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Investigating Antibiotic Resistance In Urban Agricultural Environment Using Phenotypic, Genomic, And Metagenomic Tools

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INVESTIGATING ANTIBIOTIC RESISTANCE IN URBAN AGRICULTURAL ENVIRONMENT USING PHENOTYPIC, GENOMIC, AND METAGENOMIC TOOLS

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DEDICATION

This work is dedicated to

My parents - Ayesha Bagum and Mafizur Rahaman

My wife – Mahzabeen Rahman

My sister – Sultana Razia Ruma

My nieces – Mahjabeen Sultana Nishat and Tasnuva Tabassum Ame

And

My Ph.D. advisor Dr. Yifan Zhang

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ARG- Antibiotic Resistance Genes

BHI - Brain Heart Infusion

CARD – Comprehensive Antibiotic Resistance Database

CDC – Center for Disease Control and Prevention

CLSI - Clinical and Laboratory Standards Institute

DNA - Deoxyribonucleic Acid

EPA – Environmental Protection Agency

ESBL – Extended-spectrum beta-lactamase

HGT – Horizontal Gene Transfer

LOQ – Limit of Quantification

MDR - Multidrug-resistant

MIC - Minimal Inhibitory Concentration

MRG – Metal Resistance Genes

ORF – Open Reading Frame

PAH – Polycyclic Aromatic Hydrocarbons

PCR - Polymerase Chain Reaction

PMQR – Plasmid-mediated Quinolone Resistance

RDP – Ribosomal Database Project

RNA - Ribonucleic Acid

USDA – United States Department of Agriculture

UNDP - United Nations Development Programs

US - United States of America

WGS – Whole-genome sequencing

XRF – X-ray fluorescenc

BACKGROUND OF THE STUDY

Increasing evidence has shown that the evolution and spread of antibiotic residence in the environment contribute to the occurrence of antibiotic resistance in clinical settings. This suggests an urgent need to minimize the public health risks due to the environmental exposure of antibiotic resistance.

Soil microorganisms can be a significant pool of antibiotic resistance since many species are capable of producing antibiotic substances (D'Costa *et al.*, 2006; Martinez 2008; Popowska *et al.*, 2012) and are naturally antibiotic resistant themselves. Meanwhile, as urban agriculture is gaining popularity nationwide, the unique factors associated with urban agricultural production may have a significant impact on the persistence of these bacteria. For instance, soil contaminants such as heavy metals, antibiotic residues, pesticides, etc. stemming from current agricultural practices as well as a legacy of historical residential, industrial, and transportation practices have been suggested as antibiotic resistance selective pressure, and thus can shape the composition of antibiotic-resistant soil bacteria. Consequently, urban agricultural production offers a unique angle to investigate antibiotic resistance in the environment and will provide critical insight into the emergence and persistence of antibiotic resistance.

Vegetables can acquire biological, physical, and/or chemical contaminants from soil either by direct contact or through contaminated water. Vegetables contaminated with antibiotic-resistant bacteria can then act as a source or carrier of antibiotic resistance and have the potential to cause serious public health problems.

The overall goal of this study was to determine the nature and extent of antibiotic resistance in the urban agricultural environment. The major research questions we were trying to answer were: 1. Would phenotypic determination alone be able to describe the extent of antibiotic

resistance in the environment? 2. What is the correlation between soil contaminants such as metals and antibiotics and antibiotic resistance? 3. What is the potential of antibiotic resistance transfer in soil bacteria? 4. What is the impact of soil contaminants on vegetables produced in urban agriculture in terms of microbial profile and antibiotic resistance genes?

Urban Agriculture and its importance

Urban agriculture is becoming more and more popular both in the United States and worldwide. According to United Nations Development Programs (UNDP), cities are growing 15% of the food worldwide (Smit *et al.*, 1991). Urban agriculture includes gardens and farms which cultivate within the cities, for example, community gardens and urban farms, as well as peri-urban agriculture which sells their produces directly in the urban markets, for example, farmers markets. The socio-economic impacts of the community gardens are the most comprehensively studied among all forms of urban agriculture. In the United States most of the researched community gardens in this regard are located in the low-income neighborhood of large cities, including Detroit, Denver, New York City, and Philadelphia (Blair *et al.*, 1991; Kremer and DeLiberty 2011; Park *et al.*, 2011; Teig *et al.*, 2009). Urban agriculture has health, economic, and social impacts on urban dwellers which ultimately affects national and universal growth and development. Urban agriculture continues to grow momentum as a successful strategy to improve food security in many areas (Armstrong 2000; Blair *et al.*, 1991). Moreover, at individual level, community gardeners can easily access fresh produce from community gardens and can share excess produce with other community people, which would strengthen their bonding. Previous reports documented that, consumption of fruits and vegetables were substantially increased among garden participants (McCormack *et al.*, 2010; Brown and Jameton 2000).Urban agriculture has a direct impact on urban economy by providing skills training and creating new jobs. It is estimated that United States

Department of Agriculture (USDA)-funded community food projects has created 2,300 jobs and over 3,600 micro-businesses. In addition to its economic impact, urban agriculture affects the lives of its community people and gives them the pride of access to their own land. Urban gardening not only produces foods but also helps with community development (Holland 2004).

Antibiotic resistance is a global challenge

Antibiotic resistance is a growing public health concern worldwide. Center for Disease Control and Prevention (CDC) reported that in the U.S. alone, more than 2 million people are affected annually by antibiotic-resistant bacterial infections and more than 23000 of them die. The costs to treat these infections are increasing day by day which adversely affects the economic growth. In the U.S. approximately $20 billion are spending annually to treat the infections caused by antibiotic-resistant bacteria. As bacteria are becoming resistant to more antibiotics it is predicted that the economic burden will continue to increase in the future (Solomon and Oliver 2014).

Antibiotic resistance to almost all antibiotic classes of clinical importance has been reported, including resistance to beta-lactams, quinolones, fluoroquinolones, tetracyclines, streptogramins, and lipopeptides etc. The rate of new antibiotic discovery is very low compared to the development of drug resistance. For the last three decades no new effective antibiotics have entered the market to treat infections (Silver 2011),which presents a great challenge in disease control due to lack of effective antibiotics.

Antibiotic resistance mechanisms

Microorganisms develop antibiotic resistance through a number of mechanisms, such as mutation, increased drug metabolism and increased efflux of antibiotics (Lewis 2013). A known mechanism of antibiotic resistance is mutation of the existing DNA (Tenover 2006; Walsh 2000).

Increased drug metabolism can trigger antibiotic resistance by overexpressing drug metabolism enzymes. For example, overexpression of an enzyme called beta-lactamase can hydrolyze the beta-lactam rings, a major component of the antibiotics carbapenems and penicillin. Hydrolysis of the beta-lactam rings causes inactivation of these antibiotics (Drawz *et al.*, 2014; Poole 2004). Increased efflux of antibiotics can facilitate antibiotic resistance too. Efflux pumps can remove toxic molecules from cytoplasmic compartment to the extracellular compartment as these pumps are highly specific to a group(s) of molecules. Overexpression of these efflux pumps reduces the concentration of antibiotics inside the cell by pumping them out into extracellular compartments. Lower cellular concentration causes drug inactivity (Piddock 2006).

Horizontal gene transfer (HGT) is an important mechanism in the spreading of antibiotic resistance. HGT is a mechanism by which antibiotic resistant bacteria transfer genetic material without sexual involvement to the antibiotic susceptible bacteria when they share the same habitant (Thomas and Nielsen 2005; Tenover 2006). HGT makes antibiotic resistance genes available to other bacteria in the environment (Martinez 2012). Successful transfer of resistance genes between two bacteria by HGT requires share of a common habitat by donor and recipient bacteria (Matte-Tailliez *et al.*, 2002; Wiedenbeck and Cohan 2011), and usually occurs when donors and recipients are phylogenetically related (Philippot *et al.*, 2010; Smillie *et al.*, 2011). The efficiency of HGT can be influenced by several factors, including antibiotics (Jutkina *et al.*, 2016), metals, and biocides (Porse *et al.*, 2017).

HGT occurs via one of the three mechanisms, conjugation, transformation, and transduction. Conjugation is a process of transferring genetic material between two bacteria when they are physically in contact to each other. Conjugative machinery performs this process. Conjugation is the most studied mechanism of HGT (Norman *et al.*, 2009; Guglielmini *et al.*,

2013). It is considered the most efficient mechanism for transferring antibiotic resistance genes (ARGs) associated conjugative elements, for example, transposons or plasmids (Norman *et al.*, 2009). The transfer of mobile genetic elements conferring antibiotic resistance has been documented in bacteria of soil and water environment (Davison 1999). It has been documented that mobile genetic elements (plasmids and transposons) can be transferred between distantly related bacteria too (Roberts and Mullany 2009; Tamminen *et al.*, 2012). This broad host range indicate the importance of conjugation in spreading and accumulation of ARGs between different reservoirs. Plasmids harboring antibiotic resistance determinants can easily spread and disseminate in closely or distantly related bacteria. It has been demonstrated that the $bla_{\text{CTX-M}}$ ESBL genes can be transferred to different plasmids within *Enterobacteriace* (Canton *et al.*, 2012) and now commonly found in human pathogens (Woerther *et al.*, 2013). These genes are now ubiquitous in the environment (Hartmann *et al.*, 2012). Moreover, the transfer of antibiotic resistance containing plasmids between pathogens are disseminating resistance genes conferring resistance to many antibiotic classes, such as beta-lactams, aminoglycosides, quinolones, tetracyclines etc. (Huddleston 2014).

Transformation is a process by which bacteria can uptake naked genetic material from their surroundings. Transformation serves as crucial mechanism of horizontal gene transfer among various bacterial species. Extracellular DNA are abundant in the nature. A DNA extraction method from environmental samples was developed by Mao *et al.* indicated the abundance of extracellular DNA. This study demonstrated that environment could be a potential source of naked DNA with antibiotic resistance determinants. These extracellular DNA are readily available for transmission of antibiotic resistance by transformation (Mao *et al.*, 2014). In a separate study conducted by Chancey *et al.* was observed that, in streptococcal species, resistance determinants containing

transposons can be spread by transformation and conjugation (Chancey *et al.,* 2015). Another study demonstrated that, mobile genetic elements can be transferred between distantly related species (Domingues *et al.,* 2012).

Transduction is a process by which bacteria acquire advantageous genes mediated by bacteriophages. Study of many environmental samples suggests that bacteriophage can play a big role in the dissemination of antibiotic resistance genes, for example, the transfer of gentamicin and tetracycline was observed in enterococcus (Fard *et al.,* 2011), the transfer of beta-lactamase genes was observed in *E. coli* (Billard-Pomares *et al.,* 2014) etc. Cite our own study too. Beta-lactam resistance genes and *mec*A were detected in the bacteriophages of the sewage water samples (Colomer-Lluch *et al.,* 2011). Multiple studies have detected antibiotic resistance genes in bacteriophages isolated from wastewater (Colomer-Lluch *et al.,* 2014a; Colomer-Lluch *et al.,* 2014b; Calero-Caceres *et al.,* 2014), sludge (Calero-Caceres *et al.,* 2014) and effluent (Marti *et al.,* 2014) of wastewater treatment plants, which indicates the importance of bacteriophages as a reservoir of antibiotic resistance genes.

The Role of environmental bacteria in the dissemination of antibiotic resistance

Approximately 5×10^{30} bacteria inhabited in this world and the majority of them are non-pathogenic. Many environmental microorganisms have the ability to produce antibiotics and consequently they have also developed defense mechanisms to protect them from the antibiotics they produced. Over the past few decades, antibiotic resistance research mainly focused on pathogenic bacteria. Currently a series of investigations concluded that environmental bacteria have a significant role in the dissemination of antibiotic resistance alongside the pathogenic bacteria (Benveniste and Davies 1973; Cundliffe 1989; Marshall *et al.,* 1998). Antibiotic producing non-pathogenic and, or opportunistic pathogens in the environment could play a crucial

role in the spread and aggregation of antibiotic resistance. The role of these environmental bacteria as a reservoir of antibiotic resistome is becoming the focus of many studies (Allen *et al.,* 2010; Canton 2009; Martinez 2009b).

Many antibiotic resistance genes detected in the clinically significant microorganisms were originally identified in the environmental bacteria. *Acinetobacter* is a gram-negative bacterium commonly found in water and soil. Previously isolated *Acinetobacter* from water and soil source was mostly sensitive to antibiotics. But nowadays, *Acinetobacter* is one of the challenging antibiotic-resistant bacteria in clinical settings and difficult to treat due to their multi-drug resistance (Maragakis and Perl 2008). Antibiotic-resistant *Acinetobacter* associated infections are spreading fast worldwide. *A. baumannii* is a multidrug-resistant bacterium which possesses resistance mechanisms to many antibiotic classes. Antibiotic resistance genes can also transfer to human pathogens from environmental bacteria. The *qnr* genes, also known as quinolone resistance genes, have been detected in the chromosome of *Shewanella* bacteria commonly found in aquatic environment (Poirel *et al.,* 2012). Currently, in the clinical settings the treatment of quinolone resistance related infections is problematic due to increased resistance to this drug. Moreover, CTX-Ms originally found in *Kluyvera* species (Sarria *et al.,* 2001). *Kluyvera* species were isolated from many environmental samples, including soil and water (Forsberg *et al.,* 2012b).

Soil as a reservoir of antibiotic resistance

Soil is considered one of the major natural habitat for diverse microorganisms. It has been reported that 1g of soil contain approximately 10 billion microorganisms and over thousands of species (Knietsch *et al.,* 2003). Soil microorganisms are the prime source of antibiotic substances and from where majority of the antibiotics used in the clinical and veterinary settings have been isolated. It has been estimated that microorganisms can produce around 16,500 antibiotic

molecules. Among 16,500 molecules, 52.73% were produced by *Actinobacteria*, followed by 29.7% by fungi. Remaining 17.58% antibiotic molecules were also produced by other bacteria (Berdy 2005; Fajardo and Martinez 2008). The majority of antibiotic substances are synthesized from fungi and *Actinobacteria* of soil origin. *Streptomyces* is the most popular species of *Actinobacteria* for synthesizing natural antibiotic substances (Berdy 2005).

Neshme *et al.* found that antibiotic resistance genes are ubiquitous in the environment with a significant similarity with resistance genes detected in the clinical settings. In this study, approximately 30% of the detected resistance genes came from soil samples, indicating the significance of soil as a potential source of antibiotic resistance (Nesme *et al.,* 2014). These findings suggest that soil microorganisms intrinsically harbor antibiotic resistance determinants. In a separate study, authors demonstrated that gut microbiota and soil share a significant number of resistance genes with the potential of transferring resistance to antibiotic susceptible bacteria (Forsberg *et al.,* 2012b). There might be more shared resistance genes, but limited knowledge of known antibiotic resistance genes and lack of procedures for culturing bacteria in traditional lab settings are narrowing our knowledge in this regard. These observations indicate a possible role of soil microorganisms in the dissemination of antibiotic resistance into pathogens.

Analysis of an ancient Alaskan soil revealed the presence of DNA sequences homologues to antibiotic resistance genes found in clinical settings. This study detected several known *tet*M and beta-lactamases sequences in the studied sample. Interestingly, a vancomycin conferring operon, *van* HAX was also detected which was commonly found in the clinical settings (D'Costa *et al.,* 2011). Antibiotic-resistant bacteria were also identified from a cave soil sample. Some of the isolated bacteria were resistant to 14 antibiotics. The cave was segregated from the external

world for millions of years. Among the identified antibiotic resistance genes some of them were known, but majority of them were unknown (Bhullar *et al.,* 2012).

Investigation of antibiotic resistance phenotypes by culturing methods

Soil bacteria can be isolated and characterized by the culturing method. A comprehensive study on culturable soil bacteria was conducted by D'Costa *et al.* In this study, spore forming *Streptomyces* were isolated from various soil samples. A total of 480 *Streptomyces* strains were isolated from collected samples. Recovered strains were tested for 21 antibiotics to determine their antibiotic resistance profiles. These tested antibiotics consisted of all known classes of antibiotics, from natural to semi-synthetic to synthetic. Surprisingly, all isolated strains were multi-drug resistant, and resistance was observed to all 21 antibiotics tested (D'Costa *et al.,* 2006). Similar findings were also observed by another study. A total of 412 bacterial strains were isolated from ten soil samples collected from different sites. All known classes of antibiotics were tested on the isolated bacteria. It was observed that more than 80% of bacteria were resistant to 16 to 23 antibiotics (Walsh 2013). Antimicrobial susceptibility of culturable soil bacteria was also tested by Popowska et al. In this study, bacteria isolated from soil and compost were tested for three antibiotics by disk diffusion test, followed by their minimum inhibitory concentration (MIC). The MIC data showed coherence with the disk diffusion test. High MIC values of tested antibiotics were observed in *Chryseobacterium jejuense*, *Brevendimonas vesicularies*, and *Aeromonas salmonicida*. PCR was used to detect antibiotic resistance genes in the resistant isolates. Antibiotic resistance genes conferring resistance to tetracycline (*tet*A, *tet*B, *tet*D, *tet*O, and *tet*T), streptomycin (*aac*, *aad*A, *str*A, and *str*B), and erythromycin (*erm*C, *erm*V, *erm*X, *msr*A, *ole*B, and *vga*) were detected in the resistant isolates (Popowska *et al.,* 2012).

Culture-based methods to assess antibiotic resistance phenotypes

Antibiotic resistance of culturable environmental bacteria can be determined by multiple culture-based methods, including broth and agar dilution assays, disk diffusion, and E-tests.

Broth and agar dilution assays

Broth dilution assays are used to determine the minimum inhibitory concentration (MIC) of the targeted bacteria (Wiegand *et al.,* 2008). The MIC is the minimum concentration of an antibiotic at which no visible growth of the bacteria is observed. To determine the MIC, the targeted bacterium is incubated for a certain period of time at a specific temperature into the broth containing tested antibiotics at an increasing 2-fold concentration. After incubation the MIC is determined by measuring the optical density. Depending on the tested antibiotics the range of the tested antibiotic concentration varies. The range of the antibiotic concentration should cover to define the tested bacterium as resistant compared to the reference organism. The principle of the commercially available Sensitire (Trek Diagnostic Systems, Thermo Fisher Scientific) is also based on broth dilution. The Clinical and Laboratory Standards Institute (CLSI) guidelines are commonly used to interpret the broth dilution assays data.

Theoretically the concept of agar dilution is similar to the concept of broth dilution. In this method, different concentration of the targeted antibiotic is added to the agar plates, followed by spreading of the target bacterium onto agar plates. After incubation, the MIC is determined by visual inspection of the bacterial growth.

Disk diffusion method

In disk diffusion tests, commercially available paper disks containing specific concentration of antibiotics are used to determine the zone of inhibition. The CLSI guidelines have the interpretive zone of inhibition for reference bacteria from sensitive to resistant. The disk diffusion assays are widely used to determine antibiotic resistance of the environmental isolates

(Kumar *et al.,* 2013; Zhang *et al.,* 2014). In this method, targeted bacterium of specific concentration is spread onto agar plates. Then paper disk with specific concentration of antibiotic is placed on agar plates and incubate at respective temperature and time. After incubation, the zone of inhibition is measured in millimeter and compared to CLSI guidelines for interpretation.

E-tests

The E-test assays combine the concepts of both broth dilution and disk diffusion methods. In this assay, a commercially prepared plastic strip containing a gradient of an antibiotic is used. The impregnated strip is then placed on the tested bacterium containing agar plates. After incubation the MIC is determined by identifying the intersection between zone of inhibition and precalibrated strip.

Investigation of antibiotic resistance by culture-independent methods

Considering that less than 1% of environmental microorganisms can be cultured under lab conditions (Demaneche *et al.,* 2008; Schloss and Handelsman 2003), culturing methods may miss a large amount of information associated with non-culturable microorganisms in the environment. Applying both culture-dependent and independent tools together would be an ideal strategy to characterize soil antibiotic reistome and to understand the extent of the environmental reservoir of antibiotic resistance.

Popowska *et al*. used a culture-independent qPCR tool to study the relative abundance of antibiotic resistance genes in soil DNA. *tet*M and *tet*W genes were detected in all soil samples with almost equal abundance, although these genes were not detected by PCR in bacterial isolates from the same soil samples (Popowska *et al.,* 2012). The qPCR tool was also used to study archived soil and broad-spectrum beta-lactam resistance genes (bla_{TEM}, bla_{SHV}, bla_{OXA}, and $bla_{CTX\text{-}M}$) were detected,along with class-1 integron genes (Graham *et al.,* 2016). qPCR was also used to determine

the absolute abundance of targeted genes. A total of 12 antibiotic resistance genes (*sul*I, *sul*II, *tet*A, *teA*P, *tet*C, *tet*G, *tet*L, *tet*BP, *tet*M, *tet*O, *tet*W, and *tet*X) and 1 class-1 integron gene (*intI*1) were detected and quantified by qPCR in soil and vegetable samples (Wang *et al.*, 2014).

Metagenomics is another powerful tool to study unculturable microorganisms of environmental origin. A metagenomic soil library was prepared from four agricultural soil samples and further screened for antibiotic resistance genes. A total of 45 clones were detected in the constructed soil library conferring resistance to tetracycline, streptomycin, minocycline, kanamycin, gentamycin, chloramphenicol, amikacin, and rifampicin (Su *et al.*, 2014). Novel antibiotic resistance genes can also be discovered by metagenomics. Kelly *at el*. screened a soil metagenomic DNA library and detected 41 novel genes encoding novel protein variants, including aminoglycoside acetyltransferases, dihydrofolate dihydrofolate reductases, and rifampicin ADP-ribosyltransferases (McGarvey *et al.*, 2012). In another study, 18 soil samples were studied by functional metagenomics and screened for antibiotic resistance profiling against 18 antibiotics. This study discovered 2,895 antibiotic resistance genes, covering almost all major resistance mechanisms, and the majority of them were new(Forsberg *et al.*, 2014).

Antibiotic selective pressure in agriculture

Uncontrolled and unwise use of antibiotics is a major driving force for development and dissemination of antibiotic resistance. Widely antibiotics are used for treating diseases in animals, humans, and crops. Used antibiotics can be incorporated into soil by direct application of manures, by using wastewater or effluents for irrigation.

It is estimated that the consumption of antibiotics in agriculture could range from 63K to 230K tons worldwide. The number is projected to increase by 67% during the time period of 2010 to 2030 (Van Boeckel *et al.*, 2015). In the United States livestock uses more than 70% of the

clinical antibiotics used for human treatments. The prime purpose of antibiotic usage in livestock is growth promotion. Antibiotic usage also promotes the development of antibiotic resistance. It was reported that use of sub-therapeutic concentration of antibiotics in agriculture triggered more antibiotic-resistant bacteria and antibiotic resistance genes (Zhu *et al.,* 2013a).

A significant number of studies detected antibiotics from a variety of environmental samples worldwide, including macrolides (erythromycin, tylosin, tilmicosin), sulfonamides (sulfamethoxazole, sulfadiazine, sulfamethazine), fluroquinolones (ciprofloxacin, norfloxacin, enrofloxacin), lincosamides (lincomycin), tetracyclines (tetracycline, oxytetracycline, doxycline), thiamphenicol (chloramphenicol), trimethoprim etc. (Zhang *et al.,* 2013; Pruden *et al.,* 2012; Bartelt-Hunt *et al.,* 2011).

The use of antibiotics in animal production can directly introduce antibiotics, antibiotic-resistant bacteria, and antibiotics resistance genes in the environment. Higher abundance of antibiotic-resistant bacteria and antibiotic resistance genes were observed in conventional animal production compared to organic production (Peak *et al.,* 2007; Jindal *et al.,* 2006). Increase of antibiotic-resistant bacteria and antibiotic resistance genes also observed in manure amended soil (Zhou *et al.,* 2010). In a separate study higher concentration of sulfonamide and tetracycline resistance genes were detected in a river located near to a dairy farm (Pruden *et al.,* 2006a).

Compared to livestock use, the amount of antibiotics used in crops is much less. It is estimated that 0.2-4% of the total antibiotics consumed by agriculture is used for crops (Azevedo *et al.,* 2015). Streptomycin and oxytetracycline are the two major antibiotics used on crops in the United States (McManus *et al.,* 2002). Although they suggest that the use of streptomycin antibiotics on crops does not increase streptomycin-resistant bacteria and/ or streptomycin resistance genes on plants or adjacent soil (McManus *et al.,* 2002)., a previous study recovered

oxytetracycline-and streptomycin-resistant *Erwinia amylovora*, a plant pathogen (Stockwell and Duffy 2012), indicating a concern over antibiotic resistance in plants.

Soil contamination associated with urban agriculture

Soil contamination is an important issue in urban agriculture. Theoretically, garden soil should be contamination free, but in reality, soil has some contaminants at a natural level. These natural levels of soil contaminants in urban agriculture can be elevated by introducing contaminants from different sources. Past land use history can provide some clues of potential sources of contamination in the soil. For example, if a site was previously used for gas station or garage, fuels, lubricants, and other chemicals may have entered into soil due to poor storage practices. Metals and polycyclic aromatic hydrocarbons (PAHs) from former parking lots can also contaminate soil. Among many contaminants, lead is considered as most common contaminant in urban soil. Commercial buildings can leave lead containing paints after demolishing which eventually introduce lead to the soil. Moreover, vehicle exhaust can emit lead and PAHs into the environment. The lands near high-traffic roadways are more prone to lead and PAHs contamination. Rain runoff of roofs and other structures could also add contaminants into soil. According to Environmental Protection Agency (EPA), in the United States, 23% of the private houses built before 1980 have lead in soil at hazardous concentration (Kessler 2013).

The reduced and controlled use of antibiotics did not eliminate antibiotic resistance both in clinical and natural environments (Salyers and AmabileCuevas 1997), which indicates the need to identify alternative factors selecting antibiotic resistance.

Anthropogenic pollutants can promote the spread and accumulation of antibiotic resistance. Metal contamination is commonly found in the environment and anthropogenic-derived sources are the major causes of this contamination. Metal contamination could play an important

role in the spread and maintenance of antibiotic resistance (Summers 2002; Alonso *et al.,* 2001). Many industrial and agricultural practices could introduce metals in the environment. Production of steel, batteries, TV tubes etc. can add zinc, cadmium, cobalt, nickel, chromium, and copper into the environment. Use of bactericides, fungicides and insecticides can contaminant agricultural soil with copper and lead (Diels and Mergeay 1990; Dressler *et al.,* 1991; Mergeay *et al.,* 1985; Schmidt and Schlegel 1994).

Copper (Cu), zinc (Zn), mercury (Hg), lead (Pb), and cadmium (Cd) are major metal contaminants in soils (Han *et al.,* 2002; Nemecek *et al.,* 2011). Use of metal containing fertilizer, liquid manure, and sewage sludge are commonly practiced in agriculture. These applications can introduce those metals in the soil. Use of copper (Cu) containing pesticides can also pollute the soil. These pesticides are frequently used in agriculture due to their fungicidal and bactericidal properties (Nemecek *et al.,* 2011).

Co-selection of antibiotic resistance by metals

A number of studies documented that metal resistance coexisted with antibiotic resistance (Belliveau *et al.,* 1991; Mcentee *et al.,* 1986). Metals can co-select antibiotic resistance by two important mechanisms, co-resistance and cross-resistance (Ashbolt *et al.,* 2013; Berg *et al.,* 2010; Perry and Wright 2013).

Co-resistance occurs when antibiotic resistance genes and metal resistance genes are physically linked to each other and located on the same genetic elements, for example, plasmid, integron or transposon. This physical linkage between antibiotic resistance genes and metal resistance genes were widely observed in plasmids which promotes co-selection of antibiotic resistance genes (Chapman 2003). A study conducted by Summers et al observed the transfer of antibiotic resistance along with mercury resistance, where mercury resistance gene and antibiotic

resistance gene were linked genetically and were located on the same plasmid (Summers *et al.*, 1993). In a separate study, correlation was observed between copper resistance and macrolide resistance in *Enterococcus faecium*. Transconjugant *E. faecium* showed resistance to both copper and macrolides which indicates the successful co-transfer of copper and macrolide resistance determinants (Hasman and Aarestrup 2002). Later on, the physical linkage between copper resistance conferred by *tcr*B gene and macrolide resistance conferred by *van*A gene, was established (Hasman and Aarestrup 2005).

Integrons, which are the part of transposons, can contain resistance determinants in their gene cassettes. Integrons can acquire or transfer these resistance determinants containing gene cassettes. The class 1 integrons are clinically significant and widely found in the contaminated areas. The gene cassettes of class 1 integrons can mediate antibiotic resistance, thus class 1 integrons can play a vital role in the co-selection of resistance determinants. Environmental pollution with metals can trigger this co-selection. High abundance of class 1 integrons were observed in heavy metal contaminated environments (Rosewarne *et al.*, 2010; Wright *et al.*, 2008).

Cross-resistance is another important mechanism of co-selection of antibiotic resistance. In this mechanism two different antimicrobial agents share the same mechanism to kill the targeted cell. As a result, the cell develop resistance to one antimicrobial agent to avoid the cell damage. This development of resistance to one antimicrobial agent accompanied by development of resistance to second antimicrobial agents too, which shared the same route to destroy the cell (Chapman 2003). In one study, more antibiotic resistance was observed in *E. coli* and *Enterobacter cloacae* isolates when they were grown in the presence of metal vanadate vs in the absence of vanadate, which suggests a cross-resistance facilitated by multi-drug resistance (MDR) efflux system (Hernandez *et al.*, 1998).

Many studies investigated the co-selection of antibiotic resistance by metals in the environments. Zinc and copper concentration in the soil showed a positive correlation with beta-lactam resistance (Holzel *et al.,* 2012; Hu *et al.,* 2016). Along with beta-lactam, zinc and copper also showed a strong positive correlation with tetracycline resistance (Peltier *et al.,* 2010; Knapp *et al.,* 2010), erythromycin resistance (Knapp *et al.,* 2010), and sulphonamide resistance (Ji *et al.,* 2012). A study on 90 soil samples collected from Western Australia demonstrated a significant correlation between metal concentration and absolute abundance of antibiotic resistance genes. Elevated concentration of manganese and vanadium showed greatest significant correlation with bla_{TEM}, bla_{CTX}, bla_{OXA}, *tet*M, *tet*W, *sul*1 nad *sul*2 genes. Significant correlation was also observed between copper with *bla*TEM, *bla*OXA and *tet*M, and aluminium with *bla*TEM, *bla*OXA, *tet*M, *tet*W, *sul*2 and *sul*3 (Knapp *et al.,* 2017). Co-selection was also observed in the bacteria isolated from copper-contaminated agricultural fields. These copper resistant isolates showed higher resistance to metals including, calcium, zinc, cadmium, cobalt and to antibiotics including, streptomycin, ampicillin, spiramycin, and olaquindox (Huysman *et al.,* 1994).

CHAPTER 1: PREVALENCE OF ANTIBIOTIC RESISTANCE PHENOTYPES IN URBAN AGRICULTURAL SOILS

1. Introduction

Increasing evidence has shown that the evolution and spread of antibiotic resistance in the environment contribute to the occurrence of antibiotic resistance in clinical or urban settings (Canton 2009; Martinez 2009a; Wright 2010; Berendonk *et al.*, 2015). Many antibiotic resistance genes found in pathogenic bacteria have evolved or are acquired from environmental microbial communities (Martinez 2009b). This suggests an urgent need to understand the public health significance of the environmental antibiotic resistome. However, the extent of the environmental reservoir of antibiotic resistance is yet to be well investigated as compared to that in clinical settings. The problem is even more complicated in agriculture where different environmental conditions and agricultural practices may exert unique impact on the prevalence and persistence of antibiotic resistance.

Soil microorganisms are a significant pool of antibiotic resistance since many species are capable of producing antibiotic substances and are naturally antibiotic resistant (D'Costa *et al.*, 2006; Martinez 2008). High prevalence of antibiotic resistance phenotypes have been observed in common antibiotic categories, including those of human clinical importance, such as aminoglycocide, β-lactam, glycopeptides, macrolides, quinolones, streptogramins, tetracyclines, and trimethoprim/sulfamethoxazole. A study analyzed a morphologically diverse collection of *Streptomyces* from soil samples originating from urban, agricultural, and forest sites, and found that all strains recovered were multidrug resistant to 7 or 8 antibiotics on average, with two strains being resistant to 15 of 21 antibiotics tested (D'Costa *et al.*, 2006). Antibiotic-resistant bacteria of diverse genera recovered from agricultural, urban, and pristine soils were found to be multidrug resistant, including more than 80% of the isolates resistant to 16-23 antibiotics (Walsh and Duffy

2013). Despite the variation in resistance levels across soil types, the possibility of anthropogenic effects was ruled out, suggesting a natural habitat of antibiotic resistance in the environment. A Polish study examined soils from agricultural systems, including arable farmland, vegetable garden, fruit orchard, composted, and forest soils, and concluded that both manure- and non-manure- amended soils were contaminated with bacteria resistant to tetracycline, streptomycin, and erythromycin (Popowska *et al.*, 2012).

The aim of this Chapter was to investigate antibiotic resistome in urban agricultural soil. A total of 41 samples were extensively studied in terms of soil bacteria composition by the culturing method and high-throughput 16S rRNA sequencing as well as antibiotic susceptibility profiling by Sensititre.

2. Materials and Methods

2.1 Sample collection

A total of 41 soil samples were collected at a depth of 0-7.5 cm from an urban garden located in Detroit, Michigan, USA, in the summer of 2015. The garden used plant compost and received no manure amendment, which was a common practice in many urban gardens in the metro Detroit area. Each sample of 1 kg was pooled by five subsamples collected approximately 10 cm from each other at the center and four corners around the same sampling point. Samples were put into a zip-lock bag and transported to the lab in a cooler before bacteria isolation. The remainder of samples was stored at -80 °C for further analysis.

2.2 Isolation and identification of antibiotic-resistant soil bacteria

Fifty grams of the sample was mixed with 450 mL of brain heart infusion (BHI) (Difco, Sparks, MD) in a sterile stomacher bag. The mixture was homogenized by manual agitation. Soil bacteria were isolated using an R2A agar (Difco), supplemented with ampicillin, streptomycin, and

tetracycline, individually, at 20 µg/mL (D'Costa *et al.*, 2006). Agar plates were incubated at 30°C for 4 to 5 days and up to 5 colonies of different morphology were picked for purification.

Bacteria were identified by 16S rRNA gene sequencing. DNA was extracted using a boiling method (Hanssen *et al.*, 2004). Primers 27F (AGAGTTTGATCCTGGCTCAG) and 1492R (GGTTACCTTGTTACGACTT) were used to amplify the 16S rRNA gene (Popowska *et al.*, 2012). PCR reactions of 25 µl included 5 µl of template DNA, 0.5 µM of each primer, 1× PCR buffer, 4.0 mM of $MgCl_2$, 200 µM of each dNTP, and 1U *Taq*DNA polymerase (Promega, Madison, WI). PCR was conducted in a Mastercycler (Eppendorf, Westbury, NY) under the following conditions: 15 min at 95°C, followed by 35 cycles of 1 min at 95°C, 45 s at 50°C, and 1.5 min at 72°C, with one final cycle of 10 min at 72°C. DNA amplicons were electrophoresed on a 1.5 % agarose gel for 1.5 h at 100V and visualized under UV, followed by DNA sequencing at Eton Bioscience Laboratories, NJ. Resulted DNA sequences were analyzed using the Ribosomal Database Project (RDP) website (http://rdp.cme.msu.edu).

2.3 Antibiotic Susceptibility Testing

Antibiotic resistance phenotypes were determined by using the Sensititre Antimicrobial Susceptibility System (Trek Diagnostic Systems, Westlake, OH). Minimum Inhibitory Concentration (MIC) was measured using the Gram-positive and Gram-negative MIC plates and interpreted according to the resistance breakpoints for *Escherichia coli* (*E. coli)* and *Staphylococcus aureus* (*S. aureus)* recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines. The MICs were determined for 14 antibiotics (ampicillin, amoxicillin/clavulanic acid 2:1 ratio, azithromycin, cefoxitin, ceftiofur, ceftriaxone, chloramphenicol, ciprofloxacin, gentamicin, nalidixic acid, streptomycin, sulfisoxazole, tetracycline and trimethoprim/sulfamethoxazole) for Gram-negative bacteria and 16 antibiotics

(chloramphenicol, ciprofloxacin, daptomycin, erythromycin, gentamicin, kanamycin, lincomycin, linezolid, nitrofurantoin, penicillin, quinupristin/dalfopristin, streptomycin, tetracycline, tigecycline, tylosin tartrate and vancomycin) for Gram-positive bacteria. *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were used as reference strains.

2.4 Determination of soil microbial composition by high-throughput 16S rRNA sequencing

Fourteen soil samples were randomly selected for the determination of microbial composition. Ten grams of each soil sample was weighed into one 50 mL tube followed by shaking for homogenization. The DNA was extracted from 0.25 g of each homogenized soil sample using the DNeasy PowerSoil kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The quality and quantity of extracted DNA were assessed using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and the Qubit 3.0 fluorometer (Thermo Fisher Scientific). A 16S rDNA sequencing library was constructed using 16S Metagenomic Sequencing Library Preparation protocol (Illumina, San Diego, CA) modified by Dr. Karen Jarvis from FDA. Briefly, Omni Klentaq PCR Kit (DNA Polymerase Technology, St. Louis, MO) was used for initial PCR using locus-specific primers to amplify the V1-V3 hyper-variable region of the bacterial 16S rRNA gene (Chakravorty *et al.*, 2007; Klindworth *et al.*, 2013; Lusk *et al.*, 2012; Yarza *et al.*, 2014). The Illumina sequencing adapters and dual-index barcodes were added to the purified PCR products using Nextera XT Index Kit (Illumina) by a limited cycle PCR. Prepared libraries were sequenced on the MiSeq sequencing platform (Illumina) using paired 300-bp reads and MiSeq v3 reagents, following standard Illumina sequencing protocols. Data were analyzed using Metagenomics workflow to perform a taxonomic classification against the Greengenes database.

3. Results

3.1 Isolation and identification of soil bacteria

A total of 207 soil bacteria were recovered, including 190 Gram-negative and 17 Gram-positive. The bacteria belonged to four phyla, *Bacteroidetes* (53.10%), *Proteobacteria* (38.65%), *Firmicutes* (6.76%), and *Actinobacteria* (1.45%) (Figure 1a). Out of 27 genera identified, *Chryseobacterium* (n=70, 33.82%), *Stenotrophomonas* (n=38, 18.36%), and *Sphingobacterium* (n=26, 12.56%) were the most prevalent Gram-negative bacteria (Figure 1b). *Lysinibacillus* (n=9, 4.35%) predominated in Gram-positive bacteria. Approximately 18% of isolates belonged to other genera. Heterogeneity was observed within samples. Samples E01 and E13 had isolates belonging to multiple genera in phylum *Proteobacteria*. Bacteria from different phyla, for example, *Proteobacteria* and *Bacteroidetes*, were present in the same soil sample, including E19, E20, E35, E44, E59 and E61.

(a) (b)

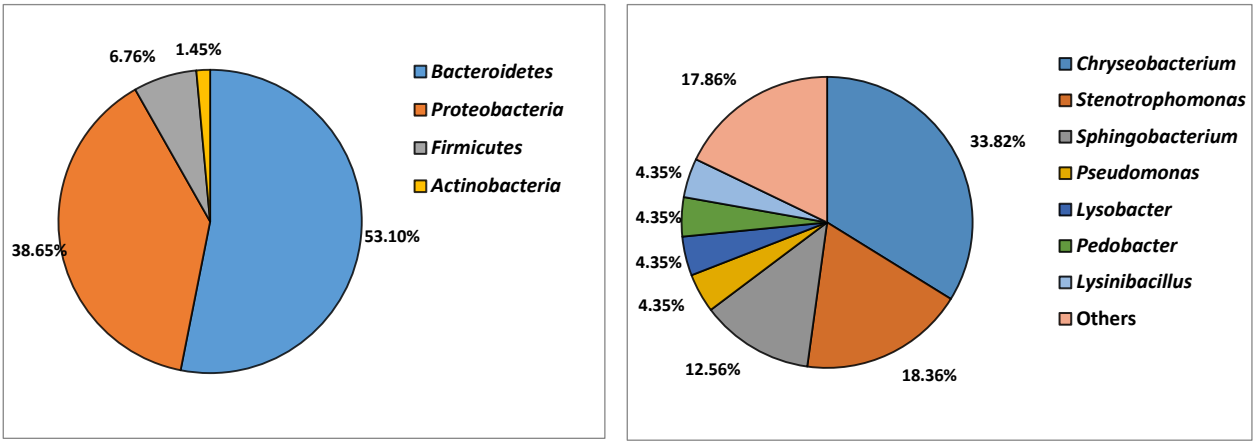


Figure 1. Microbial Composition Determined by Bacteria Identification

Legend:

(a) Phylum distribution in soil bacteria (n=207)
(b) Genus distribution in soil bacteria (n=207)

Numbers shown in the pie chart were percentages of each phylum or genus in total identified bacteria.

3.2 Antibiotic resistance phenotypes of soil bacteria

The MIC data were interpreted for nine antibiotics each for Gram-negative and Gram-positive bacteria based on the resistance breakpoints of *E. coli* and *S. aureus*. The 190 Gram-negative bacteria showed highest resistance to ampicillin (94.2%), followed by chloramphenicol (80.0%), cefoxitin (79.5%), gentamicin (78.4%), ceftriaxone (71.1%), amoxicillin/clavulanic acid (64.2%), tetracycline (51.6%), nalidixic acid (37.4%, and ciprofloxacin (33.2%). All 17 Gram-positive bacteria were resistant to gentamicin, kanamycin, and penicillin (100%). They were also resistant to erythromycin (41.2%), ciprofloxacin (29.4%), quinopristine/dalphopristine (29.4%), tetracycline (23.5%), chloramphenicol (17.6%) and vancomycin (11.8%). For those antibiotics that interpretation breakpoints were unavailable for *E. coli* or *S. aureus*, most Gram-negative bacteria fell under the highest range of MIC to azithromycin, ceftiofur, sulfisoxazole, and streptomycin, so did Gram-positive bacteria to streptomycin and lincomycin.

Top six Gram-negative genera comprised of 161 of 190 isolates (84.7%). A vast majority of these isolates fell under the highest MIC for ampicillin, amoxicillin/clavulanic acid, cefoxitin, ceftriaxone, and gentamicin (Figure 2). The MIC distribution varied by genus and antibiotic category. *Chryseobacterium* was the most common Gram-negative genus identified and also accounted for the highest percentage of antibiotic-resistant bacteria in most antibiotic categories, such as ampicillin (44.3%, 70/158), amoxicillin/clavulanic acid (54.9%, 62/113), cefoxitin (42.8%, 59/138), ceftriaxone (57.9%, 70/121), chloramphenicol (48.9%, 64/131), gentamicin (46.7%, 50/107), and tetracycline (54.7%, 70/128). However, *Chryseobacterium* was outnumbered by other genera in quinolone-resistant bacteria as evidenced by 4 *Chryseobacterium* (7.4%) versus 35 *Stenotrophomonas* (64.8%) out of 54 ciprofloxacin-resistant bacteria, and 3 *Chryseobacterium* (5.4%) versus 21 *Sphingobacterium* (37.5%) out of 56 nalidixic acid-resistant bacteria (Figure 2). *Chryseobacterium* was predominant in nalidixic acid- and ciprofloxacin-susceptible bacteria, with 63.8% (67/105) and 61.7% (66/107), respectively. As the third most popular genus identified, *Sphingobacterium* accounted for only 3/113 (2.6%), 2/121 (1.6%), and 5/54 (9.2%) in bacteria resistant to amoxicillin/clavulanic acid, ceftriaxone, and ciprofloxacin, respectively.

For Gram-positive bacteria, the top four genera covered 16 of the total 17 isolates (94.1%) and were all resistant to gentamicin, kanamycin, and penicillin (Figure 3). *Lysinibacillus* was resistant to all nine antibiotics tested, and *Microbacterium* demonstrated resistance to all antibiotics except for vancomycin. *Lysinibacillus* was the most prevalent genus in bacteria resistant to erythromycin (3 of 6), gentamicin (9 of 16), kanamycin (9 of 16), and penicillin (9 of 16). *Lysinibacillus* and *Microbacterium* were the only two genera and equally prevalent in bacteria resistant to chloramphenicol, ciprofloxacin, and quinopristine/dalfopristine. *Microbacterium* outnumbered *Lysinibacillus* by one for tetracycline resistance. The only two vancomycin-resistant bacteria were

Lysinibacillus. All three *Sporosarcina* isolates were susceptible to antibiotics except for gentamicin, kanamycin, and penicillin.

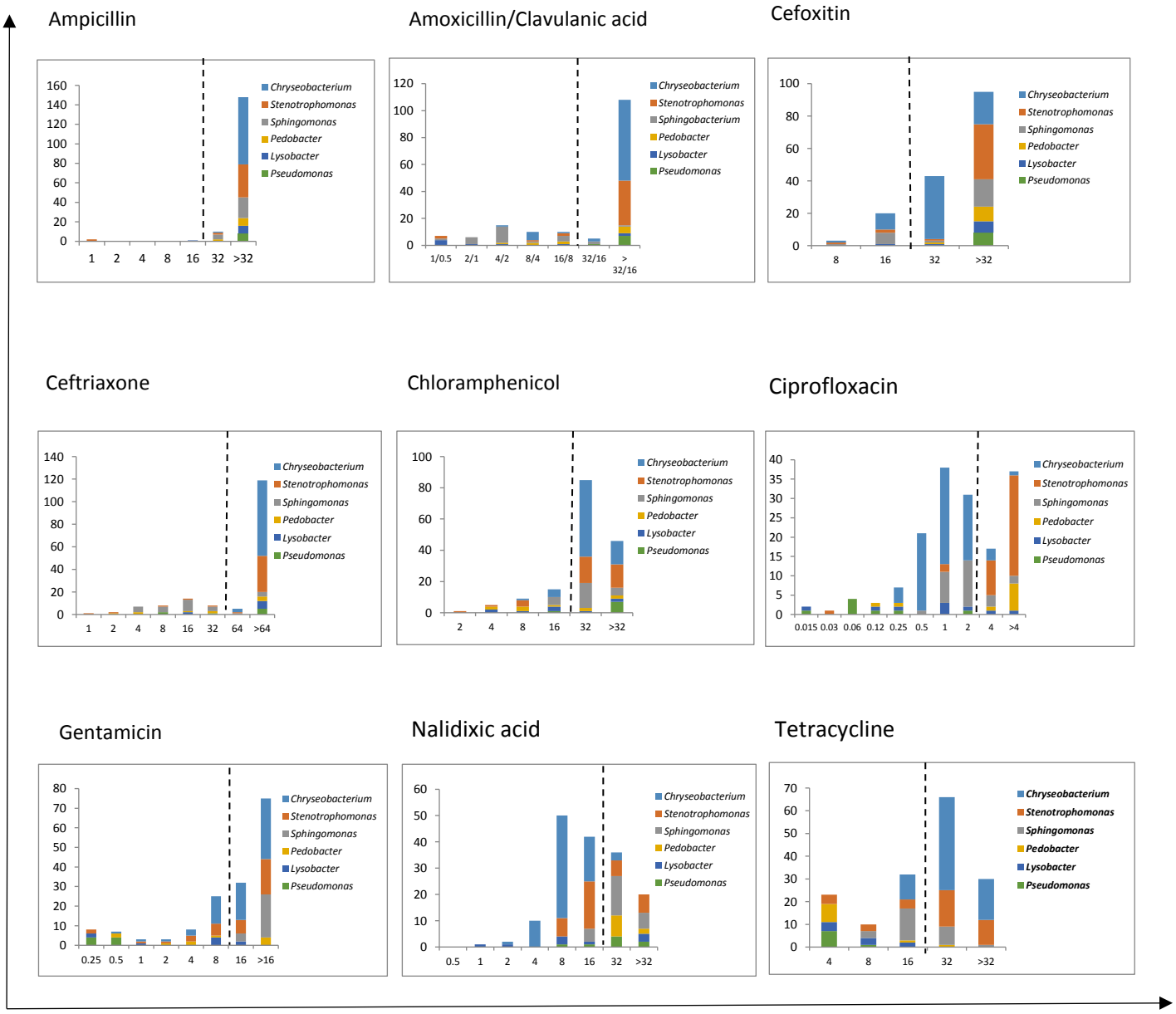


Figure 2. MIC Distribution of Top 6 Genera of Gram-negative Bacteria

Legend:

The dot line on each figure represents the resistance breakpoint for *E. coli*.

X-axis is MIC in μg/ml. Y-axis is number of isolates.

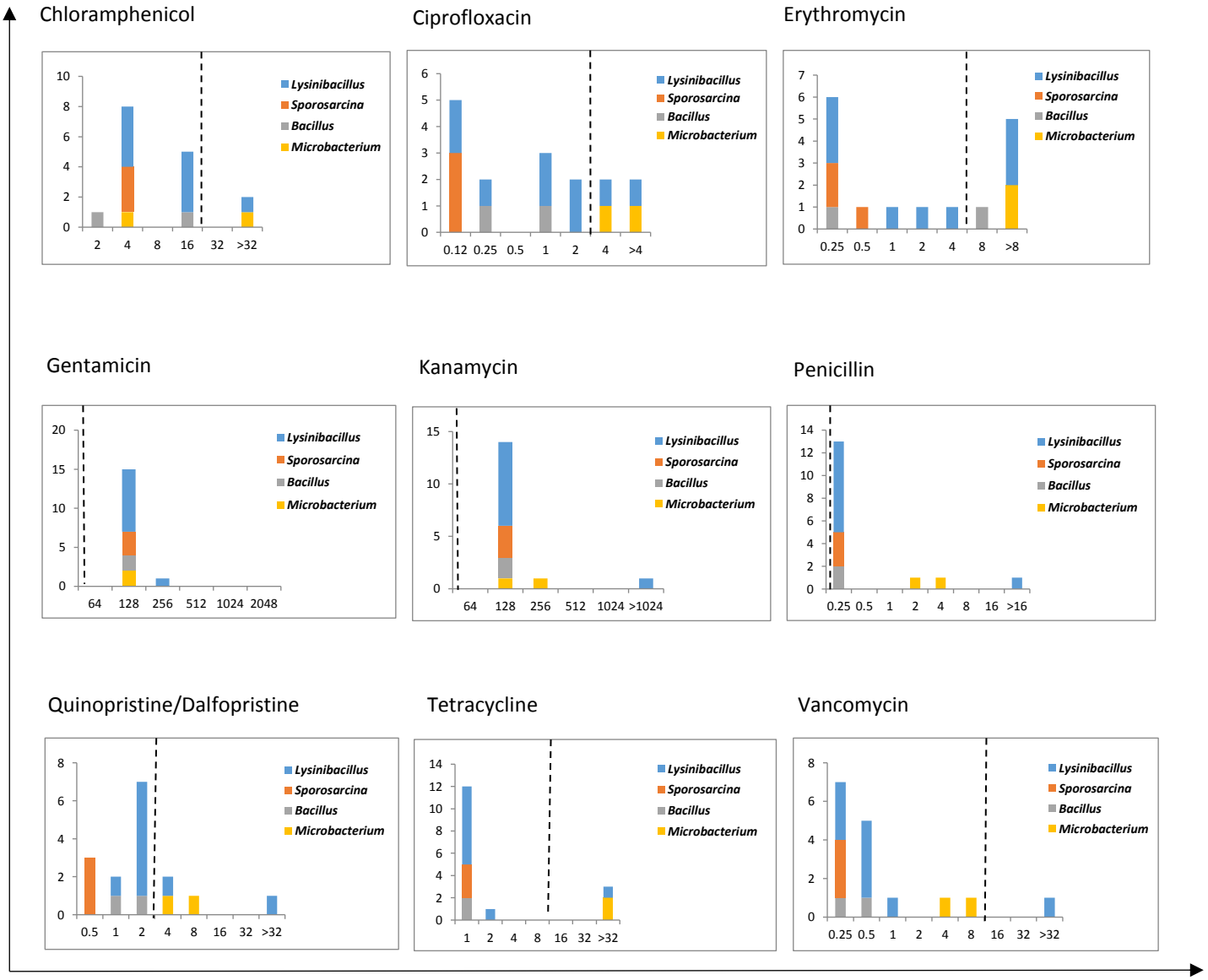


Figure 3. MIC Distribution of Top 4 Genera of Gram-positive Bacteria

Legend:

The dot line on each figure represents the resistance breakpoint for *S. aureus*.

X-axis is MIC in μg/ml. Y-axis is number of isolates.

3.3 Bacteria diversity revealed by high-throughput 16S rRNA sequencing

More than 30 phyla were present in every soil sample as revealed by 16S rRNA Sequencing. The top four phyla were the same as those identified in cultured soil bacteria, but the prevalence ranking was different. *Proteobacteria* were the most prevalent and identified in 37.32% of the reads, followed by *Actinobacteria* (19.45%), *Firmicutes* (12.28%), and *Bacteroidetes* (9.76%) (Figure 4a). *Granulicella* (2.78%), *Rhodoplanes* (2.78%), *Flavobacterium* (1.98%), *Kaistobacter* (1.85%), and *Niastella* (1.43%) were the top five genera detected (Figure 4b). Individual genera identified at very low percentages, when combined together, accounted for more than 65% of total genera. The 16S rRNA Sequencing failed to classify 8.32% of the reads to the phylum level and more than 24% to the genus level. Prevalence wise, most genera were detected in all samples, except for *Chryseobacterium* and *Enterobacter*, which were present in eight and four of 14 samples, respectively (Figure 5). Prevalent genera varied from sample to sample. E03 was extremely high in *Chryseobacterium*, *Enterobacter*, *Flavobacterium*, and *Pseudomonas*, with reads ranging from ~12K to 28K. *Granulicella* were prevalent in E05, E63, and E65, as well as *Rhodoplanes* in E05, E38, and E65.

(a) (b)

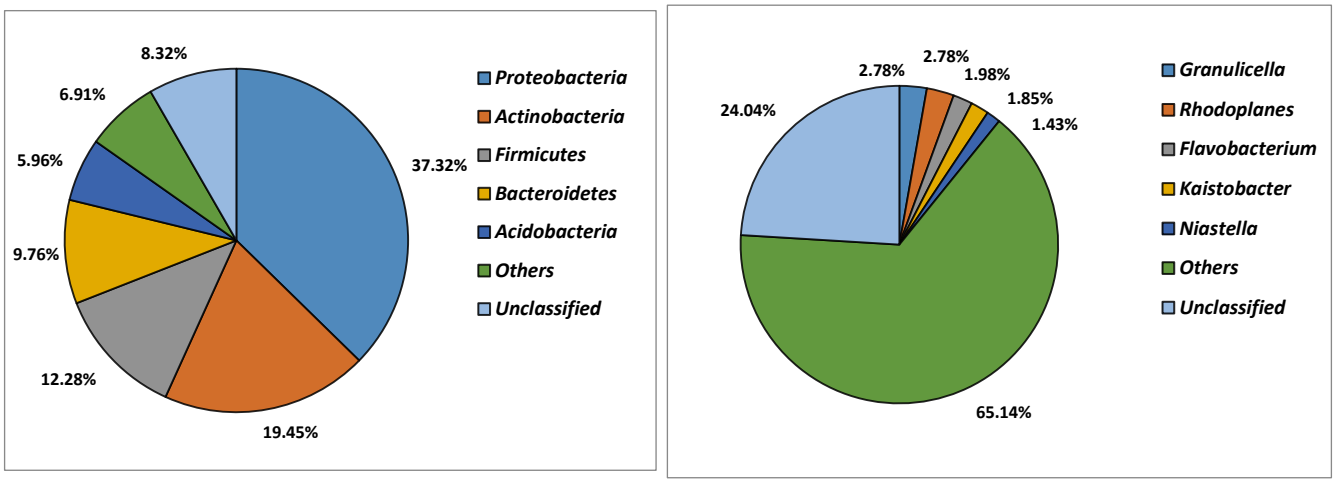


Figure 4. Microbial Composition Determined by 16S rRNA Sequencing

Legend:

(a) Phylum distribution of soil bacteria by 16S rRNA Sequencing
(b) Genus distribution of soil bacteria by 16S rRNA Sequencing

Numbers shown in the pie chart were percentages of each phylum or genus in total identified bacteria/reads.

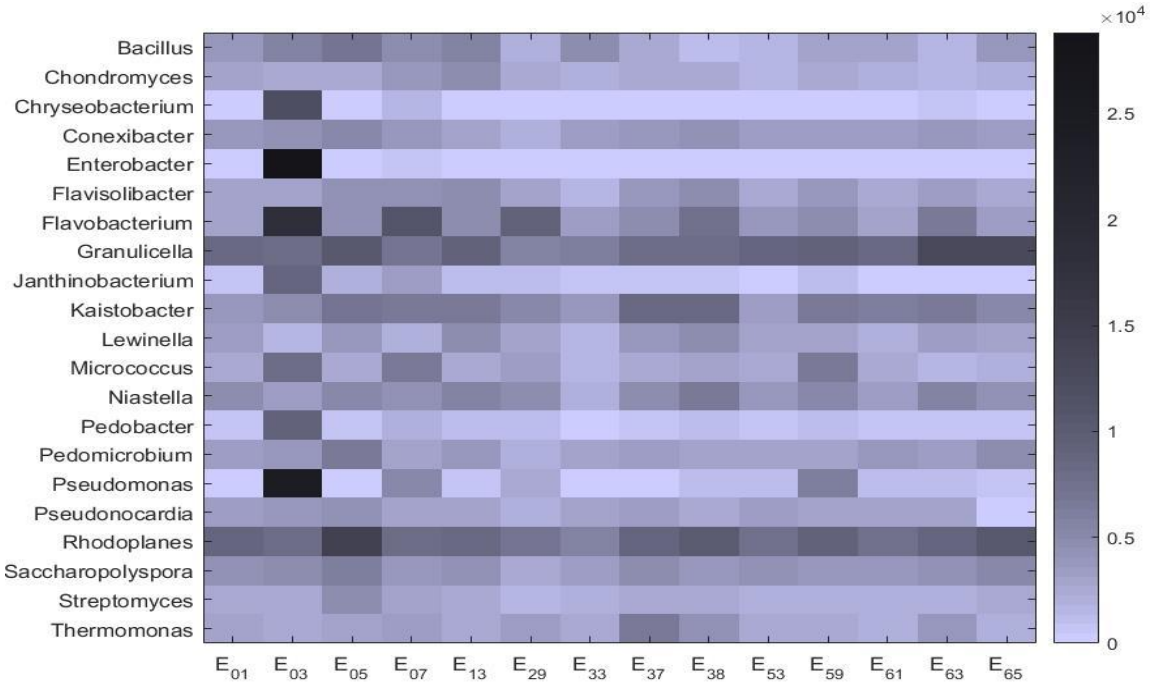


Figure 5. Bacterial Genus Distribution in 14 Soil Samples Revealed by 16S rRNA Sequencing

Legend:

The heat map was constructed by combining the top 8 most prevalent genera in each of all 14 samples. For a particular sample represented by a column, the data included the top 8 genera in that sample as well as the less common genera that were prevalent in other samples. The color gradient in the scale represents the number of the reads for respective genera. X-axis shows 14 soil samples. Y-axis shows bacterial genera identified.

Discussion

Environmental reservoir of antibiotic resistance has been drawing increasing research attention because many clinically important antibiotic-resistant microorganisms and antibiotic resistance genes originated in the environment (Berendonk *et al.*, 2015; Forsberg *et al.*, 2012a). Much research has been conducted on wastewater from agricultural and urban use or manure-amended agricultural land (Berglund *et al.*, 2015; Binh *et al.*, 2008; Pei *et al.*, 2006; Zhu *et al.*, 2013b), where antibiotic-resistant microorganisms are expected to be at high levels. However, limited information is available in US agricultural environment with minimum anthropogenic disturbance.

The data collected in this study using an urban farm model suggest that agricultural soils receiving no wastewater irrigation or manure fertilization are also rich in antibiotic resistance.

The bacterial phyla and genera identified in this study were typical of soil bacteria (Janssen 2006). The discrepancy in microbial composition between the culturing method and 16S rRNA Sequencing was not surprising as 16S rRNA Sequencing target both culturable and non-culturable microorganisms. Also, because antibiotics were added in the media during bacteria isolation, the data revealed by the culturing method were more a reflection of possible soil antibiotic resistome profile than an overall microbial structure in soil as depicted by 16S rRNA Sequencing. This could also partially explain the predominance of Gram-negative bacteria in the cultured bacteria as the outer membrane of Gram-negative bacteria are believed to provide an extra layer of protection from antibiotic inhibition and thus contributing to generally more antibiotic resistance than Gram-positive bacteria (Delcour 2009). Failure to classify 8.32% of bacteria at phylum level and 24.04% at genus level by 16S rRNA sequencing (Figure 4a and 4b) suggests that a large proportion of soil microorganisms still remain uncharacterized. Applying both the culturing and high-throughput 16S rRNA sequencing methods in this research helped achieve a more comprehensive understanding of soil microbial structure.

The predominance of *Bacteroidetes* and *Proteobacteria* identified by the culturing method was different from a Swiss report where the same four phyla were identified as in this study but *Proteobacteria* were the most prevalent and *Bacteroidetes* the least (Walsh and Duffy 2013). This might be due to differences in locations and soil samples studied. The soil bacteria recovered in this literature were from agricultural, urban, and pristine environments with a single sample tested from each site, which may have overlooked the spatial variation of the microorganisms. In fact, this was also the case for many environmental studies that investigated only a limited number of

samples (Chen *et al.*, 2016; Popowska *et al.*, 2012; Wang *et al.*, 2014). The need to consider sample variation and bacterial diversity is supported by the data generated in the current research demonstrating inter-sample variation at genus and even phylum levels as well as heterogeneity of isolates within samples.

The prevalence of antibiotic resistance phenotypes was higher than a previous study where soil bacteria recovered from manure- and non-manure-amended soils were resistant to streptomycin (42.8%), erythromycin (34.7%), and tetracycline (10.2%) (Popowska *et al.*, 2012). As soil samples collected from the current study received no agricultural or human wastes, the high prevalence implies the extent of a natural pool of antibiotic resistance. However, the contribution of soil factors from previous industrial activities to maintaining antibiotic resistance deserves further investigation. It is also worth mentioning that there are no standard breakpoints for environmental microorganisms as for clinical isolates, making it a challenge in comparing data across studies. Some researchers have used a general 20 μg/ml as the resistance breakpoint in soil bacteria and took no consideration of antibiotic variation (D'Costa *et al.*, 2006; Walsh and Duffy 2013). The current study used CLSI guidelines for *E. coli* and *S. aureus*, which addressed the antibiotic variation issue but only allowed the interpretation for those antibiotics that have resistance breakpoints. Since the implication of antibiotic resistance in clinical bacteria may not apply to bacteria of environmental origin, establishing "ecological breakpoints" or even standardized protocols in environmental investigations will be of great value for inter-study comparisons (Thanner *et al.*, 2016). To achieve this goal, more data on environmental bacterial species and isolates are the key.

Genus variation in antibiotic resistance may reflect differences of bacteria in indigenous antibiotic resistance, the tendency to acquire antibiotic resistance, or the environmental selection

on bacteria (Martinez 2009b), although it could also be related to the number variation of individual genus recovered at a given site. It was interesting to note that *Chryseobacterium* demonstrated very high rate of antibiotic resistance to most antibiotics but was mostly susceptible to ciprofloxacin and nalidixic acid. This suggests that antibiotic variation also exists, and it is important to explore the antibiotic resistance determinants for individual antibiotics as well as the potential of resistance dissemination. Identifying soil bacteria indicators towards individual antibiotics or antibiotic categories may be critical in monitoring specific antibiotic resistance in the environment (Berendonk *et al.*, 2015).

In conclusion, the study demonstrated that urban agricultural soil receiving no animal wastes or wastewater irrigation is rich in antibiotic-resistant bacteria. Future investigations are needed on the impact of previous industrial activities on soil antibiotic resistance as part of urban soil ecosystem. Isolation of soil bacteria together with high-throughput 16S rRNA sequencing on soil DNA provided more accurate information on microbial composition in soil than each method alone. The data added substantial information to the environmental database of antibiotic resistance. However, phenotypic determination of antibiotic resistance in bacterial isolates may miss the information associated with non-culturable microorganisms, which account for more than 99% of total soil microbiota. Consequently, it is also important to investigate antibiotic resistance genes in soil DNA using culture-independent methods to get a comprehensive understanding of the extent of soil antibiotic resistome.

CHAPTER 2:

QUANTIFICATION OF ANTIBIOTIC RESISTANCE GENES (ARGS) AND METAL RESISTANCE GENES (MRGS) IN URBAN AGRICULTURAL SOILS

1. Introduction

The vast majority of soil microorganisms are non-culturable, and less than 1% of soil microorganisms can be cultured in current lab settings (Demaneche *et al.,* 2008; Schloss and Handelsman 2003). Due to this limitation, culturing methods may miss a large amount of information on antibiotic resistance genes associated with non-culturable microorganisms in the soil. Culture-independent tools, for example, PCR and metagenomics, are frequently used to capture antibiotic resistance information on non-culturable soil microorganisms.

Soils are the host to diverse and abundant antibiotic resistance genes. Gene profiling has been used to understand environmental antibiotic resistome around the world. Quantitative PCR was conducted on garden soils from residential areas in Western Australia (Knapp *et al.,* 2017) and revealed the absolute abundance of tetracycline, β-lactam and sulphonamide resistance genes ranging from 0.09 to 0.37 genes/g soil. The gene abundance was similar to that commonly found in typical pristine environments as well as impacted sites. Another study identified multidrug and bacitracin resistance genes as the top two dominant gene groups from three US arable soil samples using metagenomics, despite lower gene abundance in soil compared to that in wastewater (Li *et al.,* 2015a). High abundance of antibiotic resistance genes have also been reported from urban park soils with and without reclaimed water irrigation in China (Wang *et al.,* 2014). By using a high-throughput quantitative PCR approach, the research demonstrated aminoglycoside and β-lactam resistance genes as the two most dominant gene types. Metagenomic analysis conducted in both human impacted and minimally impacted environment in China also revealed high diversity of

antibiotic resistance genotypes to aminoglycosides, β-lactams, macrolides, and quinolones (Chen *et al.,* 2016; Chen *et al.,* 2013).

There have been studies reporting the correlation between antibiotic resistance and metal or metal resistance in highly impacted areas, including biosolids and wastewater (Knapp *et al.,* 2012; Su *et al.,* 2015), lands receiving agricultural wastes (Li *et al.,* 2015b; Zhu *et al.,* 2013b; Ji *et al.,* 2012) etc. *bla*$_{TEM}$, *bla*$_{CTX}$, *bla*$_{OXA}$, *tet*M, *tet*W, *sul*1, *sul*2, and *sul*3 showed significant positive correlation with one or multiple metals, including manganese, vanadium, copper, zinc, aluminium, and nickel (Knapp *et al.,* 2017). A separate study of biogas plant residues, which are used as biofertilizers for crops cultivation, showed a strong positive correlation (p= 0.001) between overall antibiotic resistance genes (ARGs) and metal resistance genes (MRGs) (Luo *et al.,* 2017). Despite the previous findings, there is a scarcity of data on US soils, and more specifically on the soils used for urban agriculture. Moreover, negative correlation was also observed in some studies between metals and antibiotic resistance (Holzel *et al*., 2012; Knapp *et al*., 2011), suggesting variations in metals, antibiotic resistance types, and study sites.

The aim of this Chapter was to determine the prevalence of antibiotic resistance genes (ARGs) in urban agricultural soil by metagenomics. As heavy metals are commonly found in urban soils and there is increasing evidence showing the correlation of heavy metals and antibiotic resistance, metal resistance genes were also included in soil metagenomics as an attempt to elucidate the linkage between heavy metals and antibiotic resistance at the molecular level.

2. Materials and Methods

2.1 Quantification of antibiotic resistance genes and metal resistance genes by metagenomics

Metagenomic sequencing targeting common antibiotic and metal resistance genes was conducted on 21 of 41 soil samples collected in Chapter 1. Total metagenomic DNA was extracted using the Mo Bio PowerSoil DNA isolation Kit for Soil (Mo Bio, Carlsbad, CA). Sequencing

library was prepared using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina, sequenced by the Illumina HiSeq platform (Paired-End 125bp, or PE125), and assembled using SPAdes v3.7.1 (Bankevich *et al.,* 2012). Open reading frames (ORFs) of antibiotic and metal resistance genes were identified by BLAST searches using previously identified common genes as query sequences with a cutoff E-value of 10^{-4}. The genes selected were commonly studied in environmental research (Popowska *et al.*, 2012; Yan *et al.,* 2017; Unc *et al.*, 2012). The categories (genes) of antibiotic resistance were aminoglycosides (*aac*, *str*A and *str*B), β-lcatams ($bla_{\text{CMY-2}}$, $bla_{\text{TEM-1}}$, $bla_{SHV\text{-}1}$, $bla_{CTX\text{-M1}}$ and $bla_{OXA\text{-}2}$), macrolides (*emr*B, *erm*C, *erm*X and *erm*E), quinolones (*aac*6-*ib*-*cr*4, *oqx*B, *qep*A, *qnr*A, *qnr*B, *qnr*C, *qnr*D and *qnr*S), tetracyclines (*tet*A, *tet*B, *tet*C, *tet*D, *tet*H, *tet*M, *tet*O, *tet*T and *tet*W), transposons (*Tn*916, *Tn*1549, and *Tn*5397). Metal resistance genes included genes encoding resistance to arsenic (arsA, arsB, arsC, and arsR), cadmium (cad), copper (pcoA, czc), lead (pbr), mercury (mer), and zinc (zntA and zraR).

2.2 Statistical analysis

The correlations between antibiotic resistance genes (ARGs) and metal resistance genes (MRGs) were analyzed by Pearson's bivariate correlation using SPSS v. 21.0 (IBM SPSS, Chicago, IL). Independent t-test was used to determine the difference in gene abundance between antibiotic resistance genes and metal resistance genes. A *p*-value of less than 0.05 was considered statistically significant.

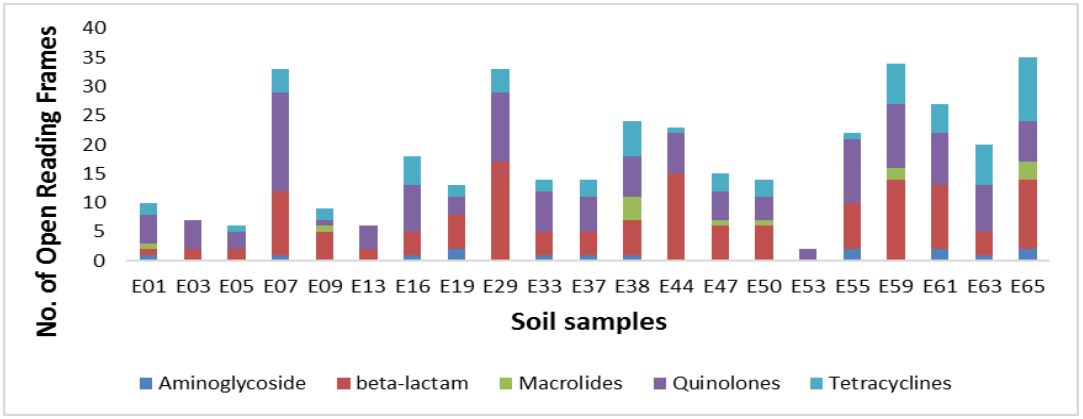
3. Results

3.1 Prevalence of antibiotic resistance genes and metal resistance genes in soil

A total of 21 (100%), 20 (95%), and 18 (85.7%) samples contained resistance genes to quinolones, β-lactams, and tetracyclines, respectively. The number of ORFs of antibiotic resistance genes ranged from 2 in sample E53 to 36 in sample E65 (Figure 6a). Quinolone, β-

lactam, and tetracycline resistance genes were among the highest percentages of ORFs identified, accounting for 37.2%, 36.6%, and 18.1%, respectively (Figure 6b). β-lactam resistance genes were positively correlated with quinolone resistance genes ($p < 0.05$), and so were β-lactam resistance genes with tetracycline resistance genes ($p < 0.05$). *qepA* and *tetA* were the most abundant quinolone and tetracycline resistance genes, respectively, and both encoded efflux pumps. All five β-lactam resistance genes (*bla*$_{\text{CMY-2}}$, *bla*$_{\text{TEM-1}}$, *bla*$_{SHV\text{-}1}$, *bla*$_{CTX\text{-M1}}$ and *bla*$_{OXA\text{-}2}$) were detected and in similar abundance. Less than 1% of the ORFs were identified as transposons. Tn*5397*, Tn*916* and Tn*1549* were found in three individual samples.

(a)



(b)

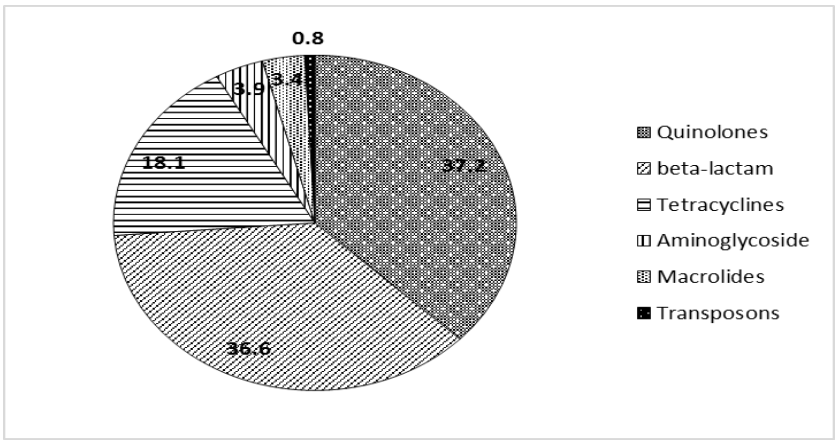


Figure 6. Illumina Sequencing Revealed Diverse Antibiotic Resistance Genes in Urban Agricultural Soil

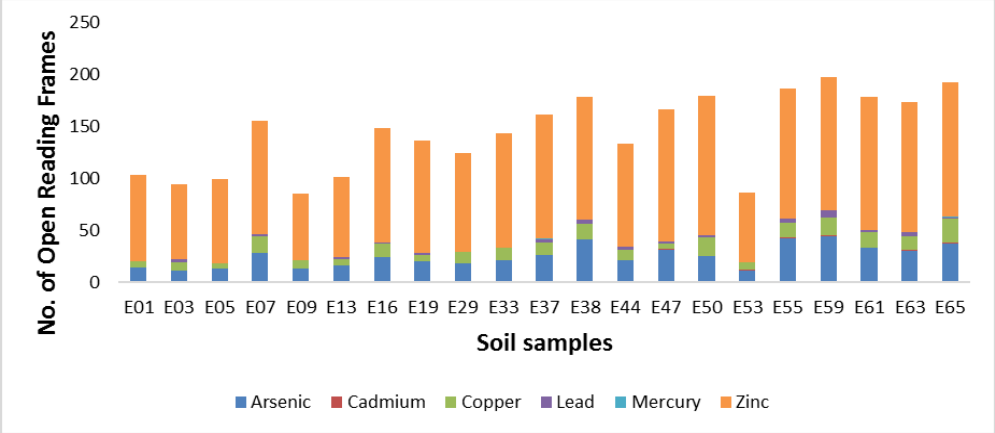
Legend:

(a) Numbers of open reading frames (ORFs) identified in 21 soil samples
(b) Compositions of individual groups of antibiotic resistance genes as well as transposons

Metal resistance genes were significantly more abundant than antibiotic resistance genes ($p < 0.01$, *t*-test) as much more metal resistance gene ORFs were observed ranging from 86 in E09

to 198 in E59 (Figure 7a). The most abundant metal resistance genes were for zinc (72.9%), followed by arsenic (17.4%), copper (8.1%), lead (1.4%), mercury (0.2%), and cadmium (0.07%) (Figure 7b). *zraA* (zinc^r), *zntA* (zinc^r), *arsR* (arsenic^r), and *czc* (copper^r) were among the most abundant ORFs.

(a)



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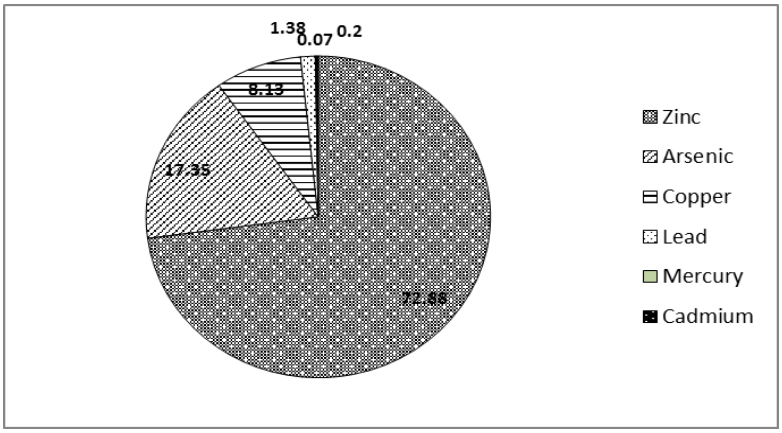


Figure 7. Illumina Sequencing Revealed Diverse Metal Resistance Renes in Urban Agricultural Soil

Legend:
(a) Numbers of open reading frames (ORFs) identified in 21 soil samples
(b) Compositions of individual groups of metal resistance genes

3.2 Correlation between antibiotic resistance genes and metal resistance genes in soil

Total antibiotic resistance genes showed a significant positive correlation with total metal resistance genes *(r* = 0.707, $p < 0.001$) (Figure 8). Positive correlation was also identified between individual metal resistance genes and antibiotic resistance genes ($p < 0.05$), though with lower correlation coefficients (data not shown). Tetracycline, β-lactam, and quinolone resistance genes were all positively correlated with zinc, arsenic, and copper resistance genes. Tetracycline resistance genes were also positively correlated with mercury resistance genes the same way as aminoglycoside resistance genes with zinc and arsenic resistance genes. No correlation was identified between lead or cadmium resistance genes and antibiotic resistance genes.

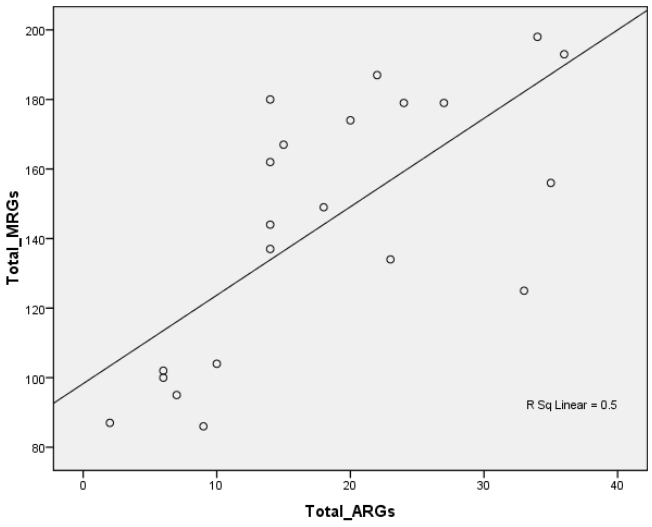


Figure 8. Correlation between Total Antibiotic Resistance Genes and Total Metal Resistance Genes

4. Discussion

The predominance of quinolone, β-lactam, and tetracycline resistance genes in both the prevalence and abundance of ORF reads suggests that urban agricultural soils are a reservoir of common antibiotic resistance genes. These results also correlated well with the high prevalence of corresponding antibiotic resistance phenotypes observed in the cultured microorganisms in

Chapter 1. However, the common aminoglycoside resistance phenotypes were inconsistent with the overall gene prevalence as identified by metagenomics. It should be noted that soil metagenomics target both culturable and non-culturable microorganisms, which reveal a more comprehensive profile of naturally-occurring antibiotic resistance genes that do not necessarily confer resistance phenotypes by the clinically-defined standard.

All quinolone resistance genes tested in this study were plasmid-mediated quinolone resistance (PMQR) genes (Yan *et al.*, 2017). While comparing to quinolone resistance caused by gene mutations in the chromosome, the level of resistance due to PMQR alone is usually lower. However, PMQR can substantially improve the occurrence of chromosomal mutations, leading to higher levels of resistance (Strahilevitz *et al.*, 2009). More importantly, PMQR can readily spread quinolone resistance through horizontal gene transfer (HGT). *qepA* encodes plasmid-mediated quinolone efflux pump. Its abundance in this study was the main contributor to the predominance of quinolone resistance genes. The prevalence of *qepA* in this study as well as in agricultural soil and aquatic environment (Li *et al.*, 2012; Yan *et al.*, 2017) suggests the ubiquitousness of the gene and the need to determine the gene prevalence in soil bacteria. Another predominant efflux gene identified in the current study was *tetA*, which encodes tetracycline efflux protein and has been detected from both soil DNA (Wu *et al.*, 2010) and soil bacteria (Srinivasan *et al.*, 2008). Taken together, the data suggest the potential importance of efflux pumps in conferring antibiotic resistance in the environment (Chen *et al.*, 2013; Chen *et al.*, 2016; Walsh and Duffy 2013) and the need to investigate the specificity of these efflux pumps, which may shed light on the underlying mechanisms of the widespread occurrence of antibiotic resistance in the environment. The positive correlation between β-lactam resistance genes and PMQR genes as well as between β-lactam resistance and tetracycline resistance genes suggest possible co-existence and co-

selection of these antibiotic resistance genes, although further investigation including a more comprehensive list of genes and additional study sites will be necessary to test this hypothesis.

The positive correlation between antibiotic resistance genes and metal resistance genes provides indirect evidence of possible co-selection of antibiotic resistance by heavy metals. The data call for further investigation on the impact of anthropogenic pollution of metals and alternative selective pressure from non-antibiotics at both phenotypic and molecular levels.

Taken together, this Chapter provided critical information complementary to what was observed in Chapter 1 on antibiotic resistance phenotypes in soil microorganisms. Phenotypic determination combined with soil metagenomics proved to be a key strategy to study the nature and extent of antibiotic resistance in the environment.

Chapter 3: Soil Contaminants, Microbial Profile, and Antibiotic Resistance in Urban Agricultural Soil and Vegetables

1. Introduction

Antibiotic-resistant bacteria in agricultural soil have the potential to contaminate vegetables grown in the field. There have been few studies on vegetables contaminated with antibiotic resistance (Holvoet *et al.,* 2013; Ruimy *et al.,* 2010). Holvoet *et al.* investigated lettuce as a vector and irrigation water and soil as a reservoir of antibiotic resistant *E. coli*. A total of 473 E. coli were isolated from 738 samples. Isolates were tested for 14 antibiotics and found that 11.4% of the isolates were resistant to at least one antibiotic with highest 7% resistance to ampicillin. Higher resistance rates were observed in the isolates recovered from the vegetables than the soil or irrigation water samples (Holvoet *et al.,* 2013). In a separate study 399 vegetables and fruits were analyzed and found to be highly contaminated with antibiotic resistant Gram-negative bacteria of soil origin. Ninty-five percent of 321 resistant bacteria belonged to Acinetobactrer, Stenotrophomonas, and Rahnella. Class A extended –spectrum beta-lactamase (ESBL) resistance was observed in 51 isolates, isolated from 13% of the tested samples (Ruimy *et al.,* 2010).

Antibiotics in soil can serve as selective pressure for antibiotic resistance and can also facilitate the transfer of ARGs to human pathogens and other microorganisms through horizontal gene transfer (HGT) (Kruse and Sørum 1994; Pruden *et al.,* 2006b). In addition, heavy metals are also potential alternative selective pressure for antibiotic resistance in agricultural soil (Stepanauskas *et al.,* 2005). A wide variety of human-driven activities are responsible for heavy metal contamination in soils and water, for example, mining, processing, and smelting (Feng *et al.,* 2010; Miclean *et al.,* 2009; Taylor *et al.,* 2010).

The aim of this Chapter was to investigate the impact of soil contaminants such as antibiotics and heavy metals on microbial profile and antibiotic resistance in urban agricultural soil and vegetables. The potential of antibiotic resistance gene transfer was also explored.

2. Materials and Methods

2.1 Sample collection

A total of 15 soil samples (5 each from E, G and O gardens) and 45 vegetable samples (21 from E, 5 from G, and 19 from O) were collected from three urban gardens (E, G and O) located in the metro Detroit area. Gardens E and G were located closer to the city center compared to garden O that was about 25 miles north of Detroit. Approximately 1 kg of each sample was collected and put into sterilized zip-lock bag. Cooler filled with ice was used to store and transport samples.

2.2 Isolation and identification of antibiotic-resistant soil bacteria

Bacteria were isolated and identified as described in Section 2.2 of Chapter 1.

2.3 Antibiotic Susceptibility Testing

Antibiotic resistance phenotypes were determined as described in Section 2.3 of Chapter 1.

2.4 Determination of soil microbial composition by high-throughput 16S rRNA sequencing

Soil microbial composition was determined as described in Section of 2.4 of Chapter 1.

2.5 Detection of Antibiotics and heavy metals

Six antibiotics that have been detected in the environment were analyzed on five soil samples collected from four corners and the center of the garden. The antibiotics included azithromycin (AZI), ciprofloxacin (CIP), erythromycin (ERY), oxytetracycline (OTC), sulfamethoxazole (SMZ), and trimethoprim (TRI) (Berglund 2015; Wellington *et al.*, 2013). The QuEChERS extraction method was used for antibiotic extraction according to AOAC Official

Method (2007.01) (Lehotay 2007). All compounds were determined simultaneously on a ACQUITY UPLC HSS T3 (100Å, 1.8 µm, 2.1 mm × 50 mm, Waters) column using electrospray ionization (ESI) in positive-ion mode by multiple reactions monitoring (MRM). The mobile phase composition was (A) water with 0.1% formic acid and (B) methanol. The gradient began at 10% of B with a flow of 0.5 mL/min. The percentage of B was linearly increased to 28% in 3.0 min, 43% in 1.5 min, and 100% in 0.5 min, and held for 2.0 min, followed by a re-equilibration time of 1.9 min (total running time = 9.0 min). The column temperature was maintained at 40 (±1) °C. This analytical method was validated by linearity, limit of quantification (LOQ), accuracy, precision, and recovery tests.

The concentration of four heavy metals Lead (Pb), Zinc (Zn), Strontium (Sr) and Rubidium (Rb) were determined in 15 soil samples (5 from each garden) by X-ray fluorescence (XRF). For XRF analysis, soil samples were sieved at 250 µm particle sizes. A hand-held Thermo Scientific XLTj-793 NITON energy-dispersive XRF analyzer was used for analysis. Each soil sample was analyzed three times to reduce the error, and the data for each sample were stored in a dedicated library.

2.6 Conjugation experiment

Conjugation experiment was designed to demonstrate the horizontal transfer of tetracycline resistance in antibiotic-resistant bacteria. Gram-negative bacteria (tetracycline^r, kanamycin^s) and Gram-positive bacteria (tetracycline^r, rifampicin^s) were selected as donors for conjugation experiment. All donor strains were tetM positive as identified by PCR. *E. coli* DH5α (amp^r, kan^r) and *Enterococcus faecalis* JH2-2 (rif^r ,fus^r) were used as recipient strains for Gram-negative and Gram-positive isolates, respectively. Conjugation was performed by the filter mating method (Agersø *et al.*, 2006) with modifications. Briefly, overnight cultures of the donor strains were

grown in Brain Heart Infusion (BHI) broth (Difco, Sparks, MD) containing tetracycline (15 µg/ml). The recipients *E. coli* DH5α and *Enterococcus faecalis* JH2-2 were grown in BHI broth containing 50 µg/ml of kanamycin and rifampicin, respectively. The mixture was then placed on a 0.45-µm-pore-size filter and incubated on BHI agar plates (Difco) at 30°C for two days. The filter was washed and vortex-mixed in BHI broth. For Gram-negative bacteria the mating mixture was spread onto BHI agar containing tetracycline (15 µg/mL) and kanamycin (50 µg/ml), and for Gram-positive bacteria the mating mixture was spread onto BHI agar containing tetracycline (15 µg/mL) and rifampicin (50 µg/ml). Transconjugants were confirmed by *tetM* PCR.

2.7 Whole-genome sequencing

A total of 24 isolates, 8 from each garden, were selected for whole-genome sequencing. Genomic DNA was extracted from pelleted bacterial cells using a commercial DNeasy PowerSoil Kit (QIAGEN, Valencia, CA). The quality and quantity of genomic DNA was determined with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE) and a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Wilmington, DE), respectively. The paired-end libraries were prepared with a Nextera DNA Flex Library Prep Kit (Illumina, San Diego, CA) together with Nextera DNA CD Indexes (Illumina, San Diego, CA) for tagmentation of the input DNA (300-400ng) and addition of the indexing primers by PCR amplification, according to the manufacturer's protocol.

The individual libraries (5 µl each) were pooled together. The quantity and quality of the single pooled library was determined with a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Wilmington, DE) and a 2100 Bioanalyzer (Agilent, Santa Clara, CA). The pooled library (15.9 ng/µl, average size 586 bp) was diluted to 4 nM with resuspension buffer included in Nextera DNA Flex Library Prep Kit (Illumina, San Diego, CA), and then denatured with freshly prepared 0.2 N

NaOH, according to the Denature and Dilute Libraries Guide (Illumina, Document # 15039740). A denatured low-concentration spike-in (1%) of PhiX control v3 was combined with the denatured library (diluted to 12 pM with prechilled HT1) and loaded into a MiSeq reagent cartridge version 3 (Illumina, San Diego, CA). Sequencing by synthesis of the paired-end 300 bp reads was conducted using an on-site MiSeq platform (Illumina, San Diego, CA).

FastQC.0.11.6 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to analyze the quality of read data. Trimmomatic.0.36 (www.usadellab.org/cms/?page=trimmomatic) (Bolger *et al.*, 2014). was employed to remove the adaptors and low quality bases from the reads, using the following options: LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36. The trimmed reads were assembled using the de novo assembler SPAdes.3.11.1 (http://bioinf.spbau.ru/spades) (Bankevich *et al.*, 2012), using default parameters with a broad range of k-mer values (from 21 to 127). Several in-house shell scripts were written to automate the analyses for multiples samples (available upon request from BI). The assembled contigs for each genome were used as queries against the Comprehensive Antibiotic Resistance Database (CARD, http://arpcard.mcmaster.ca/) to identify antibiotic resistance genes (McArthur *et al.*, 2013).

2.8 Statistical analysis

The correlations between antibiotic resistance phenotypes and antibiotics, as well as between antibiotic resistance phenotypes and metal concentrations were analyzed by using SPSS v. 21.0 (IBM SPSS, Chicago, IL). Antibiotic resistance phenotypes between soil and vegetables

3. Results

3.1 Isolation and identification of bacteria from soil and vegetable

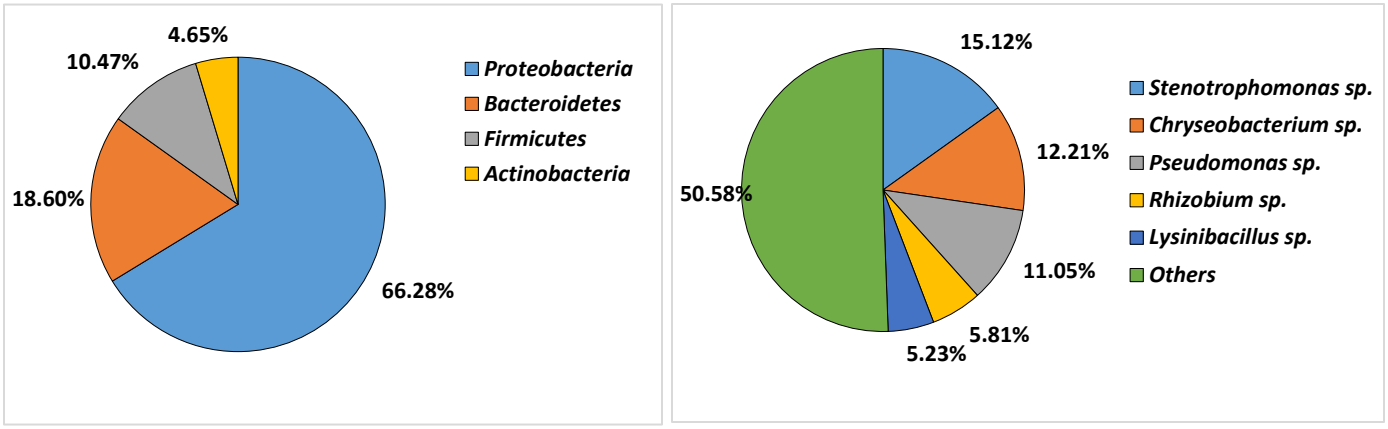
A total of 226 bacteria were isolated, including 54 from 15 soil samples (13 Gram-positive and 41 Gram-negative) and 172 from 45 vegetable samples (33 Gram-positive and 139 Gram-negative). Overall Gram-negative bacteria (n=180) were predominant over Gram-positive bacteria (n=46) (Table 1). Bacteria isolated from vegetables belonged to four phyla, *Proteobacteria* (66.28%), *Bacteroidetes* (18.60%), *Firmicutes* (10.47%), and *Actinobacteria* (4.65%) (Figure 9a), and belonged to 29 genera (Figure 9c). The major bacteria genera identified were *Stenotrophomonas* (n=26, 15.12%), *Chryseobacterium* (n=21, 12.21%), *Pseudomonas* (n=19, 11.05%), *Rhizobium* (n=10, 5.81%), and *Lysinibacillus* (n=9, 5.23%). The remaining genera combined comprised 50.58% (n= 87) of total isolates (Figure 9b). The same four phyla were identified in bacteria isolated from soil, including *Proteobacteria* (62.96%), *Firmicutes* (16.67%), *Bacteroidetes* (14.81%), and *Actinobacteria* (5.56%) (Figure 10a), and belonged to 21 genera (Figure 10c). The major bacteria genera identified were *Stenotrophomonas* (n=9, 16.67%), *Lysinibacillus* (n=7, 12.96%), *Pseudomonas* (n=6, 11.11%), Lysobacter (n=6, 11.11%), and *Chryseobacterium* (n=4, 7.41%). The remaining genera combined comprised 40.74% (n= 22) of the identified bacteria (Figure 10b).

Table 1. Gram characteristics of isolated bacteria from soil and vegetables

Sample	Gram-positive	Gram-negative	Total
Soil	13	41	54
Vegetables	33	139	172
Total	46	180	226

(a)

(b)



(c)

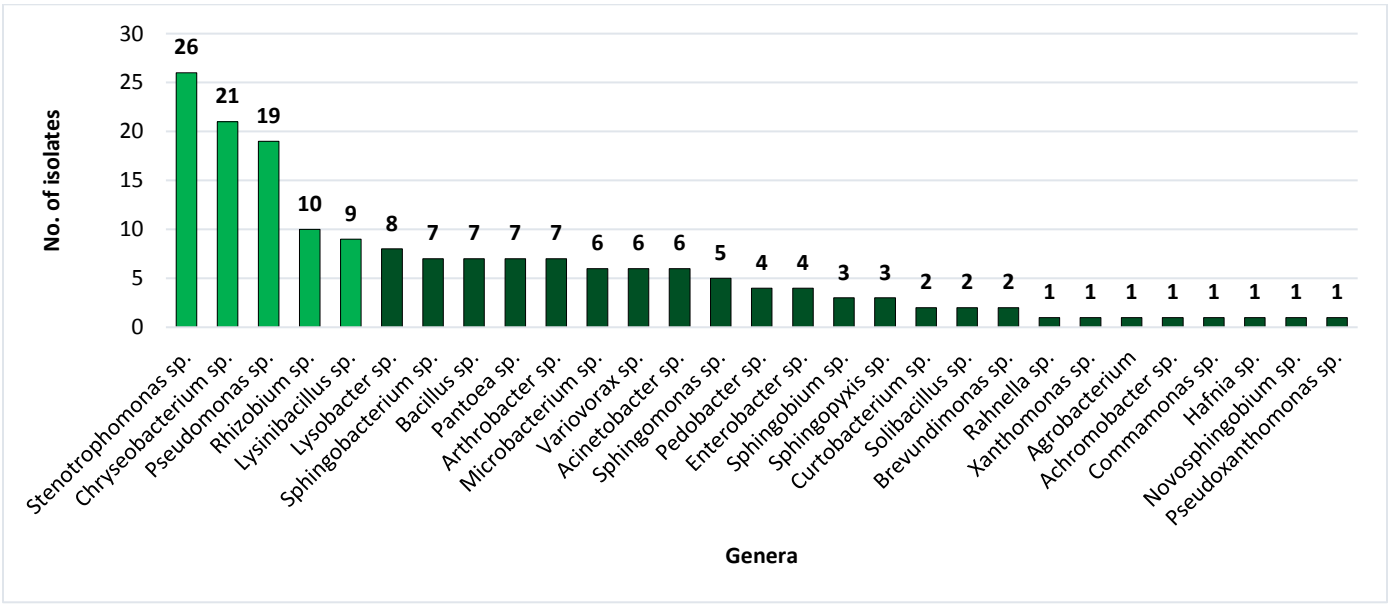


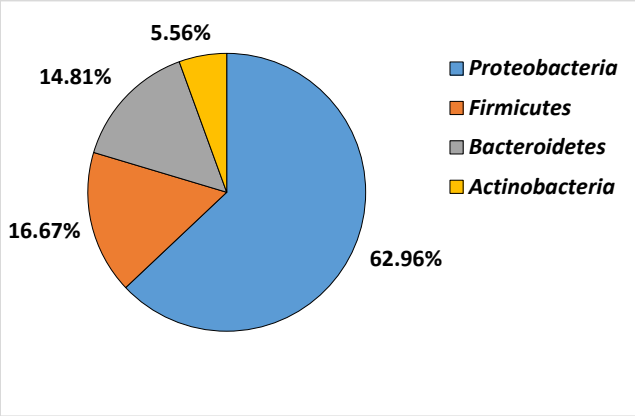
Figure 9. Microbial Composition in Vegetable Samples Determined by Bacteria Identification

Legend:

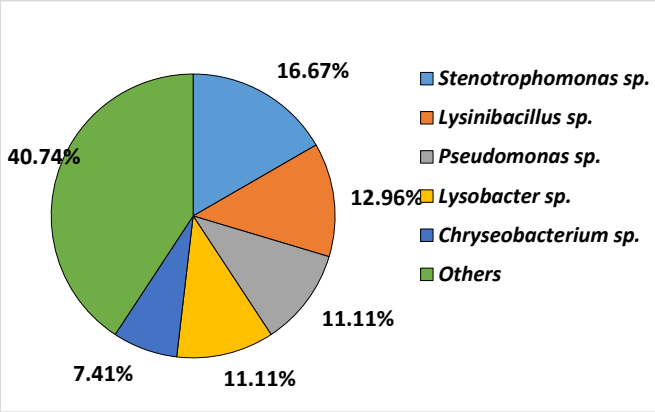
(a) Phylum distribution in vegetable bacteria (n=172)
(b) Genus distribution in vegetable bacteria (n=172)
(c) Genus composition including top 5 genera and others in vegetable bacteria (n=172)

Numbers shown in the pie chart were percentages of each phylum or genus in total identified bacteria in vegetables.
Numbers shown on top of the bars were number of the isolates identified for corresponding genus.
Dark green bars represent the "Others" category of the figure 9b.

(a)



(b)



(c)

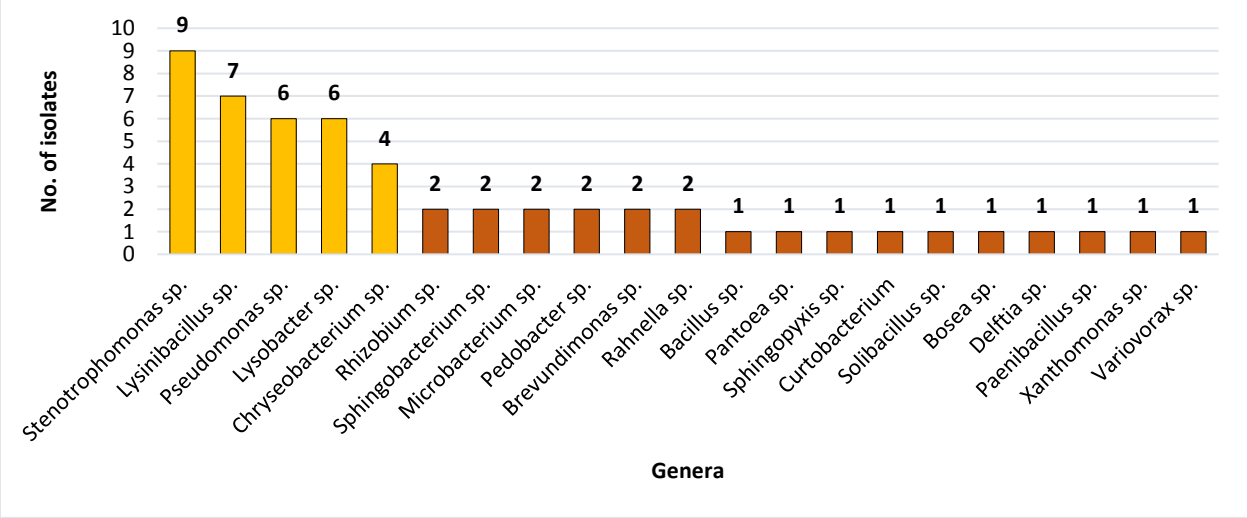


Figure 10. Microbial Composition in Soil Samples Determined by Bacteria Identification

Legend:

(a) Phylum distribution in Soil bacteria (n=54)
(b) Genus distribution in Soil bacteria (n=54)
(c) Genus composition including top 5 genera and others in Soil bacteria (n=54)

Numbers shown in the pie chart were percentages of each phylum or genus in total identified bacteria in soil.
Numbers shown on top of the bars were number of the isolates identified for corresponding genus.
Dark orange bars represent the "Others" category of the figure 10b.

3.2 Antibiotic resistance phenotypes of soil and vegetable bacteria

For Gram-negative bacteria the MIC data were interpreted based on the available resistance breakpoints of *E. coli*. Fourteen antibiotics were tested for Gram-negative bacteria. Resistance breakpoints were available for nine antibiotics. Similarly, the MIC data of Gram-positive bacteria were interpreted based on the available resistance breakpoints of *S. aureus*. A total of sixteen antibiotics were tested for Gram-positive bacteria and resistance breakpoints were available for nine antibiotics.

Gram-negative bacteria isolated from vegetables showed highest resistance to cefoxitin (85.61%), followed by ampicillin (82.01%), amoxicillin/clavulanic acid (69.06%), chloramphenicol (64.03%), ceftriaxone (57.55%), gentamicin (46.04%), nalidixic acid (35.97%), tetracycline (35.97%), and ciprofloxacin (34.53%). Gram-negative bacteria isolated from soil showed highest resistance to ampicillin (95.12%), followed by cefoxitin (82.93%), ceftriaxone (70.73%), amoxicillin/clavulanic acid (63.41%), chloramphenicol (58.54%), nalidixic acid (58.54%), ciprofloxacin (46.34%), gentamicin (43.90%), and tetracycline (24.39%) (Figure 11).

We have observed a variation in the percent of bacteria resistant to tested antibiotics between soil Gram-negative bacteria and vegetable Gram-negative bacteria. For some antibiotics soil bacteria showed higher percentage than vegetable bacteria, and vice versa. Higher percentage of bacteria was observed for ampicillin (95.12%, n=39), ceftriaxone (70.73%, n=29), nalidixic acid (58.54%, n=24), and ciprofloxacin (46.34%, n=19) resistance in soil bacteria than vegetable bacteria, 82.01% (n=114), 57.55% (n=80), 35.97% (n=50), and 34.53% (n=48), respectively. In

vegetables, Gram-negative bacteria resistant to cefoxitin (85.61%, n=119), amoxicillin/clavulanic acid (69.06%, n=96), chloramphenicol (64.03%, n=89), and tetracycline (35.97%, n=50) were more abundant than the soil samples (Figure 11).

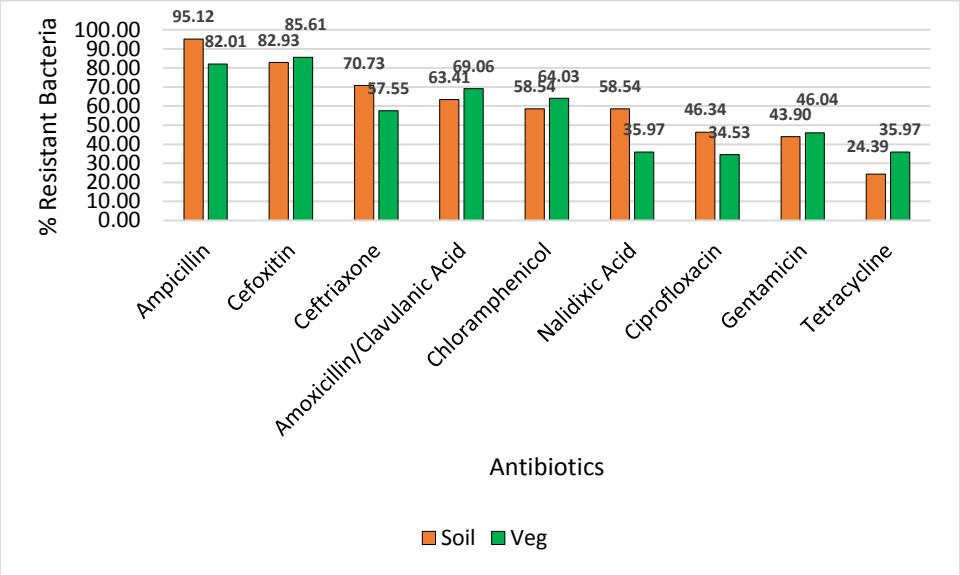


Figure 11. Prevalence of Antibiotic Resistance in Gram-negative Bacteria

Legend

Orange bars: Antibiotic resistance prevalence in Gram-negative bacteria isolated from soil (n=41)

Green bars: Antibiotic resistance prevalence in Gram-negative bacteria isolated from vegetables (n=139)

All Gram-positive bacteria (100%) recovered from vegetables were resistant to penicillin, gentamicin, and kanamycin. They were also resistant to erythromycin (30.30%), ciprofloxacin (24.24%), quinopristine/dalphopristine (12.12%), tetracycline (12.12%), chloramphenicol (6.06%) and vancomycin (6.06%). All Gram-positive bacteria (100%) isolated from soil were resistant to three antibiotics- penicillin, gentamicin and kanamycin. They were also resistant to

erythromycin (38.46%), ciprofloxacin (30.77%), quinopristine/dalphopristine (23.08%), and chloramphenicol (15.38%) (Figure 12).

Difference in percent resistant bacteria for tested antibiotics was also observed in Gram-positive bacteria recovered from soil and vegetables. No variation was observed between soil and vegetable bacteria for penicillin, gentamicin, and kanamycin as all of the isolates (100%) were resistant. For remaining six antibiotics, higher percent of soil bacteria were resistant to four antibiotics; erythromycin (38.46%, n=5), ciprofloxacin (30.77%, n=4), quinopristin/dalphopristin (23.08%, n=3), and chloramphenicol (15.38%, 2), than vegetable bacteria. Interestingly, tetracycline (12.12%, n=4) and vancomycin (6.06%, n=2) were detected only in vegetables, not in any soil samples.

For those antibiotics that interpretation breakpoints were unavailable for *E. coli* or *S. aureus*, most Gram-negative bacteria fell under the highest range of MIC to azithromycin, ceftiofur, streptomycin, sulfisoxazole, and trimethoprim/sulfamethoxazole, so did Gram-positive bacteria to daptomycin, lincomycin, linezolid, nitrofurantoin, streptomycin, tigecycline and tylosin tartrate.

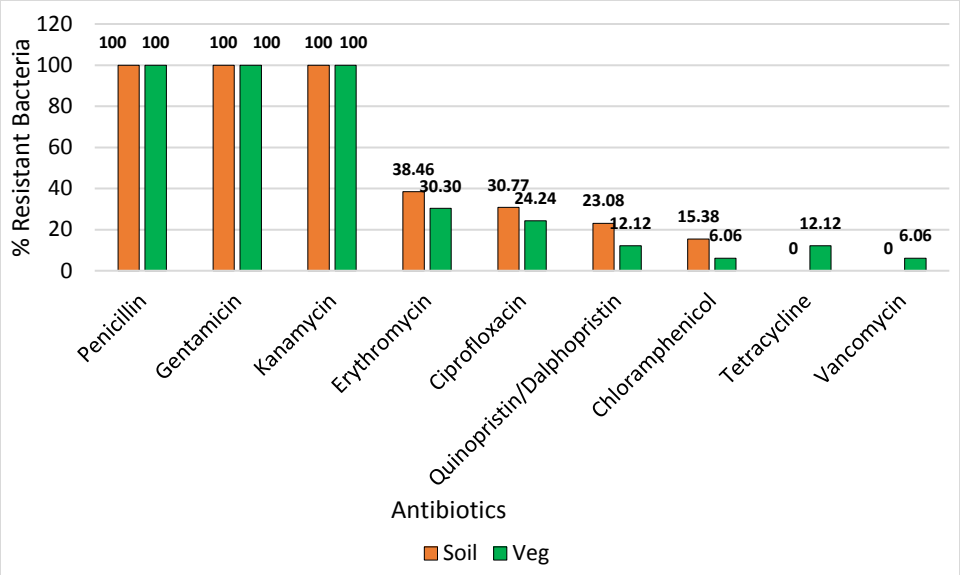


Figure 12. Prevalence of Antibiotic Resistance in Gram-positive Bacteria

Legend

Orange bars: Antibiotic resistance prevalence in Gram-positive bacteria isolated from soil (n=13)

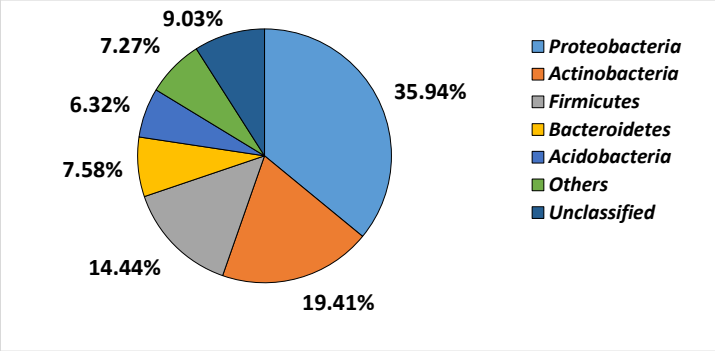
Green bars: Antibiotic resistance prevalence in Gram-positive bacteria isolated from vegetables (n=33)

3.3 Bacteria diversity in soil revealed by high-throughput 16S rRNA sequencing

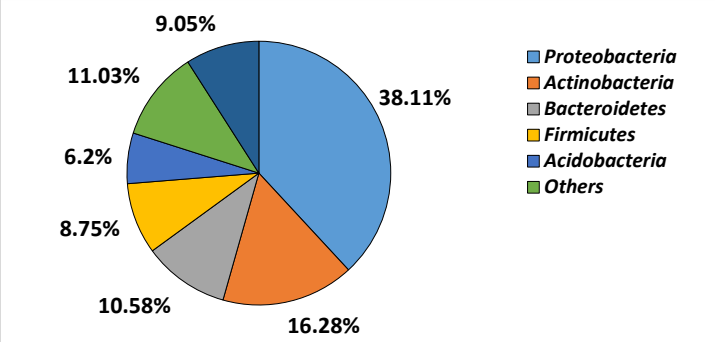
16S rRNA sequencing identified numerous phyla in each soil sample. On average, each soil sample in Garden E harbored more than 30 phyla. *Proteobacteria* were the most prevalent phylum and identified in 35.94% of the reads, followed by *Actinobacteria* (19.41%), *Firmicutes* (14.44%), *Bacteroidetes* (7.58%), and *Acidobacteria* (6.32%). 16S rRNA sequencing failed to classify 9.03% of the reads to the phylum level (Figure 13a). The top five phyla identified in Garden G were comprised 79.92% of the total reads,with *Proteobacteria* being the most prevalent phylum and identified in 38.11% of the reads, followed by *Actinobacteria* (16.28%), *Bacteroidetes* (10.58%), *Firmicutes* (8.75%), and *Acidobacteria* (6.20%). Over 9% of the reads were unclassified at phylum level. Other than top five phylum the remaining phylum accounted for 11.03% of the

reads (Figure 13b). Top five phyla identified in Garden O were *Proteobacteria* (36.25%), *Actinobacteria* (18.23%), *Bacteroidetes* (10.05%), *Acidobacteria* (8.42%), and *Firmicutes* (8.24%). The top five phyla were the same as those identified in Gardens E and G, but the prevalence order was different. In Garden O, the fourth and fifth ranks were occupied by *Acidobacteria*, and *Firmicutes*, but in the garden G, the same ranks were occupied by *Firmicutes* and *Acidobacteria*, respectively. The percentage of unclassified reads at phylum level in the garden O was 10.41%, which was higher than garden G (9.05%) and garden E (8.32%) (Figure 13c)

(a)



(b)



(c)

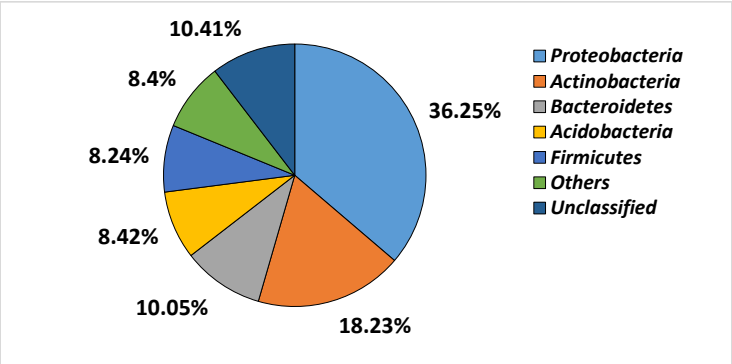


Figure 13. Microbial Composition Determined at Phylum Level by 16S rRNA sequencing

Legend:

(a) Phylum distribution of soil bacteria by 16S rRNA sequencing in E garden
(b) Phylum distribution of soil bacteria by 16S rRNA sequencing in G garden
(c) Phylum distribution of soil bacteria by 16S rRNA sequencing in O garden

Numbers shown in the pie chart were percentages of each phylum in total identified reads.

3.4 Antibiotic detection

A total of six antibiotics-azithromcin, ciprofloxacin, erythromycin, oxytetracycline, sulfamethoxazole, and trimethoprim were tested in soil and vegetable samples. Except trimethoprim, all other five antibiotics were detected both in soil and vegetable samples of three gardens. Trimethoprim was detected only in soil of the garden G at a concentration of 2.66 µg/kg. Azithromycin was detected in the soil of garden O at a concentration of 33.89 µg/kg, which is marginally higher than the concentration of the vegetable of garden E (33.39 µg/kg). Ciprofloxacin and erythromycin concentration was higher in vegetables than soils. Ciprofloxacin was detected in vegetables of two gardens, garden E (34.55 µg/kg) and Garden O (19.46 µg/kg), but detected in only soil of O garden (31.51 µg/kg). Highest 13.87 µg/kg of erythromycin was detected in vegetables compared to 5.63 µg/kg in soils. In soils, oxytetracycline concentration ranges from 30.95 to 32. 26 µg/kg, whereas in vegetables the range was 14.12 to 41.12 µg/kg. Not much variation was observed in sulfamethoxazole concentration between soils and vegetables (Table 2).

Table 2. Concentrations (µg/kg) of Antibiotics Detected in Soil and Vegetables

Antibiotics	Soil (µg/kg)			Vegetable (µg/kg)		
	Garden E	Garden G	Garden O	Garden E	Garden G	Garden O
Azithromycin	< LOQ	< LOQ	33.89	33.39	< LOQ	25.31
Ciprofloxacin	< LOQ	< LOQ	31.51	34.55	< LOQ	19.46
Erythromycin	5.39	5.63	3.11	6.48	3.02	13.87
Oxytetracycline	30.95	31.43	32.26	41.12	14.12	22.83
Sulfamethoxazole	5.34	5.63	3.11	6.32	2.71	4.77
Trimethoprim	< LOQ	2.66	< LOQ	< LOQ	< LOQ	< LOQ

LOQ = Limit of Quantification

3.5 Occurrence of heavy metals in soil

In this study four heavy metals, namely lead (Pb), zinc (Zn), strontium (Sr), and Rubidium (Rb) were tested and detected in the soils of all three gardens. The lead concentration in O garden (40.99 ± 19.44 ppb) was low compared to G (133.38 ± 64.03 ppb) and E (145.19 ± 9.11 ppb) gardens. Zinc concentration varies from 141.09 ± 69.57 ppb in the O garden to 192.11 ± 24.28 ppb in the E garden. Zinc concentration in E garden superseded both G and O gardens. Overall, G garden had high concentration of strontium and rubidium over E and O gardens. (Table 3). Lead concentration was significantly different between gardens with a *p*-value of 0.008. No significant difference was observed for zinc, strontium, and rubidium concentrations between gardens (Table 4).

In summary, the concentration of three out of four metals tested in this study were low in the garden O compared to other two gardens, E and G. Only rubidium concentration was slightly higher than the garden E, but was lower than the garden G.

Table 3. Average Concentration (ppb) of Heavy Metals Detected in Soil and Vegetables in All Three Gardens

	Gardens		
Element	**E**	**G**	**O**
	Mean (± SD) ppb	**Mean (± SD) ppb**	**Mean (± SD) ppb**
Lead (Pb)	145.19 (± 9.11)	133.38 (± 64.03)	40.99 (± 19.44)
Zinc (Zn)	192.11 (± 24.28)	189.17 (± 66.79)	141.09 (± 69.57)
Strontium (Sr)	129.91 (± 7.75)	131.37 (± 12.17)	126.64 (± 7.48)
Rubidium (Rb)	46.37 (± 1.87)	66.18 (± 4.41)	49.72 (± 9.81)

Table 4. ANOVA-Analysis to Determine Differences in Concentrations of the Heavy Metals Between and within Gardens

Metals	Difference	Significance (*p*-value)
Lead (Pb)	Between Gardens	0.008
	Within Gardens	---
Zinc (Zn)	Between Gardens	0.412
	Within Gardens	---
Strontium (Sr)	Between Gardens	0.741
	Within Gardens	---
Rubidium (Rb)	Between Gardens	0.004
	Within Gardens	---

*ANOVA (Analysis of Variance)

3.6 Correlation between antibiotic resistance and antibiotics and metals

Correlation between different categories of antibiotic resistance (aminoglycosides, beta-lactam, tetracycline, and quinolones) and antibiotic concentrations (sulfamethoxazole, oxytetracycline, erythromycin, and ciprofloxacin) were tested. Beta-lactam resistance was positively correlated with ciprofloxacin concentration ($p = 0.026$). Beta-lactam resistance was also positively correlated with tetracycline resistance ($p = 0.013$), and quinolone resistance ($p = 0.036$) (Table 5). No correlation was observed between antibiotic resistance and metal concentration. The only positive relationship was observed between lead concentration and zinc concentration ($p = 0.003$) (Table 6).

Table 5. Correlation between Antibiotic Resistance and Antibiotic concentrations

		Aminoglycoside	Beta-lactam	Tetracycline	Quinolones	Total Antibiotic Resistance	Sulfamethoxazole	Oxytetracycline	Erythromycin	Ciprofloxacin
Aminoglycoside	Pearson Correlation	1	0.318	0.790	0.632	0.737	0.110	0.316	-0.116	-0.043
	Sig. (2-tailed)		0.313	**0.002**	**0.028**	**0.006**	0.733	0.317	0.719	0.895
Beta-lactam	Pearson Correlation	0.318	1	0.690	0.607	0.770	-0.202	0.098	-0.277	0.637
	Sig. (2-tailed)	0.313		**0.013**	**0.036**	**0.003**	0.530	0.762	0.384	**0.026**
Tetracycline	Pearson Correlation	0.790	0.690	1	0.761	0.899	0.047	0.135	-0.028	0.372
	Sig. (2-tailed)	**0.002**	**0.013**		**0.004**	**0.000**	0.884	0.676	0.932	0.234
Quinolones	Pearson Correlation	0.632	0.607	0.761	1	0.945	-0.242	0.140	-0.273	0.409
	Sig. (2-tailed)	**0.028**	**0.036**	**0.004**		**0.000**	0.450	0.665	0.390	0.186
Total Antibiotic Resistance	Pearson Correlation	0.737	0.770	0.899	0.945	1	-0.163	0.185	-0.257	0.445
	Sig. (2-tailed)	**0.006**	**0.003**	**0.000**	**0.000**		0.613	0.564	0.421	0.147
Sulfamethoxazole	Pearson Correlation	0.110	-0.202	0.047	-0.242	-0.163	1	-0.603	0.091	-0.525
	Sig. (2-tailed)	0.733	0.530	0.884	0.450	0.613		0.038	0.777	0.079
Oxytetracycline	Pearson Correlation	0.316	0.098	0.135	0.140	0.185	-0.603	1	-0.478	0.282
	Sig. (2-tailed)	0.317	0.762	0.676	0.665	0.564	0.038		0.116	0.375
Erythromycin	Pearson Correlation	-0.116	-0.277	-0.028	-0.273	-0.257	0.091	-0.478	1	-0.065
	Sig. (2-tailed)	0.719	0.384	0.932	0.390	0.421	0.777	0.116		0.841
Ciprofloxacin	Pearson Correlation	-0.043	0.637	0.372	0.409	0.445	-0.525	0.282	-0.065	1
	Sig. (2-tailed)	0.895	0.026	0.234	0.186	0.147	0.079	0.375	0.841	

*Bold numbers indicate significance at $p < 0.05$

Table 6. Correlation between Antibiotic Resistance and Metal Concentrations

		Aminoglycoside	Beta-lactam	Tetracycline	Quinolones	Total Antibiotic Resistance	Sr	Rb	Pb	Zn
Aminoglycoside	Pearson Correlation	1	0.318	0.790	0.632	0.737	0.161	-0.088	-0.019	0.216
	Sig. (2-tailed)		0.313	**0.002**	**0.028**	**0.006**	0.617	0.785	0.952	0.500
Beta-lactam	Pearson Correlation	0.318	1	0.690	0.607	0.770	0.291	-0.004	-0.407	-0.129
	Sig. (2-tailed)	0.313		**0.013**	**0.036**	**0.003**	0.359	0.990	0.189	0.688
Tetracycline	Pearson Correlation	0.790	0.690	1	0.761	0.899	0.211	0.002	-0.104	0.179
	Sig. (2-tailed)	**0.002**	**0.013**		**0.004**	**0.000**	0.509	0.994	0.748	0.578
Quinolones	Pearson Correlation	0.632	0.607	0.761	1	0.945	-0.072	-0.157	-0.152	0.353
	Sig. (2-tailed)	**0.028**	**0.036**	**0.004**		**0.000**	0.825	0.625	0.636	0.261
Total Antibiotic Resistance	Pearson Correlation	0.737	0.770	0.899	0.945	1	0.102	-0.105	-0.217	0.223
	Sig. (2-tailed)	**0.006**	**0.003**	**0.000**	**0.000**		0.752	0.745	0.497	0.487
Sr	Pearson Correlation	0.161	0.291	0.211	-0.72	0.102	1	0.133	-0.127	-0.106
	Sig. (2-tailed)	0.617	0.359	0.509	0.825	0.752		0.680	0.693	0.742
Rb	Pearson Correlation	-0.088	-0.004	0.002	-0.157	-0.105	0.133	1	0.246	0.000
	Sig. (2-tailed)	0.785	0.990	0.994	0.625	0.645	0.680		0.441	0.999
Pb	Pearson Correlation	-0.019	-0.407	-0.104	-0.152	-0.217	-0.127	0.246	1	0.770
	Sig. (2-tailed)	0.952	0.189	0.748	0.636	0.497	0.693	0.441		**0.003**
Zn	Pearson Correlation	0.216	-0.129	0.179	0.353	0.223	-0.106	0.000	0.770	1
	Sig. (2-tailed)	0.500	0.688	0.578	0.261	0.487	0.742	0.999	**0.003**	

*Bold numbers indicate significance at $p < 0.05$

3.7 Conjugation experiments

A total of 55 Gram-negative and 2 Gram-positive tetracycline-resistant bacteria were tested for conjugation. Forty of 57 total were able to transfer tetracycline resistance to the recipient. Out of 55 Gram-negative isolates, 28 were from *Stenotrophomons*, followed by, 17 from *Chryseobacterium*, 5 from *Sphingobacterium*, and one each from remaining genera. Around 72 % (20/28) *Stenotrophomonas* isolates successfully transferred tetracycline resistance to the recipient at a transfer rate ranged from 2.28 x 10^{-4}/recipient cell to 3.36 x10^{-3}/recipient cell. Conjugation was successful in 64.7% *Chryseobacterium* isolates at a transfer rate ranged from 8.40 x 10^{-4}/recipient cell to 2.43 x 10^{-3}/recipient cell. Four out of five (80%) *Sphingobacterium* isolates were positive for conjugation. The transfer rate ranged from 7.20 x 10^{-4}/recipient cell to 1.81 x 10^{-3}/recipient cell. All remaining isolates also successfullytransferred tetracycline resistance into recipient cells. Both Gram-positive bacteria, *Microbacterium* and *Curtobacterium*, were positive for conjugation and the transfer rate was 1.75 x 10^{-4}/recipient cell and 2.64 x 10^{-4}/recipient respectively (Table 7).

Table 7. Conjugation Results of 40 Soil Isolates from Three Gardens

Garden	Source	Donor (*Tet*^{r}) (Number of isolates)	Conjugation rate (range)
E	**Soil**	*Chryseobacterium* sp. (6)	9.15×10^{-4} - 2.43×10^{-3}
		Curtobacterium sp. (1)	2.64×10^{-4}
		Dyadobacter sp. (1)	1.53×10^{-3}
		Lysobacter sp. (1)	8.85×10^{-4}
		Microbacterium sp. (1)	1.75×10^{-4}
		Sphingobacterium sp. (3)	7.20×10^{-4} – 1.69×10^{-3}
		Stenotrophomonas sp. (14)	2.28×10^{-4} – 3.66×10^{-3}
		Variovorax sp. (1)	1.44×10^{-3}
	Vegetable	*Chryseobacterium* sp. (1)	1.14×10^{-3}
		Stenotrophomonas sp. (1)	1.33×10^{-3}
G	**Soil**	*Chryseobacterium* sp. (1)	8.40×10^{-4}
	Vegetable	*Chryseobacterium* sp. (1)	1.87×10^{-3}
		Sphingobacterium sp. (1)	1.81×10^{-3}
		Stenotrophomonas sp. (1)	1.53×10^{-3}
O	**Soil**	*Pseudomonas* sp. (1)	1.44×10^{-3}
		Stenotrophomonas sp. (1)	2.43×10^{-3}
	Vegetable	*Chryseobacterium* sp. (1)	1.60×10^{-3}
		Stenotrophomonas sp. (3)	8.80×10^{-4} - 1.75×10^{-3}

3.8 Whole-genome sequencing revealed the presence of antibiotic resistance genes

A number of antibiotic resistance genes were detected in the sequenced isolates. Efflux pumps were the most common genes identified and found in # of 24 sequences. Resistance genes related to efflux pump AdeIJK, were detected in *Acinetobacter calcoaceticus*, which confer resistance to multiple drugs, including beta-lactams, tetracycline, chloramphenicol, pyronine, lincosamides, erythromycin, fluoroquinolones, novobiocin, fusidic acid, rifampin, acridine, trimethoprim, safranin, and sodium dodecyl sulfate. Chryseobacterium showed the presence of *lnd*-4 and *cgb*-1 b-lactamase resistance genes which can confer resistance to landomycin and beta-lactams, respectively. Genes related to multidrug-resistant Mex pump, for example, *mex*B, *mex*F, *mex*K, and *mex*W, were detected in *Lysobacter gummosus* and *Pseudomonas fluorescenns.* Quinolone

resistance gene *oqx*B and erythromycin resistance gene *erm*B were identidied in *Pantoea* isolates. *Rhizobium* isolates showed the presence of *cat* gene which confers resistance to chloramphenicol. Multidrug-resistant gene *crp* was detected in *Rahnella* and *Pantoea*. Stenotrophomonas isolates showed the presence of oqxB gene conferring resistance to quinolone, and smeD, smeE, and smeF genes conferring resistance to carbapenem. Rifampin, tetracycline, and vancomycin resistance genes were detected in Gram-positive isolates, *Bacillus* and *Microbacterium* (Table 8).

The presence of antibiotic resistance genes seemed to be independent of resistance phenotypes. There were isolates with antibiotic resistance genes identified demonstrating pan susceptible to all antibiotics tested. Isolates showing multidrug resistance phenotypes were also found to carry no antibiotic resistance genes.

Table 8. Identified Antibiotic Resistance Genes by Whole-Genome Sequencing in 24 selected isolates

Bacteria	Identified Antibiotic Resistance Genes (ARGs)	Resistance Phenotypes
Acinetobacter calcoaceticus	*oxa*, *ade*G, *adc*, *abe*S, *ade*H, *abe*M, *ade*J, ade*K*, *ade*I	AMP, AUG2, AXO, CHL, FOX, GEN,
Chryseobacterium lathyri	*Ind*-4	AMP, AUG2, AXO, CHL, FOX, GEN, NAL, TET
Chryseobacterium sp.	None	AMP, AUG2, AXO, CIP, FOX, GEN, NAL, TET
Chryseobacterium sp.	*cgb*-1 b-lactamase	AMP, AUG2, AXO, CIP, CHL, FOX, GEN, TET
Lysobacter gummosus	*sme*E, *mex*K	AMP, AXO
Lysobacter sp.	*sme*E	AMP, AUG2, AXO, CHL, CIP, FOX, TET,
Pantoea agglomerans	*oqx*B, *emr*B, *crp*	AMP, AUG2, AXO, CHL, FOX, GEN, NAL,
Pantoea sp.	*oqx*B, *emr*B, *crp*	NONE
Pseudomonas fluorescens	*mex*B, *mex*K, *mex*W, *mex*F	AMP, AUG2, AXO, CHL, FOX, NAL
Rahnella sp.	*crp*	AMP, AUG2, AXO, FOX, , NAL, TET
Rhizobium radiobacter	*cat*	AMP, AUG2, CHL, FOX, NAL
Rhizobium sp.	*cat*	CHL, FOX
Rhizobium sp.	*cat*	AMP, CHL, NAL
Sphingobacterium faecium	None	AMP, CHL, FOX, GEN, NAL, TET
Sphingobacterium sp.	None	AMP, CHL, FOX, GEN, NAL
Stenotrophomonas maltophilia	*oqx*B, *sme*D, *sme*E, *sme*F	AMP, AUG2, AXO, CIP, FOX, GEN, TET
Stenotrophomonas maltophilia	*sme*D, *sme*E, *sme*F, *oqx*B	AMP, AUG2, AXO, CIP, FOX, GEN, TET
Stenotrophomonas maltophilia	*oqx*B, *sme*D, *sme*E, *sme*F	AMP, AUG2, AXO, CIP, FOX, GEN, NAL, TET
Stenotrophomonas sp.	*oqx*B, *sme*D, *sme*E, *sme*F	AMP, AUG2, AXO, CIP, FOX, GEN, TET
Bacillus bataviensis	*rph*B	GEN, KAN , PEN
Lysinibacillus fusiformis	None	CIP, ERY, GEN, KAN, PEN
Lysinibacillus sphaericus	None	GEN, KAN , PEN
Microbacterium sp.	*tet*42, rifampin resistance	CIP, DAP, ERY, GEN, KAN, LIN, NIT, PEN, SYN, TET
Microbacterium sp.	*tet*42, *van*RO	CIP, CHL, ERY, GEN, KAN, PEN, SYN, TET

AMP=Ampicillin, AUG2=Amoxicillin / clavulanic acid 2:1 ratio, AXO=Ceftriaxone, CIP=Ciprofloxacin, CHL=Chloramphenicol, FOX=Cefoxitin, GEN=Gentamicin, KAN=Kanamycin, NAL=Nalidixic Acid, PEN=Penicillin, SYN=Quinupristin / dalfopristin, TET=Tetracycline

4. Discussion

In this study, the phyla and genera identified by both culturing and high throughput 16S rRNA sequencing represents general soil bacteria (Janssen 2006). Top predominant phylum identified by culturable and 16S rRNA sequencing were similar, but dissimilarly was observed ranking wise. A noticeable discrepancy was observed between these methods at genus level, which was inevitable as culturable bacteria represents only 1% of total bacterial diversity in soil (Demaneche *et al.*, 2008; Schloss and Handelsman 2003), and 16S rRNA sequencing captures both culturable and non-culturable microorganisms. Moreover, variation in dominant phylum and genus was also observed among gardens. Soil attributes and land use could have caused this variation(Jesus *et al.*, 2009). The 16S rRNA sequencing of this study identified *Proteobacteria* as the most prevalent phylum in all three gardens, which was different than the findings of Brazilian Atlantic Forest soils where *Acidobacteria* was the most prevalent. More than 9% of the bacteria were unidentified at phylum level in this study which was higher than one of the most biodiversity hot spot in the world where 6.5% of the bacteria were unidentified (Faoro *et al.*, 2010). This high percentage of unidentified bacteria suggests urban agricultural environment as a potential habitat for diverse bacteria.

High antibiotic resistance phenotypes were observed in this study, which suggests the inherited presence of antibiotic resistance determinants in the environmental bacteria. During literature search we found very limited number of studies focused on culturable soil bacteria (Walsh 2013; D'Costa *et al.*, 2006; Wright 2010). These studies reported similar findings of the current study where majority of the isolated bacteria were multi-drug resistant to all known antibiotic classes

currently available in the market, from natural to semi-synthetic to synthetic. However, it should be noted that there are no standard breakpoints for environmental microorganisms as for clinical isolates, making it a challenge in comparing data across studies. Some researchers have used a general 20 µg/ml for all antibiotics as the resistance breakpoint in soil bacteria ((Walsh and Duffy 2013; D'Costa *et al.,* 2006). The current study used CLSI guidelines for *E. coli* and *S. aureus*, which addressed the antibiotic variation but only allowed the interpretation for those antibiotics that have resistance breakpoints. Since the implication of antibiotic resistance in clinical bacteria may not apply to bacteria of environmental origin, establishing "ecological breakpoints" or even standardized protocols in environmental investigations will be of great value for inter-study comparisons (Thanner *et al*., 2016). To achieve this goal, collecting more data on environmental bacterial species and isolates is the key.

Isolates recovered from both soil and vegetables showed higher antibiotic resistance phenotypes. We have noticed a small variation in the prevalence of antibiotic resistance phenotypes between soil and vegetables. Interestingly, for some antibiotics we have observed higher antibiotic resistance prevalence in vegetable isolates compared to the soil isolates. For example, Gram-negative isolates from vegetables showed higher prevalence for cefoxitin, amoxicillin/clavulanic acid, chloramphenicol, gentamicin, and tetracycline, compared to the isolates of soil. Similarly, in Gram-positive bacteria, the prevalence of tetracycline in vegetable isolates exceeded the prevalence of soil isolates. These findings suggest that, along with soil, vegetables can function as a reservoir of antibiotic resistance, and in some cases even more potential than soil.

The low antibiotic concentrations detected in this study were an indication of little or no influence of external antibiotics on the soils. The sporadic presence of antibiotics can be mainly

due to the antibiotic-producing soil bacteria (Martinez 2008) in contrast to agricultural soils adjacent to agricultural production that had antibiotics detected at the scale of mg/kg (Ji *et al.,* 2012). Although the antibiotic concentrations reported in the current study were below what would normally be needed to select antibiotic resistance (Berglund 2015), the role of antibiotic residues as regulatory substances and signaling molecules should be explored because sub-inhibitory antibiotics may be able to mediate the dissemination of antibiotic resistance, especially since high prevalence of antibiotic resistance is common in the environment.

Antibiotics were frequently detected in this study in the vegetable samples, although variation in concentration was observed between gardens. This variation can be explained by antibiotics uptake mechanism by vegetables. Irrigation practices of the gardens can cause this variation as vegetables acquire antibiotics by water transport and passive absorption mechanisms. Water solubility of the antibiotics in soil can also cause this variation as all antibiotics are not equally soluble in water (Hu *et al.,* 2010). The concentration of antibiotics detected in the vegetable samples in this study was in agreement with another study where antibiotic concentrations were ranges from 0.1 -532 µg/kg. The concentration of antibiotics they detected in the leafy vegetables were higher than the root vegetables (Hu *et al.,* 2010), but we found root vegetables accumulated more antibiotics than the leafy vegetables. Close contact of the root vegetables with soil could be the reason of high antibiotic concentration.

It has been argued that metal contamination can play a role in the dissemination of antibiotic resistance into pathogenic bacteria (Summers 2002; Summers *et al.,* 1993; Alonso *et al.,* 2001). A number of studies documented that metal resistance coexisted with antibiotic resistance (Belliveau *et al.,* 1991; Mcentee *et al.,* 1986). In our study, no significant correlation was observed between metal concentrations and antibiotic resistance. Zinc and lead concentrations in soil

samples were below the normal range detected by a national agricultural soil survey conducted throughout the USA. This national survey carefully selected a total of 3045 soil samples from major agricultural production areas in the USA by avoiding the areas of possible anthropogenic and other contaminations and found that average concentration of zinc and lead were 42.9 mg/kg and 10.6 mg/kg, respectively (Holmgren *et al.,* 1993); whereas we found maximum 0.192 mg/kg of zinc and 0.145 mg/kg lead in our study sites. This is a great indication of soil safety in urban agriculture in metro Detroit despite that there is currently no legal requirement for soil testing before opening an urban garden/farm. Very low concentrations of metals might be another reason for them not showing positive correlations with antibiotic resistance as it is generally accepted that high concentration of metals can co-select antibiotic resistance by co-resistance (Hasman and Aarestrup 2002) or cross-resistance (Hernandez *et al.,* 1998).

Bacteria from diverse genera were able to transfer tetracycline resistance via conjugation, which suggests that environmental bacteria have the potential to spread antibiotic resistance to pathogenic bacteria if they share a common habitat (Matte-Tailliez *et al.,* 2002; Wiedenbeck and Cohan 2011). Not much variation was observed in conjugation rate between different genus and different gardens. Variation in conjugation rate was also minimum between soil and vegetable isolates, indicating that antibiotic-resistant isolates of vegetable originhave the similar potential of disseminating antibiotic resistance genes.

Efflux pumps seemed to play an important role in conferring multidrug resistance in soil bacteria. This is evidenced by the identification of multidrug efflux pump genes, including *ade*I, *ade*J, *ade*K, *abe*M, *abe*S, and *mex*B by whole-genome sequencing. Over-expression of these pumps can occur if they are exposed to any homologous substrates specific to the pump. Increased expression of these genes can be associated with antibiotic resistance to multiple drugs (Webber

and Piddock 2003). *Acinetobacter baumannii*, a recently emerging clinical pathogen, were frequently found to carry *ade*I, *ade*J, and *ade*K genes (Kor *et al.,* 2014). Similar genes were detected in this study in *Acinetobacter calcoaceticus*, which suggests a possible public health significance, as AdeIJK pump confers resistance to beta-lactams, tetracycline, chloramphenicol, pyronine, lincosamides, erythromycin, fluoroquinolones, novobiocin, fusidic acid, rifampin, acridine, trimethoprim, safranin, and sodium dodecyl sulfate (Damier-Piolle *et al.,* 2008). The *sme*F gene, related to smeDEF efflux pump, was also detected in all *Stenotrophomonas maltophilia* isolates which are considered as an emerging opportunistic pathogen. A previous study reported that, over-expression of *sme*F gene can facilitate multidrug resistance in *Stenotrophomonas maltophilia* (Zhang *et al.*, 2001). We have found *mex*B gene, which encode Mex pump, in one of our isolates, *Pseudomonas fluorescence*. Mex pumps were reported as a contributor of antibiotic resistance in clinically important *Pseudomonas aeruginosa* (Poole 2000). Over-expression of Mex pump can promote resistance to beta-lactams, fluoroquinolones, trimethoprim, and chloramphenicol (Chuanchuen *et al.*, 2001). Antibiotic resistance genes detected in environmental bacteria in this study proves that both environmental and clinical bacteria share many common genes, which is an indication of their possible public health significance and that these resistance genes may have arisen from environmental origins.

However, whole-genome sequencing findings failed to correlate with resistance phenotypes in some cases. *Rhizobium* sequence data showed the presence of *cat* which confers chloramphenicol resistance, but phenotypic data showed resistance to multiple antibiotics. This discrepancy can be explained by the database bias due to the presence of novel and unidentified resistance genes. Also, gene search was based on the most stringent criterion (E value = 0) and gene sequences not showing 100% match may have been left out. On the opposite side, the

identification of antibiotic resistance genes in pan susceptible isolates could be due to the failure of gene expression.

The identification of antibiotic-resistant bacteria from vegetables produced in urban gardens suggests the potential of antibiotic resistance transfer along the food chain.

Finally, urban agricultural environment is a rich source of antibiotic-resistant bacteria and antibiotic resistance genes. Vegetables grown in or on the soil can acquire antibiotic-resistant bacteria from soil and can act as a potential reservoir of antibiotic resistance in the spreading of antibiotic resistance. The mixed observation on the correlation between antibiotic resistance and antibiotics and metals calls for further research on specific soil contaminants at various concentrations. More gardens with possible anthropogenic pollution need to be investigated to establish the relationship between antibiotic resistance and environmental pollutants. The presence of clinically relevant antibiotic resistance genes in environmental bacteria and their potential of transferring antibiotic resistance indicate the necessity to explore this understudied source to better understand the presence and persistence of antibiotic resistance in the environment.

CONCLUSIONS

This study demonstrated that urban agricultural soil and vegetables have a diverse population of antibiotic resistance phenotypes and genotypes. Phenotypic determination of microbial profile and antibiotic resistance combined with high-throughput 16S rRNA sequencing, whole genome sequencing, and metagenomics proved to be a great strategy to study the nature and extent of antibiotic resistance in the environment. The positive correlation between antibiotic resistance genes and metal resistance genes provides indirect evidence of possible co-selection of antibiotic resistance by heavy metals. The data call for further investigation on the impact of alternative selective pressure from non-antibiotics at both phenotypic and molecular levels.

Soil bacteria isolated from urban agricultural environment were able to transfer antibiotic resistance via conjugation, suggesting their great potential of spreading antibiotic resistance in the environment and through the food chain. Efflux pumps may play an important role in conferring multidrug resistance in soil bacteria. The data added substantial information to the environmental database of antibiotic resistance and opened a new venue to understanding the anthropogenic impact on antibiotic resistance in food production environment.

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ABSTRACT

INVESTIGATING ANTIBIOTIC RESISTANCE IN URBAN AGRICULTURAL ENVIRONMENT USING PHENOTYPIC, GENOMIC, AND METAGENOMIC TOOLS

by

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Urban agricultural environment can be an important reservoir of antibiotic resistance and have great food safety and public health indications. This study was to investigate antibiotic-resistant bacteria and antibiotic resistance genes in urban agricultural environment using phenotypic, whole genome sequencing, and metagenomic tools. Three urban community gardens from metro Detroit were studied in two phases.

First phase of this study recovered a total of 207 soil bacteria from 41 soil samples collected from an urban agricultural garden. The most prevalent antibiotic resistance phenotypes demonstrated by Gram-negative bacteria was the resistance to ampicillin (94.2%), followed by chloramphenicol (80.0%), cefoxitin (79.5%), gentamicin (78.4%), and ceftriaxone (71.1%). Gram-positive bacteria were all resistant to gentamicin, kanamycin, and penicillin. Genes encoding resistance to quinolone, β-lactam, and tetracycline were the most prevalent and abundant in the soil. *qepA* and *tetA*, both encoding efflux pumps, predominated in quinolone and tetracycline resistance genes tested, respectively. Positive correlation ($p < 0.05$) was identified among groups of antibiotic resistance genes and between antibiotic resistance genes and metal resistance genes.

Second phase of this study isolated a total of 226 bacteria from 15 soil samples and 45 vegetable samples from all three urban gardens. Multidrug resistance was identified. The percentages of resistant bacteria to some antibiotics (cefoxitin, amoxicillin/clavulanic acid, chloramphenicol, gentamicin, and tetracycline) were higher in vegetables than those in soil. Transfer of tetracycline resistance by conjugation was observed in bacteria of both soil and vegetable origin. Efflux pump genes were common in soil bacteria as identified by whole genome sequencing. For example, *ade*I, *ade*J, *ade*K, *mex*B, *mex*K, and *mex*F were detected in antibiotic-resistant bacteria.

The concentrations of soil contaminants detected in this study were either below what would normally be needed to select antibiotic resistance (antibiotics) or below the EPA recommended level (metals). Although no significant correlation was observed between antibiotic resistance and heavy metals, the positive correlation between antibiotic resistance genes and metal resistance genes at the genomic level still suggests the need to explore the possible co-selection of antibiotic resistance by heavy metals.

The data demonstrated a diverse population of antibiotic resistance phenotypes and genotypes in urban agricultural soil and vegetables. Phenotypic determination together with soil metagenomics can be a valuable tool to study the nature and extent of antibiotic resistance in the environment. Due to the increasing evidence of the public health implication of naturally-occurring antibiotic resistance and the scarcity of environmental data in this regard, establishing ecological breakpoints for environmental antibiotic resistance interpretation and identifying environmental indicators to monitor antibiotic resistance appear to be important research areas to pursue.

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- High Prevalence of Antibiotic Resistance Associated with Urban Agricultural Environment with the Potential of Horizontal Gene Transfer (poster), submitted to International Association for Food Protection (IAFP) Conference, Salt Lake City, UT, 2018.
- Antibiotic Resistance Reservoir in Urban Agricultural Soils (oral), International Association for Food Protection (IAFP) Conference, Tampa, FL, 2017.
- Isolation of Antibiotic-resistant Soil Bacteria from a Detroit Urban Garden (poster), International Association for Food Protection (IAFP) Conference, St. Louis, MO, 2016.
- Prevalence of Antibiotic-resistant Soil Bacteria and Antibiotic Resistance Genes in the Urban Agricultural Environment (oral), Genomics Symposium, Wayne State University, Detroit, MI, 2016.

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