.0	99.2 ± 31.1
Tylosin	89.1 ± 1.5	68.7 ± 6.7	79.9 ± 5.3
Ciprofloxacin	58.0 ± 4.6	53.5 ± 5.4	6.9 ± 0.8
Tetracycline	42.9 ± 4.8	29.0 ± 3.3	2.7 ± 1.2
Cephalexin	82.0 ± 2.6	62.9 ± 3.2	57.2 ± 3.3

[a] The soil samples include both the rhizosphere and bulk soil samples.

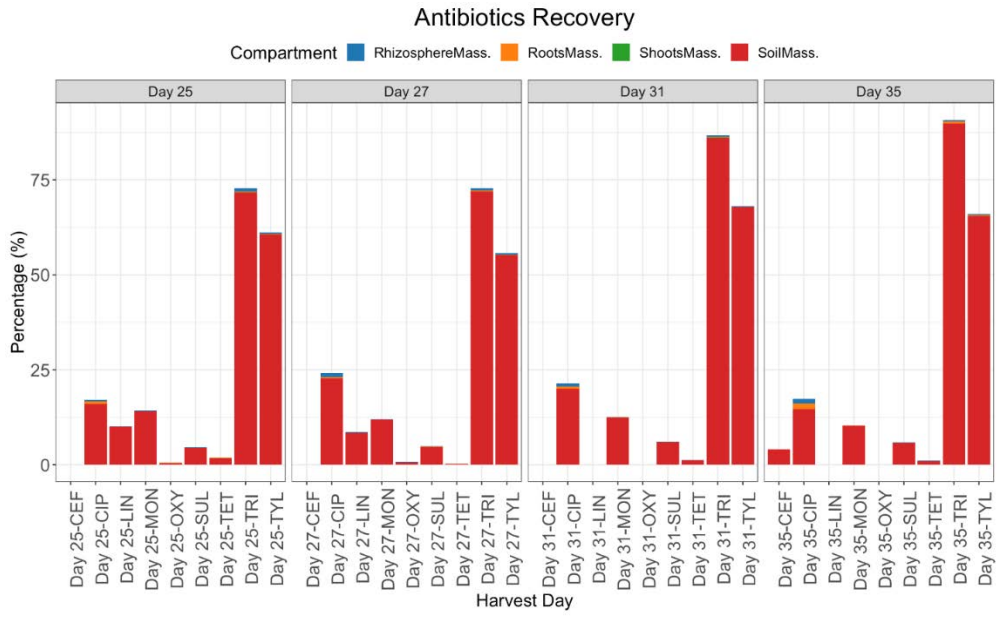

Figure B1: Measured mass recoveries of antibiotics in this study. CEF – cefalexin, CIP – ciprofloxacin, LIN – lincomycin, MON – monensin sodium, OXY – oxytetracycline, SUL – sulfamethoxazole, TET – tetracycline, TRI – trimethoprim, and TYL – tylosin.

Table B3: Root concentration factors based on antibiotic concentrations in the bulk soil (RCF_{bs}) and rhizosphere soil (RCF_{rs}), and root-to-shoot translocation factors (TF). N.A. means nonavailable due to non-detectable cephalexin concentration.

Antibiotics	RCF_{rs}	RCF_{bs}	TF	*p* value (Comparison between RCF_{rs} and RCF_{bs})
Ciprofloxacin	10.5 ± 2.6	126.0 ± 85.1	0.03 ± 0.02	1.46E-03
Lincomycin	1.0 ± 0.5	2.1 ± 1.1	1.46 ± 0.89	1.24E-02
Monensin Sodium	17.7 ± 10.3	7.3 ± 3.2	0.63 ± 0.51	1.60E-02
Oxytetracycline	10.7 ± 7.0	114.6 ± 42.7	0.36 ± 0.3	4.31E-05
Sulfamethoxazole	2.3 ± 1.0	7.0 ± 2.5	0.64 ± 0.4	1.19E-04
Tetracycline	30.9 ± 46.1	300.4 ± 257.6	0.29 ± 0.09	1.51E-02
Trimethoprim	11.8 ± 2.9	10.5 ± 2.4	0.04±0.01	3.23E-01
Tylosin	4.0 ± 1.4	3.1 ± 0.7	0.05±0.04	1.32E-01
Cephalexin	N.A.	N.A.	N.A.	N.A.

Appendix C: CHAPTER IV Supplementary Materials

Bacterial Communities and Antibiotic Resistance Genes in the Lettuce-Soil System upon Exposure to Anthropogenic Antibiotics

Table C1: Physiochemical properties of antibiotics used in this study.

Antibiotics	Molecular Weight (g/mol)[a]	Water Solubility (mg/L)[a]	pKa[a]	logKow[a]
Sulfamethoxazole	253.28	610	1.6,5.7	0.89
Trimethoprim	290.32	400	7.12	0.91
Lincomycin	406.54	927	7.6	0.2
Oxytetracycline	460.43	313	3.57,7.49,9.44[c]	-0.9
Monensin Sodium	692.87	slightly soluble[b]	4.3[c]	5.43[c]
Tylosin	916.1	5	7.73	3.27[d]
Ciprofloxacin	331.34	30000	6.09,8.74	0.28
Cefalexin	347.39	slightly soluble	5.2,7.3	0.65
Tetracycline	444.43	4	3.3,7.68,9.69	-1.37

[a] From TOXNET database: http://toxnet.nlm.nih.gov/index.html, [b] Reference [102], [c] From Guidechem database: http://www.guidechem.com/reference/dic-20635.html, and [d] From ChemSpider database: http://www.chemspider.com/Chemical-Structure.10606106.html

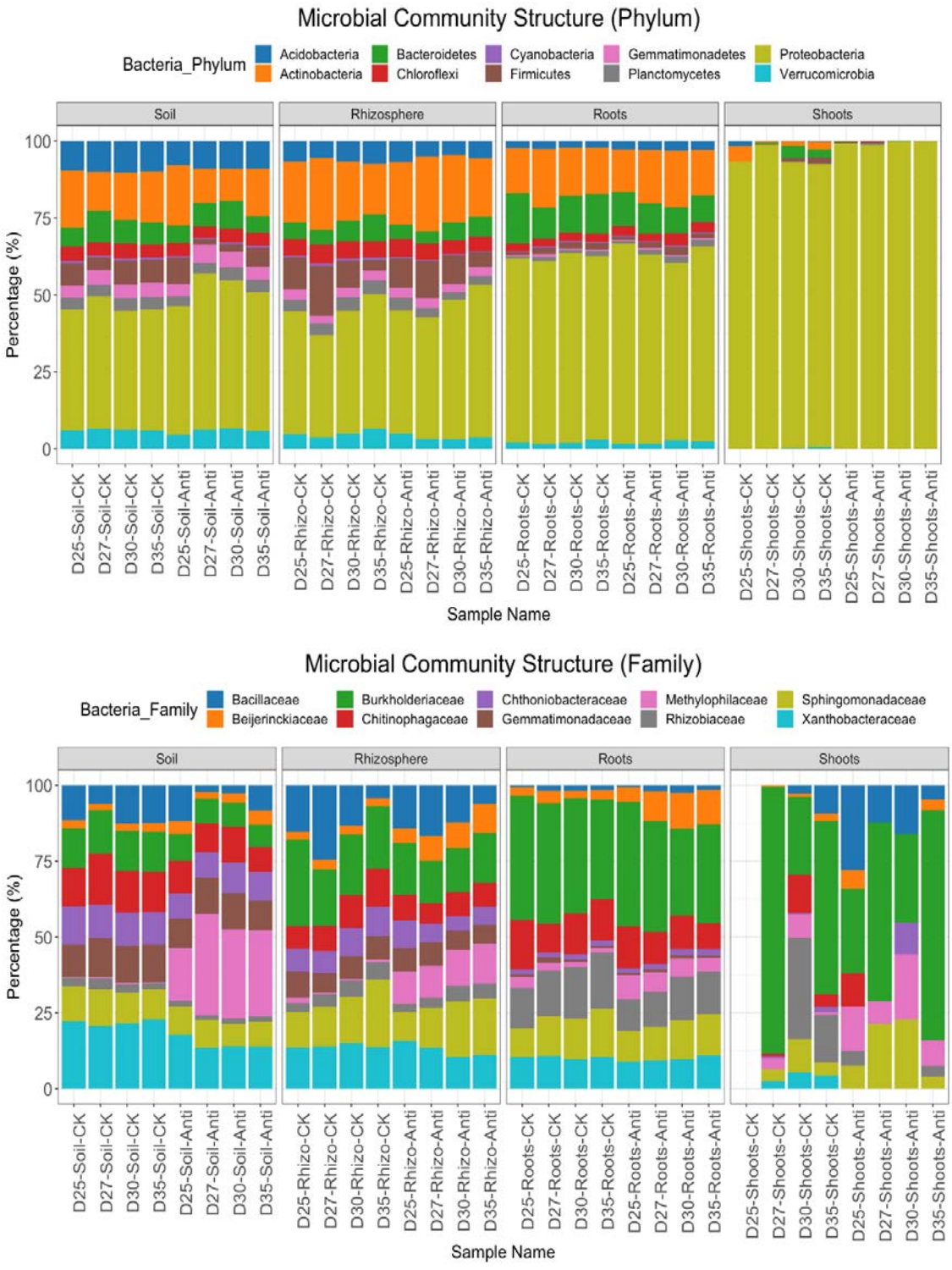

Figure C1: Bacterial community composition at the phylum and family levels. The sum of the percentages of the top 10 phyla or families were 100%. Each bar represents the fraction of each bacteria phylum and family.

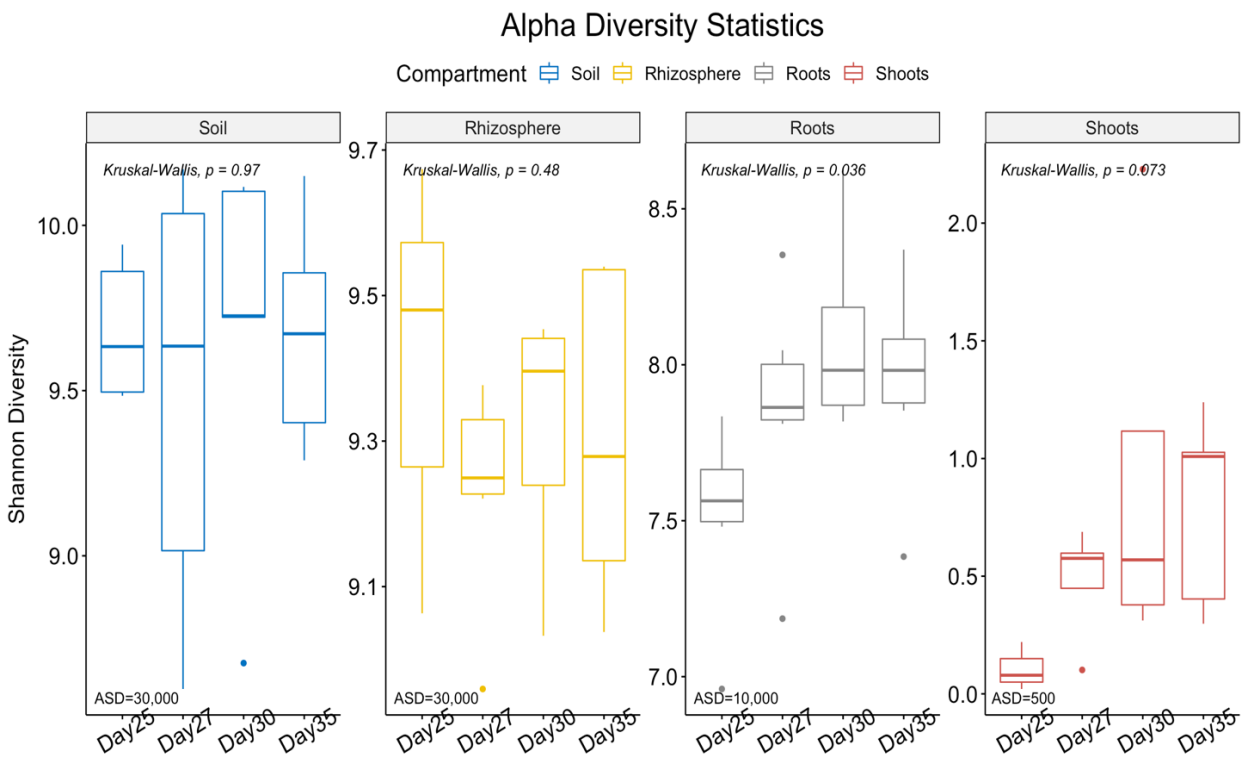

Figure C2: Bacterial alpha diversity of samples collected on four sampling days.

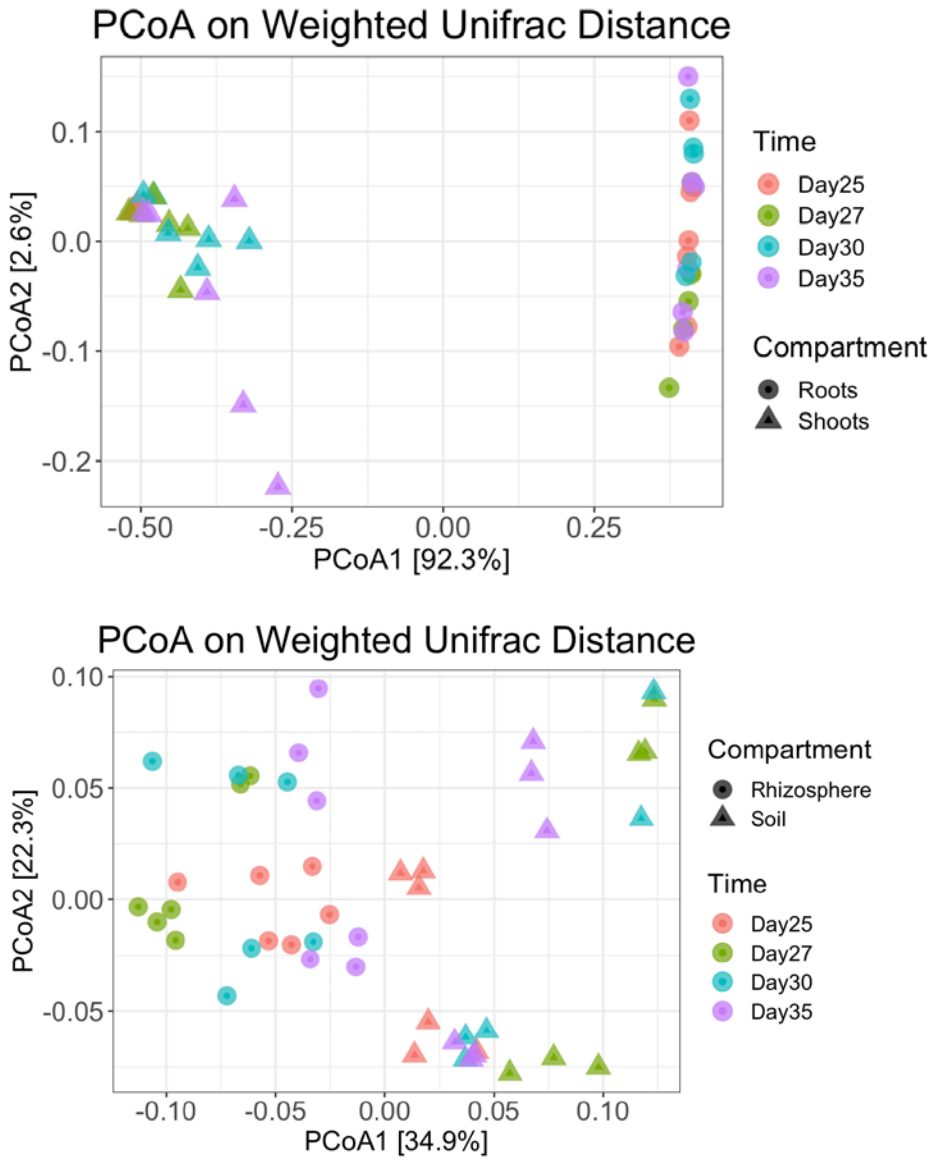

Figure C3: Bacterial beta diversity matrix of the samples collected on four sampling days.

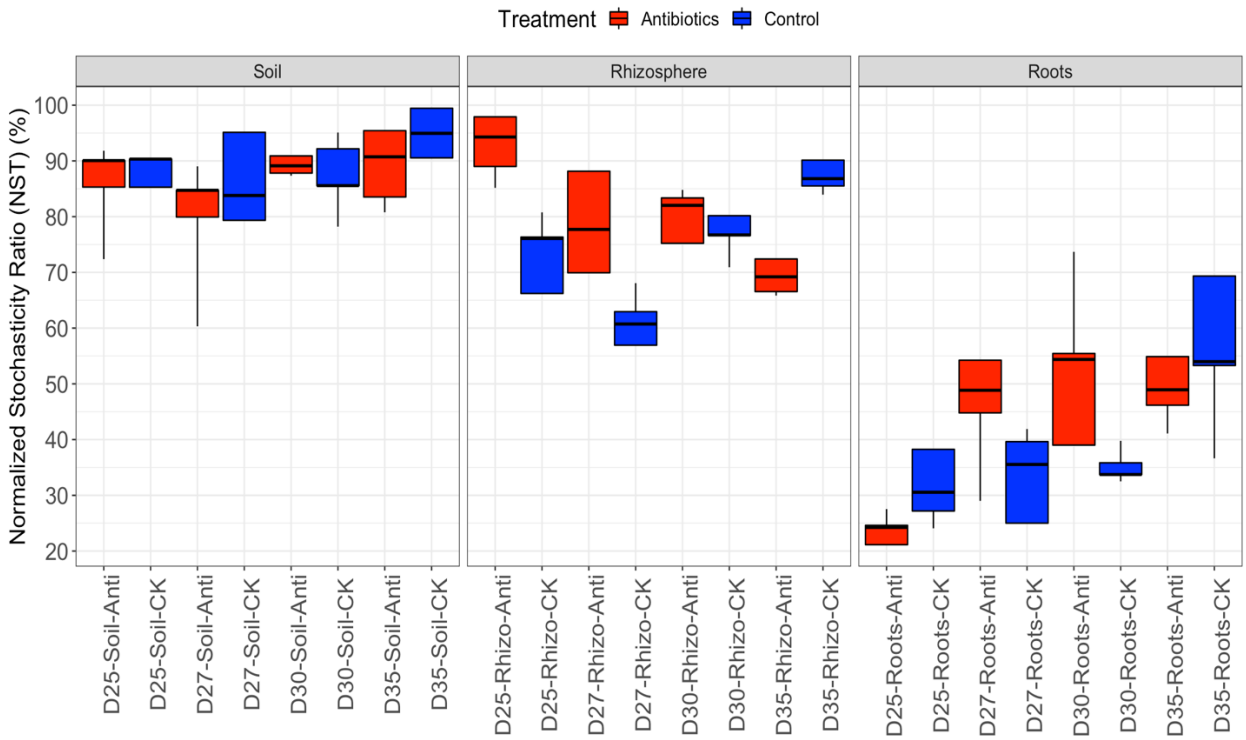

Figure C4: Normalized stochastic ratio of samples collected on four sampling days.

Table C2: Primer set for 16S rRNA, antibiotic resistance genes (ARGs) and mobile genetic elements (MGEs). Selected from [158]

Gene target	Primer number	Function	Class	Forward primer	Reverse Primer
aac3-Via	1510	deactivate	Aminoglycoside	GTGTCCGTCGCCAAGGA	GGTGACGGCCTTGTCGA
bla1	39	deactivate	Beta Lactam	GCAAGTTGAAGCGAAAGAAAAGA	TACCAGTATCAATCGCATATACACCTAA
cphA	46	deactivate	Beta Lactam	GCGAGCTGCACAAGCTGAT	CGGCCCAGTCGCTCTTC
blaSFO	121	deactivate	Beta Lactam	CCGCCGCCATCCAGTA	GGGCCGCCAAGATGCT
blaTEM	1512	deactivate	Beta Lactam	CGCCGCATACACTATTCTCAG	GCTTCATTCAGCTCCGGTTC
qepA_1_2	1201	qepA_1_2	Fluoroquinolone	GGGCATCGCGCTGTTC	GCGCATCGGTGAAGCC
emrD	64	efflux	MDR	CTCAGCAGTATGGTGGTAAGCATT	ACCAGGCGCCGAAGAAC
mepA	245	efflux	MDR	ATCGGTCGCTCTTCGTTCAC	ATAAATAGGATCGAGCTGCTGGAT
tolC	298	efflux	MDR	GGCCGAGAACCTGATGCA	AGACTTACGCAATTCCGGGTTA
tnpA	202	MGE	Transposase	CCGATCACGGAAAGCTCAAG	GGCTCGCATGACTTCGAATC
IncP_oriT	342	MGE	Plasmid-inc	CAGCCTCGCAGAGCAGGAT	CAGCCGGGCAGGATAGGTGAAGT
intI1F165_clinical	359	MGE	Integrase	CGAACGAGTGGCGGAGGGTG	TACCCGAGAGCTTGGCACCCA
ISPps1-pseud	369	MGE	Insertional	CACACTGCAAAAACGCATCCT	TGTCTTTGGCGTCACAGTTCTC
ISSm2-Xanthob	370	MGE	Insertional	TGGATCGACCGGTTCCAT	GCTGACCGAGCTGTCCATGT
intl3	1522	MGE	Integrase	CAGGTGCTGGGCATGGA	CCTGGGCAGCATCACCA
oleC	285	efflux	MLSB	CCCGGAGTCGATGTTCGA	GCCGAAGACGTACACGAACAG
strB	177	protection	Sulfonamide	GCTCGGTCGTGAGAACAATCT	CAATTTCGGTCGCCTGGTAGT
tetPB	502	efflux	tetracycline	TGGCAAGACGAGTTTGACTGA	GATCGCTCCACTTCAGCGATAA

Table C3: Sum of relative abundance in each ARGs class (log2 transferred).

	Aminoglycoside	Beta Lactam	Fluoroquinolone	MDR	MGE	MLSB	Sulfonamide	Tetracycline
D25-Shoot-CK	NA	NA	NA	-10	NA	NA	NA	NA
D30-Shoot-CK	NA	NA	NA	-7.4	-16.2	NA	NA	NA
D35-Shoot-CK	NA	NA	NA	-6.9	-14.4	NA	NA	NA
D25-Shoot-Anti	NA	NA	NA	-7.6	NA	NA	NA	NA
D30-Shoot-Anti	NA	-15.8	NA	-6.8	NA	NA	NA	NA
D35-Shoot-Anti	NA	-15.3	NA	-6.7	-16	NA	NA	NA
D25-Root-CK	-10.5	-12.9	-13.3	-6	-14.1	-11.6	NA	NA
D30-Root-CK	-9.9	-12.4	-12.3	NA	-14.2	-10.4	NA	NA
D35-Root-CK	-10.9	-13	-12.7	-12	-12.2	-9.7	NA	NA
D25-Root-Anti	-10.1	-12.7	-12.5	-13	-11.2	-10	NA	NA
D30-Root-Anti	-9.4	-12.6	-11.7	NA	-11.1	-9.7	NA	NA
D35-Root-Anti	-9.9	-13.5	-11.8	NA	-11.4	-9.9	NA	NA
D25-Soil-CK	-8.4	-13.2	-11.1	-11	-12.7	-9.2	NA	-12.8
D30-Soil-CK	-9.3	NA	-12.1	-12	-13.2	-10.3	NA	-15.2
D35-Soil-CK	-8.4	NA	-11.9	NA	-12.3	-10.6	NA	-14.3
D25-Soil-Anti	-8.9	-15.7	-12.7	-15	-12.1	-9.9	NA	-15.1
D30-Soil-Anti	-10	-15	-13.1	-13	-12.8	-11.1	NA	NA
D35-Soil-Anti	-5.7	NA	-8.7	NA	-10.2	-7.9	NA	-11.7
D25-Rhizo-CK	-8.7	-13.4	-11.2	-13	-11.8	-10.5	NA	-14.1
D30-Rhizo-CK	-9.3	-14	-13.2	-13	-12.2	-9.5	-13.7	NA
D35-Rhizo-CK	-9.8	-12.3	-12.7	-15	-12.4	-10.4	-14.2	NA
D25-Rhizo-Anti	-9.1	-14	-11.7	-13	-11.6	-9.7	NA	NA
D30-Rhizo-Anti	-8.4	-12.5	-11.1	-13	-11.3	-9.9	NA	NA
D35-Rhizo-Anti	-9.2	-14.2	-11.9	NA	-11.3	-10.2	NA	NA

Table C4: Correlation tests between top 10 families, antibiotic concentrations, and ARGs/MGEs for different niches (bulk soil, rhizosphere soil, lettuce roots, and lettuce shoots).

Variable 1	Varaiable 2	ρ	p value
	Bulk Soil		
Xanthobacteraceae	Methylophilaceae	-0.98	3.31E-05
Burkholderiaceae	Methylophilaceae	-0.90	2.01E-03
Bacillaceae	Methylophilaceae	-0.88	3.85E-03
Sulfamethoxazole	Sphingomonadaceae	-0.81	1.49E-02
Trimethoprim	Xanthobacteraceae	-0.76	2.80E-02
Sulfamethoxazole	Burkholderiaceae	-0.76	2.80E-02

Table C4 (cont'd)

Sphingomonadaceae	Methylophilaceae	-0.76	2.80E-02
Trimethoprim	Burkholderiaceae	-0.74	3.66E-02
Antibiotics total	Burkholderiaceae	-0.74	3.66E-02
Antibiotics total	Xanthobacteraceae	-0.71	4.65E-02
Bacillaceae	Sphingomonadaceae	0.71	4.65E-02
Bacillaceae	Burkholderiaceae	0.71	4.65E-02
Tylosin	Chthoniobacteraceae	0.71	4.65E-02
Sphingomonadaceae	Burkholderiaceae	0.74	3.66E-02
Sulfamethoxazole	Methylophilaceae	0.74	3.66E-02
Antibiotics total	Methylophilaceae	0.74	3.66E-02
Sulfamethoxazole	Trimethoprim	0.76	2.80E-02
Tylosin	Antibiotics total	0.79	2.08E-02
Xanthobacteraceae	Sphingomonadaceae	0.79	2.08E-02
Trimethoprim	Methylophilaceae	0.79	2.08E-02
Trimethoprim	qepA_1_2	0.83	4.16E-02
Sulfamethoxazole	Antibiotics total	0.86	6.53E-03
Xanthobacteraceae	Burkholderiaceae	0.88	3.85E-03
Lincomycin	qepA_1_2	0.89	1.88E-02
Bacillaceae	Xanthobacteraceae	0.90	2.01E-03
Trimethoprim	Antibiotics total	0.93	8.63E-04
Ciprofloxacin	qepA_1_2	0.94	4.80E-03
qepA_1_2	aac3-Via	0.94	4.80E-03
qepA_1_2	intl3	0.94	4.80E-03
aac3-Via	intl3	1.00	0.00E+00
	Rhizosphere soil		
Sulfamethoxazole	cphA	-1.00	0.00E+00
Lincomycin	cphA	-0.90	3.74E-02
Ciprofloxacin	Xanthobacteraceae	-0.90	9.43E-04
Antibiotics total	Xanthobacteraceae	-0.87	2.50E-03
Lincomycin	MonensinSodium	-0.81	1.49E-02
MonensinSodium	Sphingomonadaceae	-0.79	2.08E-02
Tylosin	Chitinophagaceae	-0.77	1.59E-02
Sulfamethoxazole	MonensinSodium	-0.76	2.80E-02
Trimethoprim	MonensinSodium	-0.76	2.80E-02
Sulfamethoxazole	aac3-Via	-0.75	1.99E-02
Beijerinckiaceae	Enterobacteriaceae	-0.74	3.66E-02
Lincomycin	Xanthobacteraceae	-0.72	2.98E-02
Bacillaceae	Beijerinckiaceae	-0.70	3.58E-02
Beijerinckiaceae	Chthoniobacteraceae	-0.68	4.24E-02
Trimethoprim	Gemmatimonadaceae	-0.67	4.99E-02

Table C4 (cont'd)

Beijerinckiaceae	Xanthobacteraceae	-0.67	4.99E-02
Antibiotics total	Chthoniobacteraceae	-0.67	4.99E-02
Sulfamethoxazole	Beijerinckiaceae	0.67	4.99E-02
Gemmatimonadaceae	Chthoniobacteraceae	0.67	4.99E-02
Ciprofloxacin	Beijerinckiaceae	0.70	3.58E-02
Bacillaceae	Enterobacteriaceae	0.71	4.65E-02
aac3-Via	Bacillaceae	0.72	2.98E-02
Trimethoprim	Tylosin	0.73	2.46E-02
Lincomycin	Tylosin	0.73	2.46E-02
Antibiotics total	Sphingomonadaceae	0.73	2.46E-02
Enterobacteriaceae	Chthoniobacteraceae	0.74	3.66E-02
Sulfamethoxazole	Antibiotics total	0.77	1.59E-02
Gemmatimonadaceae	Xanthobacteraceae	0.78	1.25E-02
oleC	qepA_1_2	0.79	3.62E-02
oleC	aac3-Via	0.79	2.08E-02
Sulfamethoxazole	Ciprofloxacin	0.80	9.63E-03
Lincomycin	Sphingomonadaceae	0.80	9.63E-03
Xanthobacteraceae	Chthoniobacteraceae	0.85	3.70E-03
Lincomycin	Ciprofloxacin	0.87	2.50E-03
Lincomycin	Antibiotics total	0.87	2.50E-03
Tylosin	Antibiotics total	0.87	2.50E-03
Ciprofloxacin	Antibiotics total	0.90	9.43E-04
cphA	aac3-Via	0.90	3.74E-02
ISPps1-pseud	Gemmatimonadaceae	0.90	3.74E-02
Sulfamethoxazole	Sphingomonadaceae	0.90	9.43E-04
Sulfamethoxazole	Lincomycin	0.93	2.36E-04
MonensinSodium	bla1	1.00	0.00E+00
intI1F165_clinical	ISPps1-pseud	1.00	0.00E+00
cphA	qepA_1_2	1.00	0.00E+00
intI1F165_clinical	aac3-Via	1.00	0.00E+00
cphA	intl3	1.00	0.00E+00
	Lettuce roots		
Sulfamethoxazole	Tylosin	-0.92	5.07E-04
Xanthobacteraceae	Methylophilaceae	-0.88	1.59E-03
Oxytetracycline	Methylophilaceae	-0.87	2.50E-03
Ciprofloxacin	Methylophilaceae	-0.87	2.50E-03
Beijerinckiaceae	Chitinophagaceae	-0.87	2.50E-03
Antibiotics total	Methylophilaceae	-0.83	5.27E-03
MonensinSodium	qepA_1_2	-0.82	2.34E-02
Bacillaceae	Burkholderiaceae	-0.82	7.22E-03

Table C4 (cont'd)

Sulfamethoxazole	Oxytetracycline	-0.78	1.25E-02
Sulfamethoxazole	Ciprofloxacin	-0.78	1.25E-02
Beijerinckiaceae	Methylophilaceae	-0.78	1.25E-02
Lincomycin	Bacillaceae	-0.77	1.59E-02
Sulfamethoxazole	Xanthobacteraceae	-0.77	1.59E-02
Tetracycline	Bacillaceae	-0.75	1.99E-02
Tylosin	Methylophilaceae	-0.73	2.46E-02
Sulfamethoxazole	Antibiotics total	-0.72	2.98E-02
Trimethoprim	Methylophilaceae	-0.72	2.98E-02
Xanthobacteraceae	Chitinophagaceae	-0.70	3.58E-02
Sulfamethoxazole	Trimethoprim	-0.68	4.24E-02
Tylosin	Burkholderiaceae	-0.68	4.24E-02
Chthoniobacteraceae	Chitinophagaceae	-0.68	4.24E-02
Burkholderiaceae	Methylophilaceae	0.67	4.99E-02
Trimethoprim	Beijerinckiaceae	0.68	4.24E-02
aac3-Via	Chthoniobacteraceae	0.68	4.24E-02
Lincomycin	Tetracycline	0.70	3.58E-02
oleC	aac3-Via	0.70	3.58E-02
Antibiotics total	Beijerinckiaceae	0.70	3.58E-02
Trimethoprim	Xanthobacteraceae	0.72	2.98E-02
Beijerinckiaceae	Chthoniobacteraceae	0.72	2.98E-02
Tylosin	Antibiotics total	0.73	2.46E-02
qepA_1_2	aac3-Via	0.73	2.46E-02
Gemmatimonadaceae	Chthoniobacteraceae	0.75	1.99E-02
Trimethoprim	Tylosin	0.80	9.63E-03
Oxytetracycline	Antibiotics total	0.80	9.63E-03
Beijerinckiaceae	Xanthobacteraceae	0.80	9.63E-03
Ciprofloxacin	Antibiotics total	0.83	5.27E-03
Sulfamethoxazole	Burkholderiaceae	0.83	5.27E-03
Sulfamethoxazole	Methylophilaceae	0.83	5.27E-03
Antibiotics total	Xanthobacteraceae	0.85	3.70E-03
Ciprofloxacin	Xanthobacteraceae	0.88	1.59E-03
Oxytetracycline	Xanthobacteraceae	0.90	9.43E-04
Trimethoprim	Antibiotics total	0.92	5.07E-04
Oxytetracycline	Ciprofloxacin	0.93	2.36E-04
	Lettuce shoots		
MonensinSodium	Tylosin	1.00	0.00E+00
MonensinSodium	Ciprofloxacin	1.00	0.00E+00
Ciprofloxacin	emrD	0.94	4.80E-03
Tylosin	Methylophilaceae	1.00	0.00E+00

Table C4 (cont'd)

Sphingomonadaceae	Enterobacteriaceae	0.90	3.74E-02
Burkholderiaceae	Enterobacteriaceae	-1.00	0.00E+00

Appendix D: CHAPTER V Supplementary Materials

Predicting Customers' Buy and Pay Preferences for Labeled Products with Machine Learning

Descriptive statistics

The demographics mostly matched the U.S. population based on the 2010 census data, because we set quotas for 630 matching samples. There were slight differences with the 110 additional completed participants. Briefly, the survey participants reported an average age of 45 years old, with 88% completed high school. Twenty-four percent of the participants reported having a college or other advanced degree. Forty-eight percent of participants were male, and fifty-two percent were female. Survey participants self-reported as White or Caucasian (65%). Hispanic (16%), Black, or (14%) other minorities (5%). Half of the survey participants were unemployed (49%), while 37% had a full-time job, and 13% had a part-time job. The high unemployment rate might be because 12% of participants are in college years (19 – 24 years old), and 16% of participants are people older than 65 years old. Forty-two percent of participants were married, whereas 37% of participants had never married. Most of the participants lived in urban settings (79%). Around 56% percent of participants were homeowners, whereas 44% of participants rent a house or apartment. The average household size, including both adults and children, was three. Appendix Table D1 showed detailed descriptive statistics for demographics.

Food relevant habits section had eight questions. Most participants ate meat 1-2 times per day (69.5%), where only 43.5% of participants ate leafy greens at the same frequency. Approximately one-third of participants eat leafy greens less than one time per day. Over 70% of

participants always wash their fresh produce, whereas only 35.9% of them always cook fresh produce. More than half of participants (58.4%) cook their fresh produce sometimes. More than half of the participants cooked their fresh produce for better taste. Most participants liked to shop at conventional grocery stores (67.6%), and more than half of participants ate home-prepared meals (57.6%).

The foodborne disease perception section had two small questions and one big question on five pathogens. Most of our participants don't have a foodborne disease in the past two years, counting 85.5%. The majority of participants (81.6%) were aware that *Salmonella* might be present in raw chicken, and needed to be fully cooked. For USDA zero-tolerance pathogen in ready to eat food, *Salmonella*, *Escherichia coli O157:H7*, and *Listeria monocytogenens*, more than half of our participants ranked them from very severe to extremely severe. Interestingly, Norovirus, which is a common virus causing diarrhea, 23% of participants did not know this virus. The severity of Norovirus in participants' minds was a little less than 50% from very severe to extremely severe. A large group of participants thought *Staphylococcus aureus* induced foodborne disease was extremely severe (36.2%).

The participants' willingness to buy and pay more for labeled products had one set of questions with eight food labels (*Without Antibiotics*, *No Medically Important Antibiotics*, *No Growth Promoting Hormones*, *Cage Free*, *USDA Organic*, *Locally Raised*, *Generic Brand*, and *Major Brand*). Each label had two questions asking participants' willingness to buy or pay more for that specific label. Our results yes or no fluctuate around 50% for each label. Appendix Table D2 showed detailed descriptive statistics for food safety and food label block questions.

Table D1: Demographics of survey participants. Total N=740

Demographic Characteristics of the Survey Population			
	Percentage (%)		Percentage (%)
Gender		Education	
Male	48	Graduate Degree	11
Female	52	Bachelor Degree	13
		Associate degree	7
Age		High School	57
19-24	12	Less than High School	12
25-34	20		
35-49	[166]	[166]Employment	
50-64	23	Full-time	37
>65	16	Part-time	13
		Unemployed	49
Ethnicity			
White or Caucasian	65	Marital Status	
Black or African American	14	Married	42
Hispanic	16	Never Married	37
Asian	3	Divorced	12
Others	2	Separated	4
		Widowed	5
Household Size			
1	22	Residence Type	
2	29	Own a single family home	52
3	21	Rent an apartment or condo	25
4	14	Rent a single family home	19
5	6	Own an apartment or condo	4
6	3		
More than 6	5	Residence Location	
		Urban	79
		Rural	21

Table D2: Survey instrument and descriptive statistics answers. N=740

Questions and Answers	Percentage (%)
Food relevant habits	
Q1: How many times per day do you eat meat	
I do not eat meat	5.7
1-2 times per day	69.5
2-3 times per day	19.3
More than 3 times per day	5.5
Q2: How many times per day do you eat leafy greens (lettuce, spinach etc)?	
Less than one time per day	34.6
1-2 times per day	43.5
2-3 times per day	16.1
More than 3 times per day	5.8
Q3: Where do you shop for groceries most often? - Selected Choice	
Conventional grocery store chains (e.g. Meijer, Walgreens, Walmart)	67.6
Local grocery stores (e.g. farmer's market, Horrrocks, Harmon's)	17.8
Organic grocery chains (e.g. Whole Foods)	10.5
Others	4.1
Q4: How often do you wash your fresh produce?	
Always	70.3
Sometimes	25.3
Never	4.5
Q5: How often do you cook your fresh produce (vegetables)?	
Always	35.9
Sometimes	58.4
Never	5.7
Q6: If you cook your fresh produce, what is the primary reason -Selected Choice	
Tastes Better	52.3
Food Safety	34.1
Other	13.6
Q7: Do you eat more home-prepared meals or meals from restaurants?	
I eat more home prepared meals than meals from restaurants	57.6
I always eat home-prepared meals	21.4
I eat as many home prepared meals as meals from restaurants	13.4
I eat more meals from restaurants than meals prepared at home	5.8
I always eat meals from restaurants	1.9
Foodborne disease perceptions	
Q8: Have you had food-borne disease in the past 24 months?	
Yes	14.5
No	85.5

Table D2 (cont'd)

Q9: Are you aware that raw chicken in the market contains *Salmonella* and has to be fully cooked before eaten?	
Yes	81.6
No	18.4

Q10 - How severe do you believe the following food-borne diseases are? (Percentage %)

	Not at all severe	Slightly Severe	Somewhat Severe	Very Severe	Extremely Severe	I do not know
Salmonella (nontyphoidal)	3.4	7.6	22.7	23.4	38.0	5.0
E. coli (*Escherichia coli*, O157 H7)	3.1	6.8	17.3	24.6	42.4	5.8
Norovirus	4.5	6.5	16.2	21.2	28.6	23.0
Staph infection (*Staphylococcus aureus*)	4.1	6.9	17.6	25.3	36.2	4.1
Listeria monocytogenes	3.8	6.5	13.9	21.5	31.8	22.6

Participants' willing to buy and pay more for labelled products

Q11: More likely to buy (Percentage %)

	Yes	No	Do not care*
USDA-Certified Organic	56.22	19.05	24.73
Raised without antibiotics	62.7	14.46	22.84
Does Not Contain Medically Important Antibiotics	57.03	17.57	25.41
Does Not Contain Growth Promoting Antibiotics	61.89	15.81	22.3
Raised Locally	60.41	12.16	27.43
Free Range	60	12.57	27.43
Major Brand Names (e.g., Kraft)	52.84	16.49	30.68
Generic Brand Names	48.24	21.35	30.41

Q11: Willing to pay more (Percentage %)

	Yes	No	Do not care*
USDA-Certified Organic	38.92	41.76	19.32
Raised without antibiotics	42.97	38.11	18.92
Does Not Contain Medically Important Antibiotics	37.43	41.49	21.08
Does Not Contain Growth Promoting Antibiotics	40.95	40.41	18.65
Raised Locally	42.03	38.24	19.73
Free Range	40.27	38.38	21.35
Major Brand Names (e.g., Kraft)	40.27	37.97	21.76
Generic Brand Names	31.35	47.03	21.62

*Added to "no" category in Machine Learning

Table D3: Tuned parameters for each machine learning model.

	Tuned parameters	Selection pool for each parameter
Logistic Regression	**C**: float, optional (default=1.0)	c_pool = [1,0.01,0.1,10,100]
Supporting Vector Machine	**C** is a parameter of the SVC learner and is the penalty for misclassifying a data point. When C is small, the classifier is okay with misclassified data points (high bias, low variance). When C is large, the classifier is heavily penalized for misclassified data and therefore bends over backwards avoid any misclassified data points (low bias, high variance) **gamma** is a parameter of the RBF kernel and can be thought of as the 'spread' of the kernel and therefore the decision region. When gamma is low, the 'curve' of the decision boundary is very low and thus the decision region is very broad. When gamma is high, the 'curve' of the decision boundary is high, which creates islands of decision-boundaries around data points. We will see this very clearly below.	c_pool = [0.01,0.1,1,10,100,1000] g_pool = [1e-5,1e-4,1e-3,0.01,0.1,10]
Neural Network	**hidden_layer_sizes.** The ith element represents the number of neurons in the ith hidden layer. The number of layers. **solver.** The solver for weight optimization.	nn_pool = [(100,),(64,32),(32,16), (128,64),(128,64,32)] solver_pool = ['adam', 'lbfgs','sgd']
Random Forest	**n_estimators**, The number of trees in the forest; **max_depth**, The maximum depth of the tree.	n_pool = [10,20,50,100,200,300] d_pool = [None,1,2,3,4,5,6,7]

Table D4: Participants willingness to buy (WTB) labelled products using probit regression. Label abbreviation: USDA Organic, USDA; Raised Without Antibiotics, NoAnti; No Medically Important Antibiotics, NoMedAnti; No Growth Promoting Hormones, NoHormone; Locally Raised, Local; Cage Free, FreeRange; Major Brand, Brand; Generic Brand, NoBrand. Numbers in the table represent coefficient. In other words, change in z-score with one unit increase in independent variables. Positive constants in the table represented the increase in probability to dependent variables. Negative constants in the table represented the decrease in probability to dependent variables. Number in the brackets refers to standard error.

Participants' WTB using Probit Regression								
Independent variables	*Dependent variable:*							
	USDA	NoAnti	NoMedAnti	NoHormone	Local	FreeRange	Brand	NoBrand
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
Gender	0.152	0.252**	0.226**	0.175*	-0.051	0.273***	-0.039	0.148
	(0.105)	(0.106)	(0.103)	(0.105)	(0.103)	(0.105)	(0.102)	(0.103)
Ethnicity	0.322***	-0.095	0.111	0.057	-0.252**	-0.143	0.035	-0.241**
	(0.111)	(0.111)	(0.108)	(0.110)	(0.108)	(0.109)	(0.107)	(0.107)
Residence Location	0.225*	0.310**	0.325***	0.248**	0.096	0.322**	0.101	0.012
	(0.127)	(0.128)	(0.124)	(0.126)	(0.125)	(0.127)	(0.124)	(0.125)
Household Size	-0.002	0.015	0.009	0.100**	0.005	0.013	0.086*	0.081*
	(0.047)	(0.047)	(0.046)	(0.047)	(0.046)	(0.046)	(0.045)	(0.045)
Employment	0.081	-0.068	-0.0003	0.055	-0.084	-0.021	-0.044	-0.039
	(0.062)	(0.062)	(0.060)	(0.061)	(0.060)	(0.061)	(0.060)	(0.060)
Residence Type	0.047	-0.031	0.012	0.031	0.014	0.037	-0.005	0.116
	(0.104)	(0.104)	(0.101)	(0.103)	(0.102)	(0.103)	(0.101)	(0.101)
Education	0.024	-0.002	-0.055	-0.089*	-0.016	-0.016	0.018	-0.034
	(0.048)	(0.048)	(0.046)	(0.047)	(0.047)	(0.047)	(0.046)	(0.046)
Age	-0.003	-0.0002	0.002	0.009**	-0.004	0.003	0.003	0.005
	(0.004)	(0.004)	(0.004)	(0.004)	(0.004)	(0.004)	(0.004)	(0.004)
How many times per day do you eat meat	-0.028	0.072	0.064	0.019	-0.035	-0.032	0.057	0.033
	(0.081)	(0.083)	(0.080)	(0.080)	(0.080)	(0.081)	(0.079)	(0.079)

Table D4 (cont'd)

How many times per day do you eat leafy greens	-0.078	0.013	-0.061	-0.081	-0.038	-0.040	0.015	-0.089*
	(0.050)	(0.050)	(0.049)	(0.050)	(0.049)	(0.050)	(0.048)	(0.049)
Grocery store choices	0.128	0.347***	0.031	0.145	0.176	0.165	-0.074	0.249**
	(0.110)	(0.112)	(0.107)	(0.109)	(0.108)	(0.109)	(0.106)	(0.106)
How often do you wash your fresh produce	0.272***	0.206**	0.205**	0.292***	0.123	0.177*	0.060	-0.103
	(0.095)	(0.094)	(0.092)	(0.092)	(0.092)	(0.093)	(0.092)	(0.093)
How often do you cook your fresh produce	0.256**	0.077	-0.026	0.064	0.116	0.209**	0.124	0.127
	(0.102)	(0.103)	(0.100)	(0.102)	(0.101)	(0.102)	(0.099)	(0.100)
Reason Food safety	0.260**	0.346***	0.214*	0.148	0.082	0.049	0.070	-0.068
	(0.114)	(0.116)	(0.112)	(0.114)	(0.112)	(0.113)	(0.110)	(0.110)
Reason other	0.085	-0.148	-0.276*	-0.049	-0.084	-0.078	-0.340**	-0.460***
	(0.163)	(0.162)	(0.160)	(0.163)	(0.160)	(0.162)	(0.161)	(0.163)
Do you eat more home prepared meals or meals from restaurant	0.090	0.149**	0.093	0.057	0.131**	0.189***	0.042	0.094
	(0.059)	(0.059)	(0.058)	(0.058)	(0.058)	(0.059)	(0.057)	(0.058)
Have you had foodborne disease in the past 24 months	-0.221	-0.128	-0.086	-0.018	-0.064	-0.131	-0.263*	-0.447***
	(0.150)	(0.150)	(0.144)	(0.145)	(0.144)	(0.146)	(0.143)	(0.144)
Are you aware that raw chicken in the market contain *Salmonella*	-0.138	-0.002	-0.162	-0.157	-0.104	-0.200	-0.030	0.156
	(0.135)	(0.136)	(0.132)	(0.133)	(0.131)	(0.132)	(0.131)	(0.132)
Nontyphoidal *Salmonella*	0.134**	0.032	0.039	-0.017	0.058	0.060	0.105*	0.016
	(0.056)	(0.056)	(0.054)	(0.055)	(0.054)	(0.055)	(0.054)	(0.055)
Escherichia coli O157:H7	-0.072	0.074	0.024	0.099*	0.007	0.056	-0.060	0.018
	(0.058)	(0.057)	(0.056)	(0.057)	(0.056)	(0.056)	(0.056)	(0.056)
Norovirus	0.019	0.055	0.008	-0.039	-0.002	0.017	0.017	-0.030

Table D4 (cont'd)

	(0.036)	(0.037)	(0.036)	(0.037)	(0.036)	(0.037)	(0.035)	(0.036)
Staphylococcus aureus	0.034	0.057	0.036	0.045	0.056	0.032	0.104**	0.080*
	(0.045)	(0.045)	(0.044)	(0.045)	(0.044)	(0.045)	(0.044)	(0.045)
Listeria monocytogenes	0.039	-0.048	0.004	0.043	0.029	-0.018	-0.059*	0.022
	(0.036)	(0.037)	(0.036)	(0.036)	(0.036)	(0.036)	(0.035)	(0.035)
Constant	-2.249***	-1.982***	-1.358**	-2.093***	-0.856	-2.256***	-1.151**	-0.766
	(0.557)	(0.560)	(0.542)	(0.552)	(0.540)	(0.551)	(0.538)	(0.539)
Observations	740	740	740	740	740	740	740	740
Log Likelihood	-453.119	-444.542	-476.630	-456.218	-472.729	-459.750	-486.699	-481.637
Akaike Inf. Crit.	954.238	937.085	1,001.259	960.437	993.457	967.499	1,021.399	1,011.274

Note: $^{*}p < 0.1$; $^{**}p < 0.05$; $^{***}p < 0.01$

Table D5: Participants' willingness to pay more (WTP) for labelled products using the probit regression. Label abbreviation: USDA Organic = USDA; Raised Without Antibiotics = NoAnti; No Medically Important Antibiotics = NoMedAnti; No Growth Promoting Hormones = NoHormone; Locally Raised = Local; Cage Free = FreeRange; Major Brand = Brand; Generic Brand = NoBrand. Numbers in the table represent coefficients. In other words, change in z-score with one unit increase in independent variables. Positive constants in the table represented the increase in probability to dependent variables. Negative constants in the table represented the decrease in probability to dependent variables. Number in the brackets refers to standard error.

Participants' WTP using Probit Regression								
Independent variables	*Dependent variable:*							
	USDA	NoAnti	NoMedAnti	NoHormone	Local	FreeRange	Brand	NoBrand
Gender	0.035	-0.064	0.061	0.021	0.047	0.171*	0.011	0.144
	(0.106)	(0.105)	(0.106)	(0.104)	(0.103)	(0.104)	(0.104)	(0.108)
Ethnicity	0.176	-0.056	0.208*	-0.035	-0.083	-0.151	0.105	0.011
	(0.110)	(0.109)	(0.109)	(0.108)	(0.107)	(0.109)	(0.108)	(0.112)
Residence Location	0.070	0.312**	0.301**	0.244*	0.187	0.181	0.186	0.195
	(0.130)	(0.128)	(0.132)	(0.127)	(0.126)	(0.127)	(0.127)	(0.133)
Household Size	-0.123***	-0.019	-0.075	-0.069	-0.087*	-0.115**	-0.032	-0.070
	(0.047)	(0.046)	(0.047)	(0.046)	(0.045)	(0.046)	(0.046)	(0.048)
Employment	0.042	0.084	0.029	0.074	0.049	0.050	0.023	-0.014
	(0.062)	(0.061)	(0.062)	(0.060)	(0.060)	(0.061)	(0.060)	(0.064)
Residence Type	-0.155	-0.078	-0.063	-0.113	-0.025	0.011	-0.028	0.030
	(0.105)	(0.103)	(0.104)	(0.102)	(0.101)	(0.103)	(0.102)	(0.107)
Education	0.120**	0.022	-0.004	0.002	-0.013	0.040	0.060	0.017
	(0.047)	(0.047)	(0.047)	(0.046)	(0.046)	(0.047)	(0.046)	(0.049)
Age	-0.012***	-0.007**	-0.008**	-0.004	-0.005	-0.001	-0.005	-0.007*
	(0.004)	(0.004)	(0.004)	(0.004)	(0.004)	(0.004)	(0.004)	(0.004)
How many times per day do you eat meat	-0.062	0.070	0.062	0.048	0.023	0.142*	0.082	0.125
	(0.081)	(0.081)	(0.080)	(0.080)	(0.079)	(0.080)	(0.079)	(0.082)

Table D5 (cont'd)

How many times per day do you eat leafy greens	-0.127**	-0.013	-0.129**	-0.055	-0.076	-0.088*	0.003	-0.059
	(0.051)	(0.050)	(0.050)	(0.049)	(0.049)	(0.049)	(0.049)	(0.052)
Grocery store choices	0.204*	0.136	0.194*	0.128	0.228**	0.237**	0.200*	0.337***
	(0.109)	(0.108)	(0.108)	(0.107)	(0.106)	(0.107)	(0.106)	(0.109)
How often do you wash your fresh produce	0.011	0.132	0.067	0.039	0.046	0.100	-0.124	-0.007
	(0.097)	(0.096)	(0.098)	(0.094)	(0.093)	(0.095)	(0.094)	(0.099)
How often do you cook your fresh produce	0.153	0.179*	0.251**	0.222**	0.222**	0.170*	0.152	0.178*
	(0.104)	(0.102)	(0.103)	(0.100)	(0.100)	(0.101)	(0.101)	(0.105)
Reason Food safety	0.183	0.148	0.121	0.284**	0.136	0.125	0.050	0.146
	(0.113)	(0.111)	(0.113)	(0.110)	(0.110)	(0.111)	(0.111)	(0.115)
Reason other	-0.073	-0.220	-0.131	-0.047	0.109	0.056	-0.320*	-0.011
	(0.171)	(0.169)	(0.172)	(0.165)	(0.161)	(0.165)	(0.169)	(0.175)
Do you eat more home prepared meals or meals from restaurant	-0.049	0.098*	0.049	-0.023	0.040	0.104*	0.016	0.057
	(0.059)	(0.059)	(0.059)	(0.058)	(0.057)	(0.059)	(0.058)	(0.060)
Have you had foodborne disease in the past 24 months	-0.113	-0.284**	0.0003	-0.081	-0.117	-0.266*	-0.282**	-0.417***
	(0.145)	(0.145)	(0.145)	(0.143)	(0.142)	(0.143)	(0.142)	(0.144)
Are you aware that raw chicken in the market contain *Salmonella*	-0.165	-0.071	-0.128	-0.162	0.053	0.003	-0.043	0.325**
	(0.137)	(0.136)	(0.137)	(0.134)	(0.132)	(0.134)	(0.133)	(0.136)
Nontyphoidal *Salmonella*	0.097*	0.041	0.015	-0.046	0.037	0.028	0.095*	0.092
	(0.057)	(0.056)	(0.057)	(0.055)	(0.055)	(0.056)	(0.055)	(0.057)
Escherichia coli O157:H7	-0.101*	0.067	0.049	0.048	0.035	0.094	-0.035	0.005
	(0.059)	(0.058)	(0.059)	(0.057)	(0.056)	(0.058)	(0.057)	(0.059)

Table D5 (cont'd)

Norovirus	0.125***	0.014	0.048	0.008	0.019	0.001	0.017	0.065*
	(0.038)	(0.036)	(0.037)	(0.036)	(0.036)	(0.036)	(0.036)	(0.038)
Staphylococcus aureus	0.013	-0.002	-0.020	0.055	-0.046	-0.033	0.028	-0.034
	(0.047)	(0.046)	(0.047)	(0.045)	(0.045)	(0.045)	(0.045)	(0.048)
Listeria monocytogenes	-0.005	0.056	0.043	0.026	0.067*	0.054	0.009	0.001
	(0.037)	(0.036)	(0.036)	(0.036)	(0.036)	(0.036)	(0.035)	(0.037)
Constant	-0.007	-1.756***	-1.378**	-0.898*	-1.028*	-1.796***	-0.700	-1.291**
	(0.554)	(0.551)	(0.556)	(0.542)	(0.538)	(0.549)	(0.542)	(0.567)
Observations	740	740	740	740	740	740	740	740
Log Likelihood	-442.311	-460.601	-448.630	-471.769	-479.687	-467.063	-470.176	-421.393
Akaike Inf. Crit.	932.622	969.203	945.259	991.538	1,007.373	982.126	988.352	890.786

Note: $^{*}p < 0.1$; $^{**}p < 0.05$; $^{***}p < 0.01$

Table D6: Machine learning model results. WTB, participants' willingness to buy; WTP, participants' willingness to pay more

Labels	Logistic Regression	Supporting Vector Machine	Neural Network	Random Forest
USDA Organic WTB	0.612 ± 0.052	0.615 ± 0.046	0.612 ± 0.051	0.619 ± 0.036
Raised Without Antibiotics WTB	0.649 ± 0.087	0.643 ± 0.085	0.643 ± 0.078	0.647 ± 0.089
No Medically Important Antibiotics WTB	0.585 ± 0.073	0.554 ± 0.098	0.565 ± 0.058	0.573 ± 0.068
No Growth promoting Hormones/Antibiotics WTB	0.601 ± 0.079	0.619 ± 0.093	0.597 ± 0.093	0.622 ± 0.082
Raised Locally WTB	0.608 ± 0.061	0.592 ± 0.055	0.581 ± 0.063	0.6 ± 0.055
Free Range WTB	0.623 ± 0.052	0.619 ± 0.035	0.619 ± 0.039	0.612 ± 0.044
Major Brand WTB	0.512 ± 0.056	0.554 ± 0.048	0.55 ± 0.062	0.511 ± 0.046
Generic Brand WTB	0.557 ± 0.053	0.524 ± 0.08	0.555 ± 0.067	0.514 ± 0.052
USDA Organic WTP	0.649 ± 0.034	0.657 ± 0.039	0.635 ± 0.037	0.643 ± 0.042
Raised Without Antibiotics WTP	0.607 ± 0.037	0.645 ± 0.052	0.595 ± 0.036	0.614 ± 0.055
No Medically Important Antibiotics WTP	0.619 ± 0.057	0.634 ± 0.061	0.607 ± 0.05	0.642 ± 0.052
No Growth promoting Hormones/Antibiotics WTP	0.562 ± 0.046	0.585 ± 0.037	0.536 ± 0.035	0.578 ± 0.045
Raised Locally WTP	0.562 ± 0.063	0.57 ± 0.052	0.542 ± 0.071	0.57 ± 0.058
Free Range WTP	0.599 ± 0.062	0.597 ± 0.043	0.576 ± 0.053	0.603 ± 0.043
Major Brand WTP	0.619 ± 0.066	0.612 ± 0.069	0.607 ± 0.051	0.596 ± 0.043
Generic Brand WTP	0.659 ± 0.083	0.668 ± 0.064	0.691 ± 0.074	0.68 ± 0.074

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BIBLIOGRAPHY

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