Effect of Soil Amendments from Antibiotic –Treated Cows on Antibiotic Resistant Bacteria & Resistance Genes Recovered from the Surfaces of Lettuce and Radishes: Field Study

By

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SCIENTIFIC ABSTRACT

Cattle are commonly treated with antibiotics that may survive digestion and promote antibiotic resistance when manure or composted manure is used as a soil amendment for crop production. This study was conducted to determine the effects of antibiotic administration and soil amendment practices on microbial diversity and antibiotic resistance of bacteria recovered from the surfaces of lettuce and radishes grown using recommended application rates. Vegetables were planted in field plots amended with raw manure from antibiotic-treated dairy cows, compost from cows with different histories of antibiotic administration, or chemical fertilizer control (12 plots, n=3). Culture-based methods, 16S rDNA amplicon sequencing, qPCR and shot-gun metagenomics were utilized to acquire an overarching view of the bacteria and resistance genes. Culture-based methodologies revealed that bacteria recovered from lettuce grown in biological soil amendments (BSAs) showed tolerance to clindamycin. Manure amendment had a significant effect on microbial community compositions characterized from both radish and lettuce relative to control samples ($p=0.02$). Total *sul1* copies were 160X more abundant on lettuce grown in manure ($p=.002$) and total *tet(W)* copies were 30X more abundant on radishes grown in manure ($p=.002$). Metagenomics revealed that lettuce grown in manure amendment acquired resistance to three more antibiotic classes than lettuce grown under other conditions. This study demonstrates that raw, antibiotic-exposed manure may alter microbiota and the antibiotic resistance genes present on vegetable surfaces. Proper composting of BSAs as

recommended by the U.S. Department of Agriculture and Environmental Protection Agency is recommended to mitigate the spread of resistance to vegetable surfaces.

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PUBLIC ABSTRACT

Antibiotics are drugs responsible for killing infectious diseases in both humans and animals. In cows, antibiotics are frequently used when they get infections in their udders. These drugs can be excreted through manure and urine and end up in the environment. Manure or composted manure is often applied as a soil amendment for crop production. The presence of antibiotics in soil may promote antibiotic resistance, meaning bacteria that carry antibiotic resistance genes (ARGs) are capable of surviving exposure to drugs that would normally kill them. Such bacteria may eventually pass their ARGs to pathogens, which then could no longer be treated effectively by antibiotics when there is an infection. Thus, there is concern that overuse of antibiotics in agriculture can contribute to reduced effectiveness of antibiotics and the growing global antibiotic resistance health crisis. This study sought to determine if prior antibiotic administration affected the antibiotic resistance of bacteria found on the surfaces of vegetables grown in soil amended with manure or compost from dairy cows. Lettuce and radishes were grown in the field in plots amended with raw manure from antibiotic-treated dairy cows, compost from cows with different histories of antibiotic administration, or a chemical fertilizer control. Mature vegetables were harvested and used to enumerate antibiotic-resistant bacterial colonies. Additionally, the 16S rRNA gene, which is a ubiquitous gene found in all bacteria, was sequenced to identify the kinds of microbes that colonized the radish and lettuce surfaces when grown under the different conditions. DNA was extracted from the bacteria collected from the

vegetable surfaces to and different methods were used to identify the kinds of ARGs present and to which kinds of antibiotics they encode resistance. The results of the study indicated that raw, antibiotic-exposed manure may increase the bacteria found on vegetables in addition to their ARGs. Proper composting of manure, as recommended by the U.S. Department of Agriculture (USDA) and the Environmental Protection Agency (EPA), is recommended to mitigate resistance and control microbial populations on fresh vegetables.

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DEDICATION

This work is dedicated to my parents, who have always had faith in my abilities even when I did not. Thank you for giving me the confidence and support to finish this degree. I love you both, endlessly.

ATTRIBUTION

Multiple contributions were made to this research from the following:

Monica A. Ponder, PhD, (Food Science and Technology Department at Virginia Tech) is currently an Associate professor and served as principal investigator of this project. Dr. Ponder assisted in the experimental design, data analysis, funding, and direction of this research project. Dr. Podner is a co-author on the manuscript in Chapter 3 for this reason.

Amy Pruden, PhD, (Civil and Environmental Engineering Department at Virginia Tech) is currently the W. Thomas Rice Professor in the Via department of Civil and Environmental Engineering, as well as the associate dean for Interdisciplinary Graduate Education. Dr. Pruden is the project director for the USDA grant 2014-05280; this grant provided a large majority of funding for the work presented in this thesis.

Giselle Guron, PhD, (Food Science and Technology Department, Civil and Environmental Department at Virginia Tech) is currently a post-doctoral fellow under Dr. Ponder and Dr. Pruden. Dr. Guron provided a large amount of technical assistance and knowledge for the molecular work completed throughout this project.

Laura Strawn, PhD, (Food Science and Technology at Virginia Tech) is currently an associate professor and extension agent on the Eastern Shore AREC. Dr. Strawn has provided guidance on experimental design and served on the advisory committee.

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LIST OF ABBREVIATIONS

ANOSIM: Analysis of Similarities

ARB: Antibiotic Resistant Bacteria

ARG: Antibiotic Resistance Gene

CDC: Centers for Disease Control and Prevention

CFU: Colony Forming Unit

Compost AB: Soils amended with composted manure of antibiotic treated cattle

Compost No AB: Soils amended with composted manure of cattle without antibiotic exposure

DNA: Deoxyribonucleic Acid

FDA: Food and Drug Administration

FSMA: Food Safety Modernization Act

HGT: Horizontal Gene Transfer

MDS: Multidimensional Scaling

MPN: Most Probable Number(s)

MRSA: Multidrug Resistant Staphylococcus Aureus

NOP: National Organic Program

OTU: Operational Taxonomic Unit(s)

qPCR: Quantitative Polymerase Chain Reaction

USDA: United States Department of Agriculture

WHO: World Health Organization

CHAPTER 1: INTRODUCTION AND JUSTIFICATION

Introduction

The World Health Organization has declared that the upsurge of antibiotic resistant bacterial infections is one of the greatest public health crises of our time (1). Bacteria that are resistant to antibiotics infect an estimated 2 million people per year in the United States, resulting in at least 23,000 deaths every year (2). Spread of antibiotic resistant bacteria may happen within healthcare settings, but also through agricultural practices. Animals that are treated with antibiotics may develop resistance in their guts, these bacteria may then be spread through the environment through feces. Drug resistant bacteria can then spread to humans through ingestion of meat from animals or uncooked fruits and vegetables.

Antibiotics administered to livestock have the capability to prevent disease, treat infection and promote growth (3). The trifecta of advantages resulting from antibiotic usage keeps livestock healthy subsequently increasing yields and revenue for farmers. The United States Food and Drug Administration (FDA) reported that around 15.58 million kilograms of antimicrobials were sold for use in food producing animals in the United States in 2015 (4). The rise in agricultural antibiotic usage has coincided with the emergence and prevalence of antibiotic resistant infections.

Livestock that have been administered antibiotics can excrete anywhere from 40-90% of some parent antibiotic compounds in their manure (5,6). Manures are routinely used as biological soil amendments (BSAs) to enhance soil fertility. The application to soil, or run-off to soil that is used to grow vegetable crops creates the potential for transfer to humans. Raw, animal based manures contain a multitude of human pathogens that have been identified as causative agents in foodborne illnesses and outbreaks (7,8). Minimal data is available on the spread of

ARB and ARGs to vegetable surfaces through this very same contact. It is also uncertain if composting raw manure reduces the transfer of ARB and ARGs to the surfaces of vegetables. Anaerobically composted manure that has reached thermophilic temperature (>131˚F) can experience a 10-100-fold reduction in some AGRs (*tet*), while experiencing increases in others (*sul*) (9). The consumption of raw vegetables that have been grown in BSAs could pose as a route in which antibiotic resistance directly enters the farm-to-fork continuum. Fruits and vegetables ranging from leafy greens to carrots, have been known to harbor a multitude of ARGs (10,11). However, no real conclusions have been drawn to determine if certain soil amendments result in higher levels of ARB and ARGs on the surfaces of fresh vegetables commonly consumed by humans.

The Food Safety and Modernization Act's Produce Safety Rule guidelines help to prevent pathogens from entering the food chain. Farmers must treat "biological soil amendments of animal origin with scientifically valid, controlled, physical and/or chemical processes or composting processes that meet or exceed specific microbial standards" (12). Farmers must reach the proper application requirements and minimum application intervals for untreated and treated BSAs of animal origin in order to reduce the risk of produce contamination with human pathogens. It is uncertain if these new guidelines will affect the incidence of ARB/ARGs in the manure of animals administered antibiotics. Further research is needed to determine if biological soil amendments from antibiotic administered animals are adding to the dissemination of antibiotic resistance in the human food chain. The project we have designed is novel in that the antibiotic history of the cattle being utilized for manure production is known; cows were administered therapeutic doses of antibiotics and manure collection occurred during peak excretion of the antibiotic or its metabolites as part of the experimental design. This project is a

subset of a larger initiative evaluating interactions between manure-based soil amendments and antibiotic resistance in the farm-to-fork continuum (13,14).

Objectives and Hypotheses

1. Compare the numbers of bacteria capable of growth on antibiotic-amended R2A media recovered from the edible surfaces of field-grown lettuce and radishes grown in soils amended with raw manure, composted manure from cows provided therapeutic antibiotics, and antibiotic-free compost:

    $H1_0$: The log CFU/g of aerobic bacteria recovered from the edible surface of the lettuce and radishes grown in field conditions will not be significantly different when grown in soils containing biological soil amendments and the chemical fertilizer control.

    $H1_a$: Amendment type (chemical fertilizer control, raw manure, composted manure from cows provided therapeutic antibiotics, compost without antibiotics) will be associated with a significant difference in the total aerobic bacteria (log CFU/g) found on the edible surface of the lettuce and radishes grown in soil with the selected amendments.

    $H2_0$: Aerobic bacteria enumerated from the edible surface of lettuce and radishes will be more abundant on the control R2A plates (log CFU/g) in comparison to the antibiotic amended plates, implying a number of plant- associated bacteria lack resistance to the antibiotic amendment.

    $H2_a$: Aerobic bacteria recovered from the edible surface of lettuce and radishes grown in soils amended with manure and composted manure from cows administered therapeutic antibiotics will show more abundant growth (log CFU/g)

on plates impregnated with ceftazidime (3rd generation cephalosporin) and clindamycin (lincosamide), implying more resistance to these antibiotics in comparison to the other in four in question.

2. Compare the classes of putative ARGs recovered from the vegetable surfaces grown in different soil amendments detected via shotgun metagenomics DNA sequencing. A more sensitive analysis using qPCR will be used to compare relative abundance of the antibiotic resistance genes, *tet(w)* and *sul1*, isolated from the edible surfaces of lettuce and radishes grown in soils amended with the different amendments defined above.

   $H1_0$: The number of *tet(w)* and *sul1* gene copy numbers recovered from the edible surface of lettuce and radishes will not significantly differ in number between amendment types.

   $H1_a$: The number of *tet(w)* and *sul1* gene copy numbers recovered from the edible surface of lettuce and radishes will significantly differ in number when grown in soils amended with different animal inputs. *tet(w)* and *sul1* gene copy numbers will be greatest from plants grown in manure or compost generated by therapeutically dosed cows.

   $H2_0$: The total number and relative abundance of ARGs identified by the shotgun DNA metagenomic sequencing annotated by the Comprehensive Antibiotic Resistance Database (CARD) from the surfaces of lettuce and radish plants will not significantly differ between amendment types.

   $H2_a$: The total number and relative abundance of glycopeptide, macrolide and beta-lactam resistance genes, which are antibiotic classes deemed critically

important by the WHO (15), identified by shotgun metagenomics coupled with the Comprehensive Antibiotic Resistance Database (CARD) will be significantly larger from the surfaces of lettuce and radish plants grown in raw manure or compost generated by therapeutically dosed cows.

3. Characterize the microbial communities of the edible surfaces of lettuce and radishes grown in various soil amendments using Illumina MiSeq sequencing of the 16S rDNA amplicons.

H_0: There will be no significant difference in the diversity (composition, evenness, and richness) of the 16S rDNA amplicons obtained from the surface of the vegetables grown in soil with the different amendments (chemical fertilizer, raw manure, composted manure from cows provided therapeutic antibiotics, compost without antibiotics).

H_1= Amendment of the soil with raw manure and compost will result in significant changes to the diversity of the bacterial 16S rDNA amplicons obtained from the edible surfaces of vegetables compared to the chemical fertilizer control. Additionally, we hypothesize increases in richness and a decrease in evenness compared to vegetables grown only using chemical fertilizers.

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CHAPTER 2: LITERATURE REVIEW

1. Food Safety Modernization Act: Produce Safety Rule

The Food Safety Modernization Act (FSMA), passed by Congress and signed by President Barack Obama in 2011, is a crucial piece of legislation that will shift the food industry's overall safety approach from a reactive to proactive standpoint. Implementation of these new preventative techniques will be particularly challenging for the produce industry which aims to provide safe, fresh produce all the way from the farm to fork. A series of mandatory produce safety standards, known as the Produce Safety Rule, have been put into effect by the FSMA protocols and are enforced by the FDA. The majority of the Produce Safety Rule focuses on eliminating routes in which pathogenic microorganisms can survive on fresh produce, ultimately preventing the occurrence of a foodborne illness outbreak. The CDC estimates that 9.4 million Americans contract a foodborne illness annually; 55,961 of these individuals are hospitalized and an additional 1,351 Americans die (1). Raw, farm-produced fruits and vegetables ranging from lettuce to melons have been attributed to 46% of foodborne illness outbreaks of known origin, between 1998-2008 (2). Leafy greens, including various lettuce species, have caused more attributed foodborne illness (22%) between 1998-2008 than any other food product (2). Lettuce is an American household staple; it is the third most consumed fresh vegetable in the United States, with 14.2 pounds being consumed per capita in 2014 (3). Though not indigenous to North America, lettuce has become the nation's leading vegetable crop in terms of value and revenue (3). Lettuce is beneficial both nutritionally and economically yet it holds constant potential for causing consumer health related hazards. Lettuce which is commonly consumed raw, can encounter many bacteria including human pathogens like *Salmonella enterica* and *Escherichia coli* (4). Foodborne illness associated with produce usually

occurs do to surface contamination from fecal matter of animal or human origin via direct contact or irrigation water (4). The Produce Safety Rule will help reduce the likelihood of a foodborne illness outbreak providing Americans with safer food products.

Increases in produce regulations may correlate to decreases in outbreak incidences stabilizing the U.S. agricultural economy. The U.S. Department of Agriculture Economic Research Services (USDA-ERS) last estimated in 2014 that foodborne illnesses are annually costing the United States economy more than $15.6 billion due to productivity loss and medical costs (5). Foodborne outbreaks are particularly detrimental for the fruit and vegetable industries which rely on seasonal sales based off harvest schedules. U.S. farmers lost $12 million in spinach sales in 2006 after a fatal *Escherichia coli* O157: H7 outbreak occurred, ultimately scaring consumers away from purchasing packaged spinach (6). A Salmonellosis outbreak thought to have originated in tomato plants in 2008 caused U.S. tomato sales to drop by $25 million; the outbreak was later traced back to jalapeno peppers added to tomato based products, proving the power of consumer perception on the economics of the produce industry (6). The detailed guidelines set in place by FSMA will help lessen the chance of deadly, economically adverse outbreaks for complying farms. Farmers will reduce risk by considering naturally occurring hazards, as well as those that may be introduced either unintentionally or intentionally from substances like soil amendments (7).

2. Soil Amendments: Vehicles for Produce Contamination

Soil amendments, which are defined as "a material incorporated into the soil that improves physical characteristics" include organic substances like manure (7). Data collected in 2013 by the USDA National Agricultural Statistics Service (NASS) determined that 4,438 U.S.

produce farms covered by the FSMA Produce Safety Rule reported using biological soil amendments (BSAs) of animal origin on their fields; 821 of these farms use raw manure correlating to 70,134 acres of land (8). The produce safety risks associated with BSAs of animal origin are quite high. *E. coli* O157:H7, a pathogenic microorganism naturally harbored in cattle, has been found to survive in compost anywhere from 77-231 days in manure-amended soil held at temperatures ranging from 5-21°C (9). BSAs of animal origin must be treated with a thermal, chemical, or biological process so that the pathogenic organisms present in the organic substance are reduced. Pathogenic microorganisms of concern highlighted in the Produce Safety Rule include *Salmonella spp., Listeria monocytogenes*, and *Escherichia coli* O157:H7 (Table 1). *E. coli* O157:H7, for example, must be undetectable at 0.3 most probable number (MPN) per gram after processing. The Produce Safety Alliance is pursuing further research to identify safe application rates for raw manures on produce fields. The use of the USDA's National Organic Program (NOP) guidelines have been suggested as the standard for BSAs until a more proven technique can be determined.

The NOP requires all farmers utilizing raw BSAs to lessen the likelihood of pathogenic contamination by requiring raw manure to sit on soil ≥ 120 days pre-harvest of produce that has direct contact with soil surfaces (10). Radishes harvested from soils amended with manure would require a 120 period from manure application until harvest because the edible taproot touches the soil amendment (10). Produce that does not have contact with soil surfaces have a reduced waiting period of ≥ 90 days pre-harvest (10). Sweet corn harvested from soils amended with manure would require a 90-day period from time of manure application until harvest because the edible portion does not encounter amendment. NOP manure guidelines do not guarantee pathogen free produce as many field studies have found culturable amounts of *E. coli* on produce

grown in the properly treated soil amendments (11, 12). Risks are greatest for root vegetables like radishes and for leafy vegetables such as lettuce where the edible portion of the plant touches the soil (13). FSMA contains acceptable validated processes for biological soil amendments of animal origin that will be applied to produce plots. One treatment known as static composting requires the biological substance to be held in aerobic conditions at a minimum of 131° F for 3 consecutive days (14). These parameters, when performed properly, result in a reduction of pathogenic organisms making a less hazardous substance known as compost (Table 2). Farmers are highly encouraged to use composted manure instead of the raw product to prevent pathogenic contamination.

Table 1: Microbial Standards That Must be Met for Biological Soil Amendments Used to Grow Fresh Produce

Pathogenic Microorganism of Concern	**Limit of Detection in Biological Soil Amendment**
Salmonella spp.	Undetectable per 4 grams (or mL) of sample using methods capable of detecting 3 most probably numbers (MPN)
Listeria monocytogenes	Undetectable (CFU) per 5 grams (or mL) of sample using methods capable of detecting 1 CFU
Escherichia coli **O157:H7**	Undetectable per 1 gram (or mL) of sample using methods capable of detecting .3 MPN

Table 2: USDA and FSMA Approved Composting Practices for Biological Soil Amendments of Animal Origin for Produce Growing

Soil Amendment	**Processing Practice**	**Application**
Manure	Time	-Manure application ≥ 120 days preharvest of produce with soil contact -Manure application ≥ 90 days preharvest of sweet corns
Static Compost	Amendment must reach minimum of 131˚ F for 3 consecutive days in aerobic conditions, followed by curing process	Can be applied to any type of cropland once parameters are met
Turned Compost	Amendment must reach minimum of 131˚F for 15 days (nonconsecutive) paired with ≥ 5 turnings in aerobic conditions	Can be applied to any type of cropland once parameters are met

Compost is organic matter that has been decomposed by microorganisms which breakdown plant and animal materials into more available forms of nutrients for plant growth (13,16). Proper composting can destroy pathogenic microorganisms reducing the numbers of human pathogens in compost in comparison to raw manure (16). Several guidelines are recommended for composting manure and vary depending on animal source and amount of manure. One major route of contamination in fruit and vegetable production is the application of improperly processed compost (17,18). Studies conducted by the University of Georgia found that *E. coli* O157:H7 was being transferred to leaf lettuce and parsley grown in fields amended with contaminated composted manure for up to 177 days after amendment application (19). Proper composting is crucial for this reason.

The transfer and survival of pathogenic organisms is an acknowledged risk when growing produce in soil amendments. The FSMA Produce Safety Rule addresses prevention of foodborne illness via contamination by soil amendments but neglects to do address the safety hazards associated with the antibiotic residues found within the biological soil amendments. Antibiotics commonly administered to livestock are poorly absorbed in the gut and can survive the digestion process. Water-soluble antibiotics, like chlortetracycline, are capable of surviving metabolism; 90% of one dose can be found in livestock urine with up to 75% of a dose being found in the feces (20,21). The excretions (manure) are then used to grow various crops and produce commodities on some agricultural sites. The antibiotics residuals excreted in the manure of animals administered drugs can diffuse into the soil and increase the selection pressure on bacteria found naturally in the soil, creating antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARG).

The entry of ARB into soil and water via manure application yields a potentially significant reservoir of antibiotic resistant genes however the mechanisms by which these organisms and genes transfer throughout the environment is relatively unknown (22,23). Many studies have determined that animals are an important source of bacterial contamination of fresh produce however few studies have sought to document an association between environmental practices of animal wastes and ARB and ARGs associated with fresh produce (22,24). The alterations associated with the environmental transfer of resistance from soil amendments to vegetable surfaces remains to be a knowledge gap in antibiotic research (25). The consumption of raw produce could be one of the direct points through which antibiotic resistant bacteria and genes enter the human food chain. The project we have designed will help uncover if the newly

instated FSMA Produce Safety Rule affects the spread of antibiotic resistance in the farm to fork continuum.

3. Antibiotic Resistance Crisis

Antibiotics are substances that inhibit the growth of or kill bacteria, treating, and preventing infection in humans and animals. Antibiotics can be made from organically derived chemicals, synthetic materials, or from substances of microbial origin (26). Various fungi and bacteria produce low doses of antibiotic compounds to outcompete other organisms within an environment (27). The mechanisms behind antibiotics are naturally occurring biosynthetic processes that have been utilized by modern medicine. Dr. Alexander Fleming pioneered the first modern antibiotic discovery when he uncovered the biocidal nature of penicillin in 1982 (28). The breakthrough made by Fleming sparked the industrialized production of antibiotics and is identified as the dawn of the antibiotic era. The antibiotic era has helped extend the lifespan of U.S. citizens and decreased morbidity and mortality in developing countries from foodborne illnesses, common infections, and poverty-linked diseases (29,30). The medically beneficial period experienced for the past 90 years is ending abruptly due to the rapid adaptability of bacteria.

Antibiotic resistance has been deemed an ancient biological process with origins dating back 500 million years (31,32). However, the selective pressures induced by industrialized antibiotics have forced microorganisms to hastily evolve to surrounding environments at an unnatural rate. The overuse of antibiotics in a plethora of industries, agricultural included, can be noted as the "single most important factor leading to antibiotic resistance around the world" (33). Every year at least 2 million US citizens acquire serious infections with bacteria that are resistant to one or more of the antibiotics designed to treat those infections. An additional 23,000 Americans die

each year as a direct result of these antibiotic-resistant infections (1). Antibiotic resistant bacterial infections have cost the US health system an estimated $21 - $34 billion dollars, yearly (1). The average antibiotic takes 10-12 years to develop; it does not take long for resistant organisms to be identified once a new drug is made available to the public (33). In fact, the world is running out of antibiotics; "between 1940 and 1962, more than 20 new classes of antibiotics were marketed. Since then, only two new classes have reached the market" (34). The amount of research, time and money that goes into the identification of new antibiotics does not outcompete the rate in which bacteria acquire resistance therefore we are facing a new era in which modern medicine may not work in times of need.

The introduction of new antibiotic compounds to the market place has significantly decreased from 20–30 new drugs per decade to 3–4 newly marketed drugs per decade (35). Biological pressure imposed by the continuous exposure to different antibiotics during clinical and agricultural applications has led to the cumulative acquisition of resistant traits in bacteria across the environment. The WHO has declared the following last resort antibiotic classes critically important for human health; 3rd and 4th generation cephalosporins, glycopeptides, macrolides, and fluoroquinolones (36). These critically important drug classes should only be utilized by those who are not receptive to any lesser treatments including lincosamides, sulfonamides and tetracyclines. The Food and Drug Administration (FDA) has also created a list of drugs that have been deemed critically important in regard to human medicine in their guidance for industry report (Table 3) (37). The FDA and WHO have created these lists so that the use of critically important drugs can be controlled; however, the U.S. agricultural industry still uses some of these drugs for animal production.

Table 3: FDA Critically Important Classes of Antibiotics, Their Usage, and Resistance Concerns

Antibiotic Class	Usage	Resistance
3rd Generation Cephalosporins (Cefotaxime, Ceftazidime, Cefdinir, etc.)	• Treats enteric pathogens responsible for foodborne and non-foodborne disease • Last resort therapy for serious diseases	Increased resistance in gram-negative bacteria largely due to cross-resistance brought on by over use of β-lactamases
Fluoroquinolones (Norfloxacin, Ofloxacin, Moxifloxacin etc.)	• Treats enteric pathogens responsible for foodborne and non-foodborne disease • Last resort therapy for serious diseases	Chromosomal mutations have resulted in resistance increases
Macrolides (Erythromycin, Azithromycin Clarithromycin)	• Treats enteric pathogens responsible for foodborne disease • Last resort therapy for serious diseases	Increased resistance in gram-positive bacteria due to mutations in efflux pump and target-sites
Sulfamethoxazole - Trimethoprim	• Treats enteric pathogens responsible for foodborne and non-foodborne disease	Recently linked with high levels of treatment failure in clinical settings

4. Antibiotic Usage in Agriculture

In 1949, Thomas Juke discovered that small doses of antibiotics increased the growth rate and health of ruminants; antibiotics have been utilized in agriculture ever since (38). Conservative estimates have concluded that worldwide, 63,151 tons of antibiotics were used for livestock production in 2010; China, Brazil, Germany, India, and the United States are currently the largest antibiotic consumers, agriculturally speaking, using half of the global total (39). The FDA estimated that in 2015, 15.58 million kilograms of antimicrobials were approved for sale and distribution for food-producing animals in the United States; 62% of these antimicrobials were considered medically important (40). The copious amount of antibiotics utilized in the U.S.

are either physically administered to animals or dispended into their water and feed for a variety of reasons. Whole herds of animals are often administered antibiotics at therapeutic levels when one member of the group displays clinical signs of disease. Antibiotics can also be administered before signs of disease even occur (subtherapeutic, prophylactic dosing) or to enhance growth rates and feed efficiency of a herd (41).

Antibiotic administration increases production rates of livestock and their products, like milk, in a multitude of ways. Biochemical processes like nitrogen excretion, phosphorylation, and protein synthesis are expedited causing an increase in mass within an animal (42). Nutrient absorption can be improved in animals exposed to antibiotics; microorganisms competing for the same nutrients as the host are eliminated increasing the efficiency of the delivered feed, causing weight gain in the animal (43). Antibiotics have also been known to alter hepatic gene expression, modifying the metabolism which causes weight gain (44). Expedited weight gain is advantageous in the agricultural industry so that it takes less time to produce an animal of appropriate size for sale. The feed conversion ratio or the efficiency of livestock to convert animal feed into the desired output (mass) is an important economic factor for producers. Animals administered antibiotics have a lower feed conversion ratio making the overall economic net gain higher. Weight gain via antibiotic administration is often coupled with improved animal health creating an additional beneficial factor for farmers

The health and quality of life of livestock determine the quality of the products they produce. Dairy cows are often administered prophylactic doses of antibiotics, including drugs from the classes lincosamide, cephalosporin, sulfonamide, to prevent disease from occurring. Lactating dairy cows enter a dry period in which they stop producing milk. Infection of the udder, known as mastitis, is seven times more likely to occur during this dry period in

comparison to the lactation period (45). Dry cow therapy is a common practice used to prevent mastitis. Single intrammamary doses of cephapirin, a first-generation cephalosporin, is administered over the dry period, which can last anywhere from 4 to10 weeks (46). It is estimated that 9 of 10 dairy cow operations in the United States practice intramammary dosing to prevent mastitis (47). Cows that have been infected with mastitis, which is caused by Gram-positive bacteria, are administered pirlimycin, a lincosamide, two times over a 24-hour period (48). It is estimated that 16% of lactating dairy cows in the U.S. receive antibiotic treatment for clinical mastitis (47). The constant administration of antibiotics to prevent and control disease allowing for a more profitable, healthy animal (49). The clear benefits of agricultural antibiotics are outweighed by heightened instances of antibiotic resistant infections in animals and humans.

5. Antibiotic Resistance in Humans and Agriculture

Multiple epidemiological investigations have proven that the extensive use of antimicrobial agents utilized in agriculture have led to the emergence and dissemination of resistant strains of human pathogens, worldwide (50-52). Cephalosporins are utilized in both clinical and veterinary settings and can be found on both the WHO and FDA critically important list. Heightened instances of resistance have been identified due to the saturation of the drugs in different parts of the environment. Cephalosporin resistant pathogens have been identified from the milk of German dairy cows commonly administered drugs for mastitis (53). Instances of ARB in animals and their byproducts are of concern; these aggressive strains can potentially be acquired humans. One such instance occurred in Nebraska, where a young boy acquired an extended-spectrum cephalosporin resistant *Salmonellosis* infection (54). The resistant strain was traced back to a specific herd of cattle located on a ranch near the young boy's house (54). Cephalosporins are commonly used for curing disease in humans and animals; cattle, swine and

poultry industries can legally administer 4th generation cephalosporins to animals while similar cephalosporins are also used to fight serious cases of surgical infections in humans (55). The crossover between clinical medicine and agricultural usage becomes deadly for those who acquire resistant infections.

Sulfamethoxazole, which is listed on the FDAs critically important list, is a sulfonamide that when coupled with trimethoprim is used to treat a wide range of ailments including urinary tract infections and ear infections in humans (56). Sulfonamides inhibit the growth of many Gram-negative and positive bacteria and is one of the most widely utilized veterinary antimicrobials used in industrialized countries (57). The sulfonamide resistance gene is generally carried via plasmid; resistance "is currently a problem of considerable clinical importance, especially in urinary tract infections. Twenty-five to 80% of E. coli strains found in these infections are normally resistant to 100-1000 times the normal minimum inhibitory concentration of sulfonamide-sensitive strains" (58). Resistance to sulfonamides have increased in human and animal E. coli isolates between 1950 and 2002 (59). Sulfonamides are still used in U.S. cattle, swine, and poultry facilities despite widespread resistance. Erythromycin, a macrolide, has had increasing levels of resistance is *Streptococcus* species in both North America and Europe (60). Macrolides are also used in the same agricultural settings as sulfonamides and cephalosporins and are commonly used to treat gram-positive infections in humans (61). Overuse of macrolides, in addition to the various antibiotics classes previously mentioned, are leading to the worldwide increases in resistance being seen today (62). The selection pressure implemented by antibiotic use in livestock creates a reservoir of bacteria harboring antibiotic resistance genes; these genes help to defend against antibiotics attempting to destroy a cell.

6. Antibiotic Resistance: Genes

The emergence of antibiotic resistance within a cell can be attributed to multiple genetic processes, the two most prominent being natural mutations and gene transfer (63). Mutations are spontaneous changes in genetic material. Mutations in genetic elements can lead to the procurement of antibiotic resistance; these mechanisms do not always result in resistance therefore they are not classified as resistance genes. The mutation of three particular gene types leads to resistance; elements that encode an antibiotic target, transporter mechanisms and the regulator genes controlling the expression of those transporter elements (64,65). Mutations causing increased efflux pump activity, misshapen antibiotic binding sites and alterations in antibiotic compounds attempting to attack the cell via enzyme formation are all forms of mutation that lead to resistance in a cell (65). Resistance-based genetic mutations have been selected for at an expedited rate because of the antibiotic era bacteria are enveloped in today.

ARGs consist of encoded DNA which allow bacteria to withstand antibiotic exposure. The transfer of ARGs, and genetic material in general, between bacteria can occur in many forms. Vertical gene-transfer occurs when genetic material is transferred from parent cell to its offspring; resistance genes are capable of being transferred in this manner. Horizontal Gene-Transfer (HGT) is of greater concern in regard to resistance. Antibiotic resistance genes have been located on transposons, integrons and plasmids which can be horizontally transferred to other bacteria of the same or dissimilar species (66). Bacteria transfer genes horizontally via transformation, transduction, or conjugation.

Transformation allows for the uptake of both chromosomal and plasmid mediated DNA floating free in the environment. The mechanisms behind transformable bacterial strains is not entirely understood but appears to be a key factor in the transfer of antibiotic resistant genes. For

instance, a non-resistant cell could pick up the genetic material of a lysed, resistant bacterial cell in the environment, gaining resistance in the process (66). Transduction occurs when a bacteriophage containing a portion of donor genome transfers the genes to a recipient cell. Nearly any DNA sequence, including antibiotic resistance genes found in a bacterial genome, can be transferred via bacteriophage (67). Conjugation, the final mechanism responsible for HGT, requires cell to cell contact in order to transfer genetic material. “Conjugation is thought to have had the greatest influence on the dissemination of antibiotic resistance genes” however recent research has revealed that transduction and transformation might contribute to the spread of resistance in a higher magnitude than originally estimated (67).

The various genetic elements responsible for antibiotic resistance, including unexpressed resistance genes, are comprised into a category known as the resistome (69). The resistome in its entirely is capable of quickly adapting to antibiotic agents in both clinical and environmental settings (70). The environmental resistome is of great interest and consists of “hot spots” were HGT is most likely to occur. The plant rhizosphere, an area in which the plant root system is associated with soil microorganisms, manure lagoons and agricultural ponds are common hot spots for many reasons (**71**). The rhizosphere is a natural region in which a multitude of varying bacterial communities are placed in close proximity, facilitating HGT (71). Manure lagoons and agricultural ponds are manmade hot spots that are constantly being exposed to antimicrobial substances that are used on agricultural lands. Human activities have increased resistance pressure on the microbes residing in these lagoons resulting in a reservoir of ARGs that would not normally exist. The genetic reservoirs can be unintentionally spread across the farm to fork continuum by farming practices like manure application. Antibiotic resistance gene levels have been known to increase in soil samples following manure application (72). Cattle manure has

also been known to harbor antibiotic resistance genes including tet(O), tet(Q), tet(W), tet(M), tet(B) and tet(L) and sul(1) (73,74). The application of manure and other biological soil amendments to crop land facilitates the spread of antibiotic resistant bacteria and genes in the environment. However, the understanding of the dissemination of these antibiotic resistance determinants via animal feces remains limited (38).

7. Antibiotic Resistance in Soil Amendments

The selective pressures applied to the environment by soil amendments like manure is vast and not entirely understood. Healthy dairy cattle generate an estimated 80 lbs of manure per day on a 1,000 lb animal unit basis (75). Dairy cow manure is commonly used in commercial crop production however its impact on the resistome of plants and soil has not largely been investigated (76). Large countries like China have been thought to produce roughly 1900 million tons of livestock manure on an annual basis (77). Such a vast amount of agricultural byproduct becomes hard to manage; utilization of manures, from sources like dairy cattle, as soil amendments provides a practical economic option for farmers who have excess amounts of waste on premises. The various amendments created from manure improve soil fertility by increasing beneficial microorganisms in the soil; farmers also benefit through the reallocation of farm resources (78). The increase in microorganisms also causes an increase in resistance genes. As previously discussed, a large portion of antibiotics are excreted by the animals they are administered to creating a large amount of selection pressure on microbes. Antibiotic resistance genes are selected for in the animal gut and further selected for in the environment through antibiotic-infused excretions. Antibiotic resistant bacteria and genes are thought to disseminate through manure application, promoting horizontal transfer of genes to soil microbiota and surrounding environment (79). The dissemination theory has been proven as raw manure

application has been shown to increase the frequency of detection, and levels of antibiotic resistance genes and mobile genetic elements in soils (80-84). Manure amendments have been shown to cause a bloom in resistance genes regardless of prior antibiotic exposure (83) while other studies found 5-fold increases in resistance genes correlated to prior antibiotic exposure (84). Genes can persist in the environment for a long-time period especially in farms were manure is constantly reapplied. Long-term applications of manure have been shown to increase the abundance of ARGs in agricultural sites and create long-term selective pressures on manure amended soils (80). In fact, manure has been shown to enrich for taxa that more commonly carry resistance genes (83). Composting, which alters taxa and reduces antibiotic residues, has been suggested to manage the release of antibiotics and antibiotic resistance genes throughout the environment (85).

The composting of manure destroys pathogenic bacteria within the soil amendment while eliminating on average 50-70% of some antibiotics compounds within the matrix (86,87). Antibiotic resistance genes, including those coding for resistance to ampicillin, tetracycline, sulfonamides and erythromycin, have been shown to decrease through the composting process; some genes become undetectable after proper composting (88-92). Improper composting in which the proper temperature (55˚C) had not been reached has shown to significantly decrease resistance genes compared to raw manure (88) suggesting that attempted composting is still more effective at reducing the spread of resistance compared to the use of raw manure. Contrastingly, some evidence suggests that aerobic composting increases abundance of antibiotic resistance genes, including those coding for resistance to tetracycline and sulfonamides (93). Increases in resistance genes were attributed to the consequent changes in bacterial communities brought on by aerobic composting. Bacteria are both selected for and destroyed composting processes;

however, those that are killed are still capable of transformation in which lysed antibiotic resistance genes can assimilated into the genome of live bacterial strains proliferating in the compost (85). Increases in resistance genes could be explained by this phenomenon. Evidence indicates that composting has the ability to reduce antibiotic resistance genes and bacteria from manure, however the type of composting and the execution of the composting process greatly affect the amount in which reduction occurs. The variable levels of reduction could explain why antibiotic resistant bacteria and genes have been detected on produce grown in soils amended with raw manure and compost.

8. Antibiotic Resistance and Fresh Produce

Antibiotic resistant bacteria and genes have been isolated from the surfaces of fruits and vegetables; consumption of raw produce has the potential to be a route in which antibiotic resistance can enter the human food chain. Unprocessed vegetables from the farm have been shown to have more quantifiable ARB than vegetables from the market place (94). However, retail market produce samples, like lettuce, tomatoes and carrots, have been found to have quantifiable amounts of ARB and ARGs present on their surfaces (94-99). Studies conducted by Hassan et all found that 76.5% of produce samples collected from Saudi Arabian markets contained bacteria with phenotypic resistance to at least one of fourteen antibiotics being selected for (96). Multi drug resistant coliforms were isolated from the surfaces of commercially processed sprouts in Germany; antibiotics are not commonly utilized in vegetable production in Germany, therefore resistance was determined to be acquired from an animal or human source (97). Studies conducted in the UK found that 70% of *Enterobacterial* strains identified from carrot surfaces were resistant to ampicillin and first and second generation cephalosporins (99). Prewashed salad mixes purchased in the same study had comparable levels of antibiotic resistant

bacteria with the addition of Beta-lactamase resistance in ten of the identified *Enterobacter* strains (99). Studies performed at North Carolina State found that *E. faecium* strains isolated from leafy greens, melons and herbs had notable resistance against erythromycin (22%), quinupristin/dalfopristin (28%), tetracycline (24%), and ciprofloxacin (28%), all of which are important in human medicine (100). U.S. and Mexican produce were found to have quantifiable levels of ARB in this experiment, further proving that antibiotic resistance is a global phenomenon capable of being spread around the world through exchanges of produce across borders (101). Antibiotic resistant bacteria, like the species discovered on market ready and farm grown produce, can ultimately transfer resistance genes via HGT to bacteria present within the human microbiome. The potential for gene transfer creates opportunities for antibiotic resistance to proliferate in the human microbiome, increasing the risk of antibiotic failure. Uncovering ways to inhibit the transfer of antibiotic resistance

Better understanding the critical controls points at which resistance can be hindered on produce surfaces starts at the farm level. The amendment in which produce grows can determine the genes that reach consumer markets. *Brassica* grown in struvite amended fields had significantly higher levels of mobile genetic elements and antibiotic resistance genes due to struvite application (80). Similar increases in resistance genes were detected in vegetables (lettuce, radishes, carrots) grown in sewage sludge (106). Organic produce, which must grow in biological soil amendments compared to conventional farms which may use artificial fertilizers, are an opportune point of study for ARG analysis. Organic and conventionally grown lettuce purchased from Chinese markets were observed to have 134 ARGs, total (80). Organic lettuce samples contained ARGs at an 8-fold higher than conventionally grown lettuce samples (80). Contrastingly, a multitude of field studies have shown that animal-based soil amendments do not

alter the levels of resistant bacteria and genes quantified from vegetable surfaces (92,104-106). The robust nature of soil microbiota have been shown to dominate the bacteria comprising the phyllosphere and rhizosphere of vegetables grown in fertilizer (107). Geographic location, climate and soil type have been shown to drive the microbiota associated with fruit and vegetable surfaces more so than soil amendments (104,107). Differences in ARB and ARGs enumerated from vegetables surfaces grown in BSAs must be studied further to determine if produce is a route in which resistance is disseminating throughout the farm-to-fork continuum.

9. Antibiotic Resistance in the Farm to Fork Continuum

The farm to fork continuum is an umbrella term used to define the pathway a food product takes in order to end up on a consumers' table. The farm to fork pathway includes every practice that takes place starting from the farm a food is grown on to the processing it receives before being sold in market. Significant evidence has linked agricultural practices that occur in the farm to fork continuum to antibiotic resistant infections in humans. However, fundamental understanding of the pathways in which antibiotic resistant bacteria and their genes transfer throughout the food chain is quite limited. ARB and ARGs are commonly identified in soil amendments and on produce samples. Further research must be conducted so that mitigation strategies can be developed to prevent agriculturally-borne antibiotic resistance from entering the human food chain. Manure and soil amendment management is a critical control point within the farm to fork continuum that could hold many solutions to the current resistance phenomenon (85). Many studies have concluded that animals are an important source of bacterial contamination on fresh produce however few studies have sought to document an association between environmental practices of animal wastes and antibiotic resistant bacteria and antibiotic resistant genes

associated with fresh produce (108-110). The present study has been conducted to fulfill this knowledge gap in research.

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CHAPTER 3: EFFECT OF SOIL AMENDMENTS FROM ANTIBIOTIC_TREATED COWS ON ANTIBIOTIC RESISTANT BACTERIA & GENES RECOVERED FROM THE SURFACES OF LETTUCE AND RADISHES: FIELD STUDY

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Abstract

Cattle are commonly treated with antibiotics that may be excreted in their urine or feces. Application of manure or composted manure containing antibiotics or antibiotic resistant bacteria (ARB) as a soil amendment may result in transfer to plants. This study was conducted to determine the effects of antibiotic administration and soil amendment practices on microbial diversity and antibiotic resistance of bacteria recovered from the surfaces of lettuce and radishes grown in field using recommended application rates. Vegetables were planted in field plots amended with raw manure from antibiotic-treated dairy cows, composted manure from cows with different histories of antibiotic administration, or chemical fertilizer control (12 plots, n=3). Culture-based methods, 16s rDNA amplicon sequencing, qPCR and shot-gun metagenomics were utilized to acquire the effect of soil amendment on the vegetable bacterial communities and associated resistance genes. Biological amendments resulted in distinct separation of bacterial communities on both vegetables compared to no amendment. Increases in clindamycin resistant bacteria, a class of antibiotics administered to cattle, were noted on lettuce grown in biological soil amendments. Additionally, vegetables grown in manure were associated with increased abundance of specific ARG copies and resistance genes to additional classes of antibiotics. Growth in compost resulted in fewer ARGS on vegetables compared to manure amended soils. This study demonstrates that raw, antibiotic-exposed manure may alter microbiota and the antibiotic resistance genes present on vegetable surfaces. Proper composting

of soil amendments as recommended by the USDA and EPA may offer a strategy to mitigate some types of ARGs.

Introduction

The accelerated dissemination of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) throughout the environment is considered one of the largest public health threats of the 21st century (1,2). Every year 2 million Americans contract bacterial infections resistant to one or more antibiotics, amplifying treatment costs and resulting in death for ~23,000 people (1). One strategy to combat this growing problem is to restrict the use of select classes of medically important antibiotics to humans (2). Antimicrobial use in agriculture is broad; from therapeutic treatment of animals, prophylactic prevention of disease and sub-therapeutic growth promotion. In 2015, over 15 million kgs of antimicrobials were distributed to food-producing animals in the USA; 62% of which were considered medically important (3). American dairy cows are commonly administered antibiotics in-between lactation periods and can produce up to 80 lbs of manure per day on a 1,000 animal per unit basis (4). Animals administered antibiotics can excrete more than 70% of some parent compounds in feces and as much as 90% in urine; excretions must be managed and often end up as soil amendments on vegetable crop fields (5-8). The consistent production of antibiotic exposed manure has put pressure on the environmental resistome, selecting for bacterial resistance.

Manure, specifically from cattle, is a known reservoir of ARB and ARGs; application of manure to soil has been shown to increase ARGs detected in soil (9-14). Composting treatments of the manure are known to reduce levels of parent antibiotic compounds, but reduction of ARB and ARGs are variable (15-19). Many studies have concluded that animal waste is a significant

source of bacterial contamination on produce however few studies have aimed to document an association between environmental practices of soil amendments and ARB and ARGs associated with fresh produce (21-23). A wide range of ARB and ARGs have been detected on both farm fresh and market ready produce (24-29). Additionally, organic produce, which must be grown in natural fertilizers like manure, have been found to have equal (30,31) or higher levels of ARG containing bacteria in comparison to conventionally grown vegetables (32). Composting of manure using a method validated to reduce pathogenic bacteria is required if the compost will be applied to soils used to grow fruits or vegetables (33). It is not known how composting affects the levels of ARB and ARGs transferred to the surfaces of vegetables grown in said amendments.

In this study, culture-dependent and independent analyses were conducted to evaluate the effect of soil amendment on the bacterial communities, especially quantities of ARB and ARGs detected on the surfaces of lettuce and radishes grown in a clay loam field that, prior to this study, had not been amended with animal amendments or antibiotics for a decade. Biological soil amendments included: raw manure from dairy cows administered pirlimycin and cephapirin, statically composted manure from cows with different antibiotic treatment histories (antibiotic administration or none during collection). Lettuce and radishes grown in soils with the different biological amendments were compared to those grown using a chemical fertilizer. We aimed to characterize the bacterial communities of the vegetable surfaces through sequencing of 16S rDNA amplicons, and enumerate antibiotic-tolerant bacteria using culturing and culture independent methods. Additionally, the classes of putative ARGs recovered from the vegetable surfaces were compared via shotgun metagenomic DNA sequencing. The results will help provide important information on the interactions between vegetables grown in antibiotic

exposed soil amendments and the prevalence of antibiotic resistance in the farm-to-fork continuum.

Materials and Methods

Field Study Design

The land utilized in this experiment was located in Virginia Tech's Urban Horticulture Center (UHC) in Blacksburg, Virginia. Soil was identified as clay loam and further analyzed by Waypoint Analytical (Richmond, Virginia) (Table S1). Prior to this project, animal amendments or antibiotics had not been intentionally applied to the soil within the past decade. The land was divided into 24 (3 m x 3 m) plots; each bordered with steel siding to reduce cross contamination of the soil amendments. The plots were treated with one of four soil amendments: raw dairy cow manure, static-compost with antibiotic exposure, static-compost without antibiotic exposure, and non-amended (Fig 1). Soil was pretreated with Roundup (glyphosate) and non-amended Inorganic Nitrogen-Phosphorous-Potassium (NPK) as it was determined that the soil nutrients were not high enough to support the growth of vegetables without the assistance of NPK. The trial occurred in early Spring, 2016. Rainfall total and average temperatures are available in Wind 2017 (34).

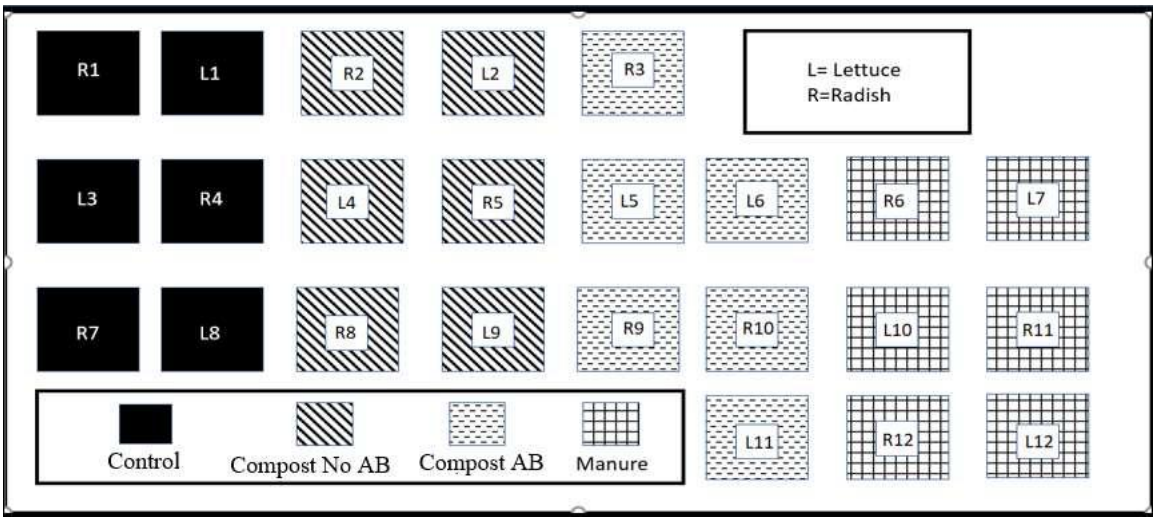

Figure 1: Diagram of vegetable plot design. Squares represent 3mx3m plots of land. Manure and compost amended plots were placed downwind of control plots to minimize the potential for aerial contamination.

Generation of Biological Soil Amendments

Manure from the pirlimycin- and cephapirin-treated dairy cows were compiled and mixed to form dairy manure with antibiotics (35). Briefly, manure from 18 dairy cattle that had received intramammary administration of pirlimycin (2, 50 mg doses 24 hours apart) and cephapirin (1 dose, 300 mg). Cattle manure was collected over a period of peak antibiotic excretion as determined by Ray 2017 (35). Manure was also collected from cows not currently treated with antibiotics. Manure for composting was combined with alfalfa hay (4:1) and sawdust (4:3) to achieve a Carbon: Nitrogen ratio of 25-30% and a moisture content of 55-65%. The same additions and ratios were reached with generate an antibiotic origin compost (Compost AB) or antibiotic-free compost, referred to as Compost No AB. Both compost treatments were developed using a forced aeration static composting approach following the FSMA guidelines and reached an internal temperature >131°F by day 2 of composting (35, 36). The core temperature of the compost pile remained thermophilic (>131°F) for 21 days.

Manure and compost were added to the vegetable plots at a rate of 6.72 Mg/ha as described by Wind 2017 (34). Manure was stockpiled for 57 days before being applied to six

vegetable plots in the raw form. NPK was added to the fertilizer control plots at rates recommended for optimal growth for radish (50 % N-50 % P-50 % K) and lettuce (125 %N -100 %P -100% K) (37). Because the manure/compost was not nutritionally sufficient alone to meet the optimal growth levels, supplemental inorganic N-P-K was also applied to the plots at rates of 50-50-20 for radish and 100-100-75 for lettuce. N-P-K, manure, and compost(s) were applied to the plots on Day 0 of the experiment as shown in Figure 1.

In Field Lettuce Production and Harvest

Lactuca sativa cv. Organic Nancy lettuce seeds (Johnny's Selected Seeds, Fairfield, Maine) were planted in horticulture vermiculite, hand-watered, and fertilized with inorganic NPK solution. After eight weeks, the seedlings were transplanted into 12 field plots (3 replicates per amendment, Fig. 1) at a stock rate of roughly 54 plants per plot. Transplanting occurred thirty days after application of soil amendments. Lettuce plants were grown until maturity (heads of 12 inches in diameter) and harvested on two separate dates, 38 and 39 days after transplanting (Fig 2a). Soil amendments had been applied 67/68 days prior to lettuce harvest.

Lettuce was harvested on two consecutive days from 6 of 12 plots randomly chosen each day. Temperatures ranged from a high of 68-60 ˚F over the two-day period. The similar average temperatures combined with 0% rainfall created minimal variability between Day 1 and Day 2 samples. The 12 lettuce plots were assigned random individual numerical values. From each plot, six heads of lettuce were selected and harvested. Heads of lettuce with evidence of decay or disease were not selected for analysis. The lettuce heads were removed from the base just above soil level using ethanol sterilized pruning shears; to minimize cross contamination gloves and shoe covers were changed between each plot. The bottom-most leaves (4-6) in direct contact with the soil were removed from the base and discarded before placing into large collection bags

which were immediately transported to the lab for analysis. Samples were processed within two hours from harvest.

Radish Production in Field and Harvest

Raphanus sativus cv. Crunchy Royal radish (Johnny's Selected Seeds, Fairfield, Maine) seeds were sown 30d after application of soil amendments (n=3 per amendment). Seeds were planted 1/8 to 1/4-inch-deep in rows that were roughly 2-3 inches apart (Virginia Cooperative Extension 2015). Radishes were harvested when market ready; when the bulbs began to push out of the soil line; 46/47 days after planting (Fig 2b). Soil amendments had been applied 74/75 days prior to radish harvest. Radishes were harvested on two consecutive days from six of 12 plots

Figure 2a: Lettuce plants were, on average, 12" in diameter upon harvest.

Figure 2b: Radish bulbs were collected after breaking through the surface of the soil line, as depicted above.

randomly chosen each day. Temperatures ranged from a high of 70 to a low of 65 ˚F and no rainfall occurred. These similar average temperatures combined with 0% rainfall created minimal variability between Day 1 and Day 2 samples. Radishes that showed visible signs of decay or plant disease were not selected for analysis. The radishes were pulled from the ground while wearing gloves and shoe covers; to minimize cross contamination gloves and shoe covers were

changed between each plot. From each plot, at least ten radishes were harvested, placed into a collection bag; these 12 bags were immediately transported to the lab for analysis. Samples were processed within two hours from harvest.

Enumeration of Aerobic Heterotrophic Bacteria

Bacteria recovered from the surface of radish taproots (75g) or lettuce leaves (25g, 2-3 inner and outer leaves) were enumerated in this experiment. The leafy green tops and fibrous root hairs of radishes were removed aseptically with scissors before processing. Bacterial cells were disassociated from the vegetables using by gently shaking at 220 rpm on a multi-purpose rotator (Fisher Scientific, Waltham, MA) for 5 minutes submersed in a solution of sterile 0.1% peptone (Difco, Becton Dickinson and Company, Franklin Lakes, NJ) with 0.1% Tween 80 (Fisher Scientific) solution. Each sample was then hand massaged for an additional 2 minutes after shaking. For both plant types, 10 ml of the suspension were serially diluted and spread-plated (100 µl) in duplicate onto 7 different types of R2A media (Difco, Becton Dickinson and Company Franklin Lakes, NJ) containing various concentrations of antibiotics (3 µg/ ml tetracycline, 10 µg/ ml ceftazidime, 25 µg/ ml, erythromycin, 25 µg/ ml clindamycin, 25 µg/ ml sulfamethoxazole, and 11 µg/ ml vancomycin) and an R2A control. Antibiotic concentrations were determined by enumeration of bacterial colonies from compost from dairy cattle treated with antibiotics on R2A of differing antibiotic concentrations. Concentrations were chosen by an observed decrease in CFU from the lowest antibiotic concentration tested. Plates were incubated at 37 °C for 24h prior to enumeration.

Nucleic acid Isolation

Immediately after enumeration, the remaining diluent was aseptically filtered through 0.22-µm 47-mm mixed cellulose esters membrane (EMD Millipore, Merck Group, Darmstadt,

Germany) to collect bacterial cells. Filters were folded four times, torn, and stored in sterile, DNase-free, O-ring screw cap tubes at -80 °C until DNA extraction. Diluent from each of the 24 samples were processed independently.

The frozen filters were placed into Lysing Matrix E tubes from the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH) with the manufacturer's sodium phosphate buffer and MT buffer. DNA lysis using physical disruption by the FastPrep® Instrument (MP Biomedicals, Solon, OH) occurred after 40 seconds at a speed setting of 6.0. The manufacturer's instructions were followed except for an additional bead beating step and 2 h incubation a room temperature, allowing for maximized cell lysing. The DNA was resuspended with 100 µL DNase/pyrogen-free water and the tubes were incubated at 55 °C for 5 min. The freshly eluted DNA was then applied to the OneStep PCR Inhibitor Removal Kit (Zymo Research Corporation, Irvine, CA) per manufacturer's directions before storing at -80 °C in DNase-free, O-ring screw cap tubes. A radish sample grown in antibiotic free compost was lost during the DNA extraction process; n=2 for Compost AB samples being analyzed throughout this experiment for this reason.

Quantification of antibiotic resistance genes

Quantitative real time PCR (qPCR) was used to determine the number of copies of 16S rDNA, *tet(w)* and *sul1* in lysates from bacterial DNA from the surface of the field grown lettuce and radishes. DNA extracts were diluted 1/10 to reduce PCR inhibition. Diluted samples were utilized in 10- µL reactions, which were created for all gene targets. 2x SsoFast EVAgreen Supermix (BioRad Laboratories, Hercules, CA), 20 ng of DNA template and 400 nM primers were combined with 2.4 µL of molecular grade water (Sigma-Aldrich, St. Louis, MO). Triplicate technical replicates of each sample were amplified along with triplicate standard curves and a negative control. The standard curve, comprised of 7, 10-fold dilutions and ranged from 10^8-10^2

gene copies/µl for 16S rRNA and 10^7-10^1 gene copies/µl for *tet(W)* and *sul1*. The negative control was comprised of molecular grade water (Sigma-Aldrich, St. Louis, MO). Samples were amplified in a CFX Connect TM Touch Real-Time PCR Detection System (BioRad Laboratories Hercules, CA). The protocol consisted of 1 cycle of 98 °C for 2 min, 40 cycles of 98 °C for 5s and annealed at various temperatures and times depending on the gene target. 16S rRNA targets were annealed at 55°C, 5 s, *tet(W)* at 61°C, 7 s and *sul1* at 71°C, 7 s followed by a melt curve.

16S rDNA Amplicon Sequencing and Analysis

Illumina 16S rDNA amplicon sequencing was performed on lettuce and radish DNA samples following the Earth Microbiome Project 16S Amplification Protocol version 4_13 (38,39). DNA samples from vegetables grown in each plot were amplified via PCR using unique barcoded bacteria-archaeal primers 515FB and 926R. The amount of DNA used for amplification was normalized to an equivalent 16S rDNA gene copy numbers between all samples before barcoded PCR amplification. Barcoded PCR was performed in triplicate for each sample; products were pooled on an equal mass basis of 200 ng and products purified using QIAquick PCR Purification Kit (QIAGEN, Valencia, CA). The final pooled product was submitted to the Genomics Research Laboratory of the Biocomplexity Institute (BI) of Virginia Tech for paired-end 300 cycle sequencing on the Illumina Miseq. PANDAseq (40) was used to stich the paired-end reads together at a quality score of >0.80 and sequence length of 372-375 bp**.** The QIIME pipeline (41) was used to annotate the reads to the Greengenes 16S rRNA gene database (42), after which mitochondrial and chlorophyll sequences were filtered out of the OTU table. The samples had a minimum number of reads of 6127 and a maximum of 40750. All samples were rarefied to 6127.

Metagenomic Analysis

Metagenomic analysis was performed on the 12 lettuce and 11 radish samples. Two lanes comprised of 23 undiluted DNA samples, were submitted to the Genomics Research Laboratory of BI. DNA (3 ng) were prepared using the Accel-NGS 2S DNA kit (SwiftBio, Ann Arbor, MI) incorporating 11 cycles of PCR to prepare libraries for high throughput sequencing on Illumina HiSeq 2500 with a high output paired-end 2×100 read length protocol. The paired-end sequence files (one file per end) were transformed into fastq format and then uploaded to MetaStorm (43). MetaStorm is an online platform that allows metagenomics data to be analyzed using a variety of databases. The Comprehensive Antibiotic Resistance Database (CARD v1.0.6) was selected in MetaStorm and used as the ARG functional annotation reference database for the read matched samples (44). The gene counts derived from MetaStorm were normalized to the abundance of 16S rRNA gene to determine the relative abundances of the total detected ARGs (45). The trimmomatic default setting was used in Metastorm, providing 80% nucleotide coverage of each read.

Statistical Analysis

JMP® Pro 12 (SAS Institute, Cary, NC) was utilized for all statistical analyses; $p \leq 0.05$ indicated statistical significance for all parametric and non-parametric tests. Plate counts between 25-250 CFU/plate were log-transformed to approximate normal distribution.

The overall effect of soil amendment type was compared using a one-way ANOVA analysis with a Tukey's post-hoc analysis to test for differences in the average log CFU/g of antibiotic-tolerant bacteria recovered off lettuce and radish surfaces. The same statistical measures (one-way ANOVA, Tukey's post-hoc analysis) were taken to determine the effect of soil amendments on the antibiotic-tolerant bacteria enumerated (log CFU/g) each individual media type.

Copies ARG (*tet(w)* and *sul1*) were normalized by dividing ARG copy numbers /16S rRNA gene copy numbers. The effect of soil amendment type on the proportion of target genes were compared using the nonparametric Wilcoxon coupled with a Steel-Dwass All Pairs test to conduct multiple comparisons. The same statistical measures were used to analyze total gene copies (*tet(w) and sul1*). Significance between samples was defined as $p \leq 0.05$.

The α-diversity estimates acquired from the16s rDNA Amplicon sequencing were calculated by analyzing the observed species, Shannon index and Chao1 values. Values were compared by using Wilcoxon coupled with a Steel-Dwass All Pairs test. Unweighted and Weighted Unifrac distances derived from the β-diversity estimates were plotted in Multidimensional Scaling (MDS) plots in PRIMER-E (version 6.1.13). β-diversity estimates were compared in PRIMER-E using analysis of similarities (ANOSIM) ($p \leq 0.10$); levels of separation were defined by Ramette (10). Overall rarefied bacterial compositions derived from 16s rDNA Amplicon sequencing were compared using nonparametric Wilcoxon coupled with a Steel-Dwass All Pairs test. The relative abundances of total ARGs from shotgun metagenomics were compared in PRIMER-E using analysis of similarities (ANOSIM).

Results

Effect of Soil Amendment on the Culturable of Antibiotic-Tolerant Bacteria

Lettuce: The total aerobic bacteria (log CFU/g) recovered from lettuce grown in biological soil amendments were comparable to plants grown in chemically fertilized plots (Table 1). Antibiotic inclusion within the R2A media did result in decreases to the number of bacteria recovered. In general, the number of antibiotic tolerant bacteria, defined here as the log CFU/g bacteria that grew on R2A impregnated with antibiotics, recovered from plants grown in fields with biological amendments was not significantly different from plants grown in control plots (Table1). The

only exception was that lettuce grown in biological soil amendments had a 2.0 log CFU/g increase in bacteria recovered on R2A with clindamycin (25µg/mL) compared to lettuce grown in control plots ($p < 0.05$, Table1).

Radish: In general, growth of radish taproots in fields amended with manure, compost or non-amended control did not significantly affect the log CFU/g bacteria recovered on R2A or R2A supplemented with six antibiotics (Table 1).

Effect of Soil Amendment on the Quantification of *tet(W)* and *sul1*

Lettuce: The total *sul1* gene copies recovered from the surfaces of lettuce plants grown in raw manure amended plots had nearly 160X more copies than plants grown in the non-amended control (p=.002) (Fig 1). Lettuce grown in AB compost had roughly 60X as many gene copies as plants grown in non-amended control (p=.002) (Fig 1) while lettuce grown in unexposed compost had roughly 40 times as many copies (p=.002) (Fig 1). When the total *sul1* gene copies were adjusted for bacterial numbers by normalizing with the 16S rRNA gene copies there was still a 16X and 13X increase for lettuce leaves grown in raw manure and AB compost respectively, compared to plants grown in the control plots (p=.01) (Fig 2). The total *tet(w)* gene copies recovered from the surfaces of lettuce plants grown in raw manure amended plots had significantly larger copies than plants grown in non-amended control (p=.04) (Fig 3). Inclusion of biological amendment in the soil resulted in an increase in numbers of 16S rDNA copies on lettuce surfaces; adjusting the *tet(w)* gene copies with respect to 16S rRNA gene copies there was a small but significant effect in comparison to non-amended control plots (p=.024) (Fig 4).

Radish: The total *sul1* gene copies recovered from the surfaces of radish taproots grown in raw manure amended plots had 22 times more gene copies than plants grown in non-amended control

(p=.014) (Fig 5). Radish plants grown in AB compost had nearly 20 times as many gene copies plots (p=.002) while radishes grown in unexposed compost had over 15 times the gene copies (p=.04) compared to plants grown in non-amended control plots. No significant differences were detected (p >.05) in the quantity of *sul1* gene copies, with respect to 16S rRNA gene copies, recovered from radish taproots grown in the various soil amendments (Fig 6). The total *tet(w)* gene copies recovered from the surfaces of radish taproots grown in raw manure amended plots had roughly 30 times more copies than plants grown in non-amended control plots (p=.002). The total *tet(w)* copy numbers recovered from the surfaces of radish taproots grown in AB compost created a 3-fold increase (p=.009) compared to radishes grown in the non-amended control plots (Fig 7). Recovered *tet(w)* gene copies, with respect to 16S rRNA gene copies, recovered from the surfaces of radish taproots grown in raw manure amended plots had a 4-fold increase in gene copies compared to radishes grown in the non-amended control (p=.01). Radish plants grown in antibiotic-exposed compost had a one-fold increase in *tet(w*) copy numbers compared to the control plots. (p=.037) (Fig 8).

16s rDNA Amplicon Sequencing

Characteristics of Sequenced Data: A total of 273,429 bacterial sequence reads were recovered from the lettuce samples; 149,010 sequence reads were obtained from radishes for a total of 422,439 bacterial reads. Sequences ranged between 6,127- 40,750; all samples were rarefied to 6,127 for this reason. The rarefaction curves derived from the sequencing data shows that lettuce samples were closer to reaching sequencing saturation (Fig 9), which is indicated by plateauing, in comparison to the radish samples (Fig 10). The deepest sequencing was achieved by lettuce samples grown in antibiotic exposed compost followed by samples grown in antibiotic free compost, control plots, and manure plots, in that order (Fig 9). Radishes grown in manure

obtained the deepest sequencing saturation out of the radish samples followed by samples grown in antibiotic exposed compost, antibiotic free compost, and control samples, in that order (Fig 10).

Influence of Soil Amendment on the Characteristics of Vegetable Bacteria

The Unweighted Unifrac distances, reflecting the β-diversity of the lettuce and radish communities, were significantly different ($p<0.001$) and strongly separated (Global $R=0.77$) based on vegetable type (Fig 11). Growth in different amendment types created a significant difference in β-diversity across all vegetable types ($p=0.02$), however the communities were only weakly separated (Global $R< 0.25$) (Fig 11). Pairwise tests determined that samples grown in antibiotic exposed compost were significantly different from samples grown in manure ($p=0.02$) and were distinctly separated ($R=0.68$). Vegetables grown in manure were significantly different from samples grown in control plots ($p=0.02$) and were distinctly separated ($R=0.48$) (Fig 11). The Weighted Unifrac distances were significantly different ($p<0.001$) and strongly separated based on vegetable type (Global $R=0.76$) but not amendment type ($p>.05$), Global $R < 0.25$) (Fig 12).

The same phyla and bacterial classes dominated the surfaces of the lettuce and radish samples in varying relative abundances. *Proteobacteria* were the most abundant phylum for both lettuce and radishes (>80%) followed by *Actinobacteria* (>4%) and *Firmicutes* (>6%) (Table 3,4). *Gammaproteobacteria* (57.3%) was the dominant class among lettuce samples while *Alphaproteobacteria* (34.6%) dominated the radish surfaces (Table 3,4). Bacterial OTUs from lettuce plants were classified into 8 bacterial orders; the same orders were present in the radish samples with the addition of *Sphingomonadales* and *Xanthomonadaceae.* Bacterial OTUs from

lettuce plants were classified into 11 families; the same families were present in the radish samples with the addition of *Xanthomonadaceae.*

Influence of Soil Amendment on Diversity of Lettuce-Associated Bacteria: The α-diversity (Shannon index, Chao1 and evenness) of the lettuce phyllosphere bacterial communities were minimally affected by the use of biological soil amendment ($p>.05$) (Table 2). Lettuce grown in manure had the highest mean Chao1 value implying that the samples were more diverse in their microbial abundance than the three other treatment types.

The Unweighted Unifrac distances of the lettuce phyllosphere amplicons were significantly different ($p=0.01$) and slightly separated (Global $R=0.35$) based on amendment type (Fig 12). However, bacterial communities of lettuce grown in manure or antibiotic exposed compost were significantly different ($p=0.10$) and clustered separately ($R=0.70$, 0.96, respectively) from lettuce samples grown in control plots (Fig 13). ANOSIM of the Weighted Unifrac distances of the lettuce samples grown in the field did not define amendment type as an overall separation factor ($p>0.10$, Global $R< 0.25$) (Fig 14). However, pairwise testing revealed that lettuce samples grown in control plots were significantly different ($p=0.10$) and slightly separated ($R= 0.33$) from lettuce gown in antibiotic exposed compost (Fig 14).

Influence of Soil Amendment on Lettuce-Associated Bacterial Community Composition: The bacterial sequences classified for lettuce samples were assigned to 13 phyla; chiefly *Proteobacteria* (93.9%), *Firmicutes* (4.38%), and *Actinobacteria* (2.39%) (Table 3). There were no significant differences between the relative abundances of detected phyla based on amendment type. Bacteria were identified from 44 different classes; *Gammaproteobacteria* had the highest relative abundance among lettuce samples (64.8%). Additionally, *Betaproteobacteria* (21.5%), *Actinobacteria* (2.30%), *Bacilli* (4.33%), and *Alphaproteobacteria* (5.89%) belonged to

the most represented classes (representing more than 1% relative abundance) identified from the lettuce samples. There were no significant differences between the relative abundances of detected classes and amendment type. The most abundant bacterial order identified from the lettuce sequences belonged to *Pseudomonadales* (49.1%); *Burkholderiales* (20.1%) and *Eneterobacteriales* (14.6%) (Table 3). *Methylophilales* (1.37%) were significantly more abundant on lettuce samples grown in the two composted amendments (p=.045, Table 3). Of 11 most represented families of bacteria, *Oxalobacteraceae* (19.5%), *Moraxellaceae* (21.5%), and *Pseudomonadaceae* (27.5%) proved to be most abundant. *Methylophilaceae* were roughly 40 times more abundant (p=.044) on lettuce samples grown in the two composted amendments compared to samples grown in manure and control plots (Table 3).

Influence of Soil Amendment on Diversity of Radish-Associated Bacteria: The α-diversity indices (Shannon index, Chao1, Evenness) were highly similar between radish samples regardless of amendment type (p>.05) (Table 2). In addition, the β-diversity of the radish bacterial communities (unweighted Unifrac distances) were not significantly different when grown in different amendment types (p>0.10, Global R< 0.25) (Fig 15). ANOSIM of the Weighted Unifrac distances revealed that amendment type created a significant difference (p=0.56) between radish samples and almost created separation (Global R=0.24) between the samples (Fig 16). Bacterial communities of radishes grown in manure were significantly different and separated from radishes grown in plots lacking biological amendments and radishes grown in antibiotic exposed compost (p=0.10) (R= 0.50, (R=0.59), respectively) (Fig 16).

Influence of Soil Amendment on Radish-Associated Bacterial Community Composition: Bacterial sequences classified for all radish samples were assigned to 21 phyla. Most of the radish sequences belonged to the phyla *Proteobacteria* (85.7%), *Actinobacteria* (7.06%), and

Firmicutes (4.42%) (Table 4). Significant differences were not detected between the relative abundances of the detected phyla. 64 classes of bacteria were identified from the radish sequences; *Gammaproteobacteria* had the highest relative abundance among radish samples (46.2%). The most represented sequences belong to the classes *Alphaproteobacteria* (26.5%), *Betaproteobacteria* (12.4%), *Actinobacteria* (6.75%), and *Bacilli* (4.31%). There were no significant differences between the relative abundances of detected classes. The most abundant order of bacteria identified from the radish sequences belonged to *Pseudomonadales* (38.5%); *Rhizobiales* (21.4%) and *Burkholderiales* (9.70 %) followed in abundance. Seven additional bacterial orders were largely represented among radish samples (Table 4). Of 12 most represented families of bacteria, *Pseudomonadaceae* (27.5%), *Rhizobiaceae* (17.5%), and *Moraxellaceae* (15.8%) proved to be most abundant. *Pseudomonadaceae* were up to 10 times more abundant on radishes grown in manure (p=.032) (Table 4) than any other amendment type.

Shot-gun Metagenomics

Identifying Classes of ARGs from Vegetable Surfaces:

A total of 299,894,788 quality-filtered reads were annotated with the MetaStorm read matching pipeline from the 23 vegetable samples. On average, lettuce samples generated 12,087,606 reads per sample while radishes generated 14,076,683 reads per sample (Table S2). Roughly 590 putative ARGs were identified. ARGs were sorted based on the class of antibiotic to which they encode resistance. ARGs were normalized to the number of 16S rDNA sequences in the sample to account for difference in sequencing depth. Samples were predicted to have a role in resistance to 21 different classes of antibiotics; chiefly commonly prescribed antibiotics such as quinolone, triclosan, and trimethoprim as well as polymyxin. The majority of the ARGs conferred simultaneous resistance to multiple antibiotics and were classified as multidrug genes

(Table 5,6). A heatmap of the relative abundance of ARGs demonstrated that distinct patterns were observed amongst vegetable type as indicated by two distinct clusters; with the exception of a single radish sample grown in antibiotic free compost (Fig 17). ANOSIM of the Bray-Curtis similarities produced from the relative abundance and types of ARGs identified distinct patterns between lettuce and radishes ($p=0.01$). While there were no significant differences in ARG profiles of vegetables grown in the different amendment types (ANOSIM $p>0.10$), the ARG profile of the manure grown vegetables were slightly separated from those grown in both the compost amended and non-amended soils ($R=0.29$, $p< 0.03$) (Fig 18). Given the distinct separation between ARG profiles from different vegetables further analysis of treatment effect was also performed within vegetable type.

The samples grown in the non-amended plots were more distinctly separated from the manure- amended samples when ARG profiles were compared within one vegetable type (Fig 19, 20: $R=0.56$, $R=.041$), respectively. Control radishes were also separated from radishes grown in antibiotic exposed compost ($R=.042$) When the relative abundance of each of the classes of ARGs detected on the surfaces of the individual lettuce and radish samples were compared between amendment types there were no significant differences (Wilcoxon $p>0.05$). Radish samples contained genes from all 21 antibiotic classes across the four amendment types. Lettuce samples grown in manure contained genes from 21 antibiotic classes, 3 of which were unique to manure-grown lettuce. Lettuce grown in control plots contained ARGs from 16 antibiotic classes (Fig 21).

Discussion

The aim of this study was to determine how agricultural practices affect the ARB and ARGs of vegetables commonly consumed raw. In the present study, distinct separations between

bacterial communities were observed when vegetables were grown in raw manure amended soils compared to non-amended soils. Unique patterns of ARGs, including resistance genes for three additional classes of antibiotics and increased levels of specific genes were observed in manure-amended soils. One of these classes, the sulfonamides drugs are commonly used in cattle (46) but were not administered to dairy cows during this study but it is possible these cows were previously treated, and resistant bacteria persisted within the gut. A more sensitive approach to the quantification of sulfonamide resistance was taken by targeting the *sul1* gene via qPCR. Normalized *sul1* gene copies compared increased 2-2.95 log increase for lettuce and radishes respectively grown in manure-amended soil, this increase is less than the 4-log increase in *sul1* gene copies in soil amended with dairy cow manure and may reflect a lower rate of transfer to the vegetable surface (47,48). However other studies of *sul1* on vegetables grown in raw manure have not historically produced as strong of a trend (12, 25). In a similar field study, manure amendment resulted in much higher concentrations (9.52 Log_{10} copies g) of *Sul1* in the soil samples with only a small fraction 2.03 (Log_{10} copies g) transferred to carrots grown in the same plots (12). Interestingly, no *Sul1* genes were above the limit of detection on lettuce and radish samples grown in the same plots (12). Vegetables processed in the present study were shaken and hand massaging in a solution containing a surfactant to promote removal of the cells by disrupting Van der Waals interaction. Other studies used pure Milli-Q water with sodium phosphate buffer and one minute of hand massaging, which might not have promoted as much surface removal and may account for smaller number of bacteria detected in those studies (12). Additionally, the present study utilized a bacterial DNA extraction kit that incorporates a vigorous physical and chemical lysis step to lyse bacteria that have been filtered onto membranes. This served three purposes: to collect more bacteria from the entire surface as

opposed to a per gram amount, to increase the yield of DNA, and to reduce the amount of contaminating DNA from plant cells. Other studies lysed directly from the vegetable surface using kits that primarily use a chemical lysis step to disrupt bacterial cells; this could have created a difference in gene detection. DNA extraction protocols have been previously shown to impact the bacterial community profile and detection of many genes (49). It is likely that the transfer rate of *sul1* genes from amendments in soils to vegetable surfaces is dependent on many factors; as studies report increases in *sul1*copies on carrots between 1-log -2 log (25). The origin of the manure may play a role in ARG presence and survival in the soil, however it is unclear if an interaction exists with transfer to the vegetable surface.

Method sensitivity is an important consideration when comparing the types and levels of ARGs. The metagenomic approach used in this study identifies over 590 different ARGs from amongst the vegetable samples. However, the frequency of detection of two ARGs *sul1* and *tet(W)* gene was reduced compared to real time PCR. Metagenomic analysis detected the *sul1* gene in only one of 23 field samples while qPCR detected ~ 10^2 gene copies. Similarly, the *tet(w)* gene was detected in one sample from metagenomic analyses but in all 23 samples when using qPCR. The data retrieved from Shot-gun metagenomics helps to give a broad overview of the ARGs present within samples but did not provide the same level of detection as qPCR. Small sample size (n=23) also inhibited interpretation of the metagenomics results in this study. Discretion is advised when interpreting Shot-gun metagenomic data for this reason. Correlations between ARGs and microbiota were possible because of these coupled genetic analyses paired with 16s rDNA Amplicon sequencing.

Bacterial communities of vegetables grown in manure-amended soil possessed more unique bacterial OTUs resulting in distinct, separable bacterial communities compared to

vegetables grown in control plots. Phyla with relative abundances less than 1% were considered rare and included *Armatimonadetes, BRC1*, *Chlamydiae*, *Chlorobe*, *Chloroflexi*, *Elusimicrobia*, *FBP*, *Fibrobacteres*, *Nitrospirae* and *Planctomycetes*. *Verrucomicrobia,* which are more common in uncultured, pristine soils (50). *Verrucomicrobia* are occasionally observed in the human gut microbiome, and frequency of detection is 40% greater in individuals treated with broad-spectrum antibiotics (51). *Fibrobacteres* are another unique group detected in the vegetable samples grown in biological amended soils; this phylum was originally thought to have been most abundant in mammalian and more frequently, rumen intestines but were recently classified as mobile, microaerophilic organisms after being detected in landfill sites and freshwater lakes (52,53). In addition to unique OTUs, significant increases in *Pseudomonadaceae* were detected on both radishes and lettuce grown in raw manure amended soils. This is not surprising to see as raw manure application has been shown to increase *Pseudomonas* spp. populations by ten-fold in field soil (54). While, metagenomics techniques used in this study did not allow discrimination of co-occurring ARGs and 16S rDNA sequences, the dominant group of *Pseudomonadacea*, comprising up to 44% of radish and 31% lettuce communities have a high frequency of carrying class 1 integrons, such as those conferring *sul*1 resistance (55). *Pseudomonas* spp. have been shown to be the largest contributors of class 1 integrons and ARGs to soils after manure slurry application *(*47, 56). While, a significant increase in *sul*1 and *tet*(W) genes could be detected on vegetables grown in manure amended soil, there was no difference in culturable bacteria on sulfonamide or tetracycline-amended agar. This may reflect presence of non-functional genes or incomplete operons.

Phenotypic tests are necessary to confirm antibiotic resistance. While differences in ARGs were detected on vegetables grown in biological based amendments, this did not translate

to significant differences in the number of cultured ARB. The lack of significant differences between amendment type and ARB detected are comparable to other studies that did not find differences between antibiotic-exposed biological soil amendments and the culturable ARB recovered from fresh produce grown (11, 12, 25). The exception was a 2 log CFU/g increase in clindamycin-tolerant bacteria from lettuce grown in the plots amended with BSAs. The dairy cows utilized in this experiment had been administered pirlimycin, a lincosamide class antibiotic; this drug is similar to clindamycin, also from the class lincosamide. This suggests that bacteria developed resistance within the gut and then persisted within the soil until it was transferred to the vegetable surface. R2A media does not select for specific bacteria and could have influenced the spread-plating results. The addition of antibiotics to the R2A media also adds a certain level of discretion of the interpretation of the spread-plating results; *Proteobacteria* have been enriched by the addition of clindamycin in experiments enumerating culturable bacteria from tomato surfaces (57). Similar enrichment could have occurred during the execution of the present study. It has been estimated that less than 1% of microbes residing in the rhizosphere and soil are culturable with the methods available today (58,59). The present study is novel in that culture based methodologies have been paired with 16s rDNA Amplicon sequencing so that more detailed conclusions could be obtained on the microbiota associated with the vegetable surfaces.

The bacterial communities of aerial vegetable surfaces have been well characterized (60-63).Plant seeds are known to contribute to the initial population of bacteria of plants as they germinate, but the bacterial members quickly alter post-germination (64). The soil serves as a major microbial reservoir; many of the OTUs identified from plant surfaces can be detected in the soil used for growth (65-67). While the communities of radish endophytes and sprouts have been studied (68-70) a void in research on the characterization of the microbial communities

associated with the radish taproot surface exists. Radish taproots are dominated by OTUs classified in the Proteobacteria phylum; within this phylum the taproot surfaces have higher prevalence OTUs belonging to the order *Psuedomonadales (up to 45%)* while seeds have been shown to have higher levels of *Pantoea* (70). The relative abundances of the communities associated with radish (*Raphanus sativus)* surfaces were dominated by the same three phyla as lettuce samples; *Proteobacteria*, *Actinobacteria*, and *Firmicutes* (Table 4). These results support the theory that soil drives major populations of bacteria recovered from plant surfaces, as the lettuce and radish plants, which are from different families, contained similar proportions of bacteria.

The composting process transforms the bacterial community of manure with elevated heat inactivating many enteric bacteria and increasing populations of thermophile bacteria (Song 2014). While the radish samples had a significant increase in *Psuedomonadaceae* when grown in raw-manure amended soils, vegetables grown in compost-amended soils had roughly 40 times more OTUs identified in the order *Methylophilales* (Table 3). *Methylophilales* have been identified as important microbes in large-scale aerobic composting processes due to biodegradation properties of the bacteria (72). The composts that amended the vegetable plots in this experiment were aerobically composted in a static manner, possibly enriching for *Methylophilales* in the process. The *Methylophilales* could have transferred to the surfaces of the lettuce plants because of this enrichment. *Methylophiles* likely originated from the manure itself, as cows and other herbivore guts, breaking down undigestible nutrients for animals to consume (73). *Methylophiles* are an important example of environmental bacteria that can become incorporated into the human gut microbiome; these versatile bacteria ultimately enter the farm-to-fork continuum via vegetable consumption, which is why determining the ARGs carried by

bacterial communities found on the surfaces of vegetables is important. *Pseudomonas spp.* are also ubiquitous throughout the environment; instances of multidrug resistance *Pseudomonas aeruigenosa* infections have been increasing world-wide at an alarming rate and are considered a serious threat by the CDC (1).

The present study shows evidence that the amendment of raw manure increases the quantity of ARGs and the classes of acquired resistance detected in bacteria recovered from vegetable surfaces grown in said amendment. Raw manure also shifts the microbial communities of the vegetables, increasing *Pseudomonadacea* populations, creating a clear separate from vegetables grown in un-amended soil. Composting seems to decrease levels of ARGs while leveling the microbial communities found on vegetables, besides *Methylophilales*, which aid in the composting process. Prior antibiotic administration to dairy cows could have been the driver of these distinct changes; more research must be conducted to determine if raw manure is responsible for the increases in ARB and ARGs or if antibiotic administration and raw manure acted in a synergistic manner. The distinct changes in microbiota and increase in ARGs is an indicator of the potential for spread of antibiotic resistance through the consumption of vegetables eaten raw.

Research Figures:
Tables

*Table 1: Enumeration Data. R2A= total aerobic bacteria. R2A CO=bacteria resistant to cefotaxime (10ug/mL). R2A V= bacteria resistant to vancomycin (11ug/mL). R2A ER= bacteria resistant to erythromycin (25ug/mL). R2A T= bacteria resistant to tetracycline (3ug/mL). R2A S= bacteria resistant to sulfamethoxazole (100ug/mL). R2A CL= bacteria resistant to clindamycin (25ug/mL). Significance denoted with **bold***.

Media Type	R2A		R2A CE		R2A V		R2A ER		R2A TET		R2A S		R2A CL	
Treatment Type	Lettuce	Radish	Lettuce	Radish	Lettuce	Radish	Lettuce	Radish	Lettuce	Radish	Lettuce	Radish	Lettuce	Radish
Raw Manure	5.64 ±0.375	5.77 ±0.366	4.66 ±0.079	4.72 ±0.443	5.33 ±0.379	4.32 ±1.08	4.88 ±0.492	3.71 ±1.13	3.00 ± 0.00	3.00 ±0.00	3.1 ±0.00	3.1 ±0.00	5.057 ±0.361	4.89 ±0.626
Compost (ABX)	5.98 ±0.139	5.56 ±0.474	5.02 ±0.201	4.076 ±0.850	5.59 ±0.830	4.64 ±0.251	4.72 ±1.40	3.80 ±1.21	3.0 3 ±0.058	3.03 ±0.058	3.56 ±0.791	3.1 ±0.00	5.533 ±0.319	3.68 ±1.01
Compost (No ABX)	5.90 ±0.423	5.86 ± 0.589	5.01 ±0.431	4.21 ±1.05	5.75 ±0.456	5.08 ±0.688	4.50 ±1.29	4.41 ±1.20	3.07 ±0.058	3.03 ±0.058	3.1 ±0.00	3.1 ±0.00	5.38 ±0.498	4.10 ±0.869
Chemical Fertilizer Control	5.23 ±0.184	5.46 ±0.352	4.15 ±0.948	4.12 ±0.973	4.80 ±0.293	4.06 ±0.747	3.1 ±0.00	3.56 ±0.884	3.00 ±0.00	3.00 ±0.00	3.1 ±0.00	3.07 ±0.0578	***3.63 ± 0.916**	4.05 ±0.826

Table 2: Average and Standard Deviation of the Shannon and Chao1 values of field vegetables grown in the four different amendment types. Evenness was calculated as E=H'/H max where H max=ln S being S the total number of species in the sample, estimated with Chao1.

Vegetable	**Amendment Type**	**Shannon Index (H')**	**Lower Chao1**	**Chao1 Mean**	**Upper Chao1 (H Max)**	**Evenness (E)**
Lettuce	Control	4.36 ± 0.53	529	653	811	0.65
Lettuce	Compost No AB	4.66 ± 0.50	479	675	950	0.68
Lettuce	Compost AB	4.35± .544	415	518	687	0.66
Lettuce	Manure	4.95± 0.46	660	834	1086	0.71
Radish	Control	6.67± 0.49	1354	1440	1526	0.91
Radish	Compost No AB	5.01± 1.15	296	511	1237	0.70
Radish	Compost AB	4.86± 3.56	317	1120	1689	0.65
Radish	Manure	4.48 ± 1.19	783	915	1173	0.63

*Table 3: Comparison of the taxonomic abundances (%) of lettuce samples. Significance is indicated by * (Wilcoxon; P<.05). Each amendment type represents average of n=3.*

Taxon	Relative Abundance (%)			
Phylum	**Fertilizer**	**Compost No AB**	**Compost AB**	**Manure**
Actinobacteria	4.24	1.62	0.85	2.84
Firmicutes	6.93	2.27	3.25	5.08
Proteobacteria	87.80	95.36	95.42	91.12
Class				
Actinobacteria	4.09	1.56	0.82	2.74
Bacilli	6.88	2.23	3.19	5.03
Alphaproteobacteria	6.10	7.69	3.49	6.27
Betaproteobacteria	24.31	17.62	19.65	24.45
Gammaproteobacteria	57.29	69.87	72.13	60.05
Order				
Actinomycetales	4.09	1.56	0.82	2.74
Bacillales	3.56	2.19	3.16	4.99
Caulobacterales	2.52	3.93	1.52	3.13
Rhizobiales	2.18	2.67	1.57	1.70
Burkholderiales	24.25	14.38	17.53	24.26
*Methylophilales**	0.05	3.17	2.10	0.16
Enterobacteriales	11.20	14.01	21.32	11.87
Pseudomonadales	45.59	53.83	49.42	47.48
Family				
Micrococcaceae	2.72	0.50	0.37	1.26
Nocardioidaceae	0.74	0.47	0.20	0.82
Bacillaceae	0.94	0.30	0.43	0.50
Caulobacteraceae	1.20	0.76	0.25	0.92
Rhizobiaceae	1.26	2.55	0.81	1.58
Sphingomonadaceae	1.95	2.55	1.48	1.44
Comamonadaceae	0.43	0.63	0.50	0.46
Oxalobacteraceae	23.80	13.62	16.99	23.71
*Methylophilaceae**	0.05	3.17	2.10	0.16
Moraxellaceae	20.91	30.87	18.05	16.24
Pseudomonadaceae	24.66	22.88	31.33	31.19

*Table 4: Comparison of the taxonomic abundances (%) of radish samples grown in various amendment types. Significance indicated by * (Wilcoxon; p<.05). Each amendment type represents average of n=3 , Compost No AB n=2.*

Taxon	Relative Abundance (%)			
Phylum	**Fertilizer**	**Compost No AB**	**Compost AB**	**Manure**
Actinobacteria	9.71	7.19	7.06	4.29
Firmicutes	6.12	2.18	6.97	2.41
Proteobacteria	80.67	87.92	82.71	91.73
Class				
Actinobacteria	9.23	6.98	6.72	4.09
Bacilli	5.92	2.14	6.79	2.39
Alphaproteobacteria	34.57	18.12	24.07	29.45
Betaproteobacteria	14.52	9.78	11.95	13.44
Gammaproteobacteria	30.65	59.62	45.98	48.57
Order				
Actinomycetales	6.98	9.23	6.72	4.09
Bacillales	2.14	5.92	6.78	2.39
Caulobacterales	0.55	2.61	1.44	0.90
Sphingomonadales	2.18	2.67	1.57	1.70
Rhizobiales	15.28	26.22	17.01	27.16
Burkholderiales	8.71	10.04	8.87	11.19
Methylophilales	0.95	4.17	2.90	2.17
Enterobacteriales	18.03	0.38	0.41	0.30
Pseudomonadales	37.69	26.59	43.06	46.59
Xanthomonadaceae	2.76	2.90	2.24	1.20
Family				
Micrococcaceae	4.17	4.33	2.79	2.12
Nocardioidaceae	2.59	1.35	2.20	0.95
Bacillaceae	1.88	0.85	1.55	1.80
Caulobacteraceae	2.60	0.55	1.44	0.90
Rhizobiaceae	20.35	11.77	12.59	25.36
Sphingomonadaceae	4.02	1.35	3.98	0.90
Comamonadaceae	6.91	5.99	6.08	2.52
Oxalobacteraceae	3.01	2.62	2.67	8.63
Methylophilaceae	4.17	0.95	2.90	2.17
Moraxellaceae	14.10	8.90	38.33	2.07
*Pseudomonadaceae**	12.49	28.79	4.72	44.52
Xanthomonadaceae	2.87	2.73	2.20	1.18

*Table 5: Relative Abundances of ARGs detected from the surfaces of lettuce samples grown in the field. ARGs are grouped by antibiotic class. No significant differences were detected (Wilcoxon p>.05, n=12). * Indicates differentiation in presence of ARG detection between antibiotic class and amendment type.*

Antibiotic Classes (ARGs)	Relative Abundances (%)			
	Control	**Compost No AB**	**Compost AB**	**Manure**
Aminocoumarin	0.04	0.08	0.10	0.08
Aminoglycoside	0.03	0.08	0.09	0.06
Bacitracin	0.01	0.03	0.05	0.03
Beta_lactam	0.01	0.05	0.06	0.03
Chloramphenicol	0.01	0.02	0.03	0.03
Elfamycin*	0.00	0.00	0.00	0.02
Fosfomycin	0.00	0.00	0.01	0.01
Fosmidomycin	0.01	0.03	0.04	0.03
Glycopeptide	0.01	0.02	0.01	0.04
MLS	0.03	0.07	0.07	0.07
Multidrug	0.43	1.10	1.35	0.98
Peptide	0.00	0.01	0.02	0.01
Pleuromutilin	0.00	0.00	0.00	0.00
Polymyxin	0.03	0.07	0.11	0.09
Quinolone	0.07	0.16	0.20	0.14
Rifampin	0.01	0.01	0.01	0.03
Sulfonamide*	0.00	0.00	0.00	0.02
Tetracenomycin*	0.00	0.00	0.00	0.01
Tetracycline	0.03	0.06	0.10	0.06
Triclosan	0.07	0.11	0.16	0.15
Trimethoprim	0.04	0.08	0.06	0.09

Table 6: Relative Abundances of ARGs detected from the surfaces of radish samples grown in the field. ARGs are grouped by antibiotic class. No significant differences were detected (Wilcoxon p>.05, n=11).

Antibiotic Classes (ARGs)	Relative Abundance			
	Control	**Compost No AB**	**Compost AB**	**Manure**
Aminocoumarin	0.05	0.12	0.03	0.06
Aminoglycoside	0.04	0.13	0.06	0.04
Bacitracin	0.01	0.06	0.01	0.02
Beta_lactam	0.04	0.14	0.26	0.06
Chloramphenicol	0.03	0.03	0.01	0.04
Elfamycin	0.00	0.00	0.00	0.00
Fosfomycin	0.00	0.02	0.00	0.02
Fosmidomycin	0.03	0.04	0.02	0.02
Glycopeptide	0.16	0.08	0.09	0.11
MLS	0.05	0.11	0.06	0.05
Multidrug	0.66	1.59	1.60	1.02
Peptide	0.00	0.03	0.00	0.00
Pleuromutilin	0.00	0.00	0.00	0.00
Polymyxin	0.06	0.12	0.03	0.06
Quinolone	0.05	0.30	0.06	0.08
Rifampin	0.06	0.04	0.04	0.05
Sulfonamide	0.00	0.03	0.00	0.00
Tetracenomycin	0.00	0.00	0.00	0.00
Tetracycline	0.16	0.15	0.16	0.11
Triclosan	0.06	0.13	0.02	0.20
Trimethoprim	0.18	0.13	0.15	0.13

Research Graphs

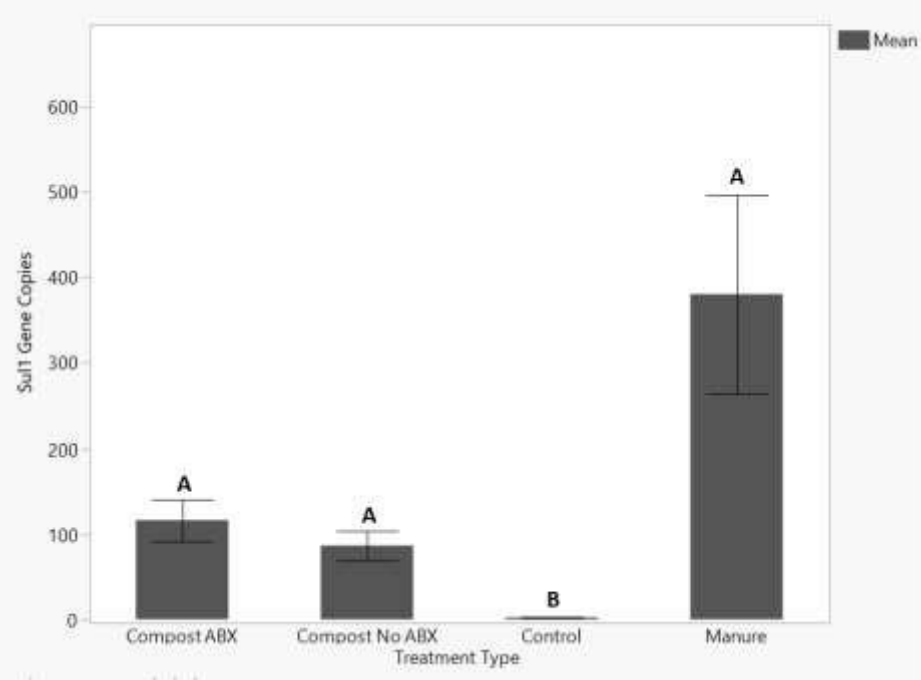

Figure 1: Effect of soil amendment type on the total sul1 gene copy numbers recovered from lettuce surfaces. Wilcoxon and Steel-Dwass were used to determine significance (p<.05). Error bars represent 1 standard error from the mean. Letters denote significant differences between soil amendment type (n=3).

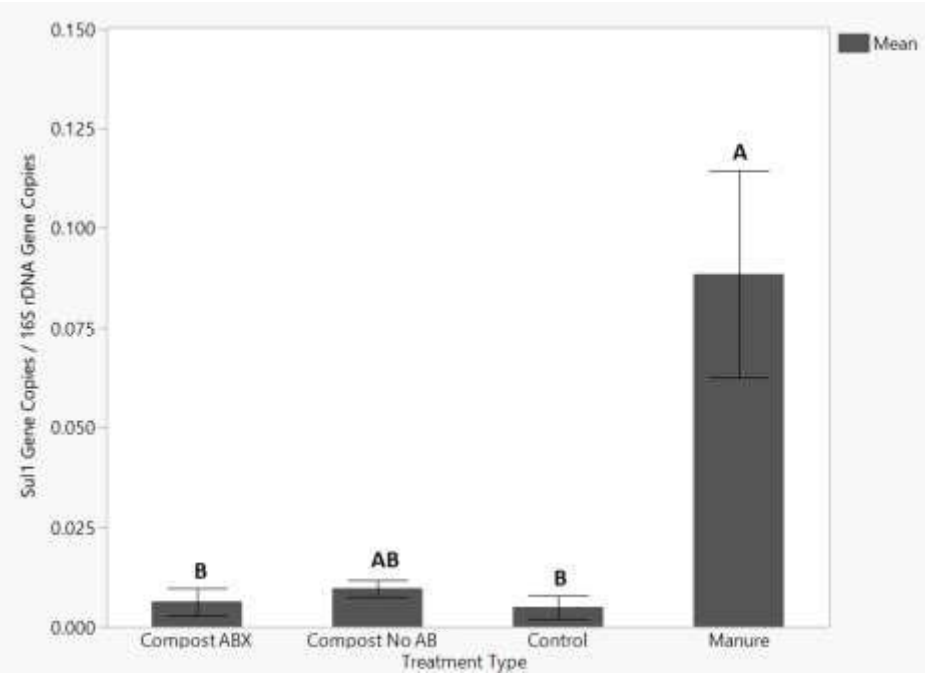

Figure 2: Effect of soil amendment on the proportion of sul1 gene copy numbers normalized by the 16s rDNA copy numbers recovered from lettuce surfaces. Wilcoxon and Steel-Dwass were used to determine significance (p<.05). Error bars represent 1 standard error from the mean. Letters denote significant differences between soil amendment type (n=3).

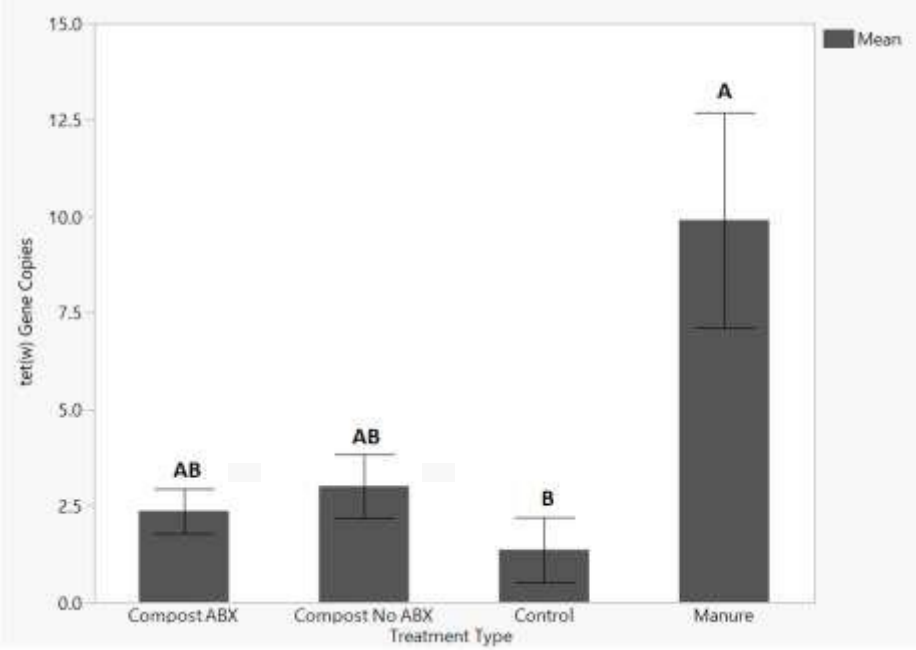

Figure 3: Effect of soil amendment type on the total tet(w) gene copy numbers recovered from lettuce surfaces. Wilcoxon and Steel-Dwass were used to determine significance (p<.05). Error bars represent 1 standard error from the mean. Letters denote significant differences between soil amendment type (n=3).

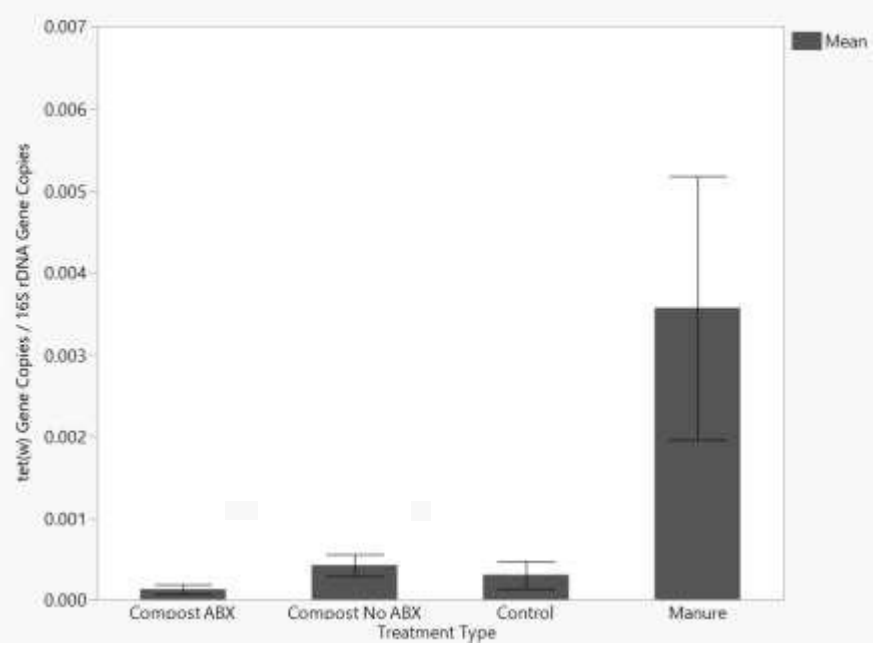

Figure 4: Effect of soil amendment on the proportion of tet(w) gene copy numbers normalized by the 16s rDNA copy numbers recovered from lettuce surfaces. Wilcoxon and Steel-Dwass were used to determine significance (p<.05). Error bars represent 1 standard error from the mean (n=3).

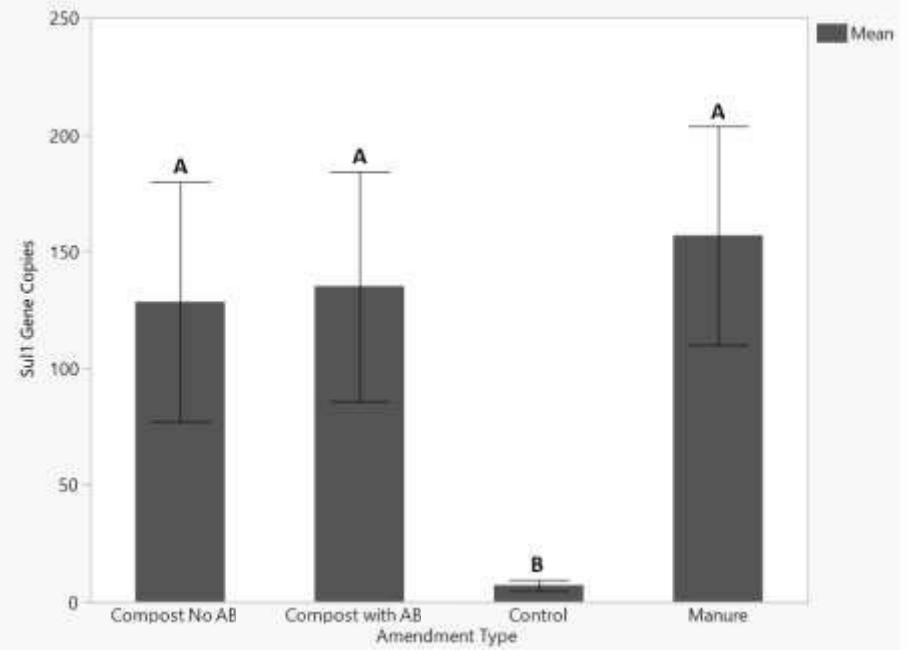

Figure 5: Effect of soil amendment type on the total sul1 gene copy numbers recovered from radish surfaces. Wilcoxon and Steel-Dwass were used to determine significance (p<.05). Error bars represent 1 standard error from the mean. Letters denote significant differences between soil amendment type. (n=3, n=2 Compost AB).

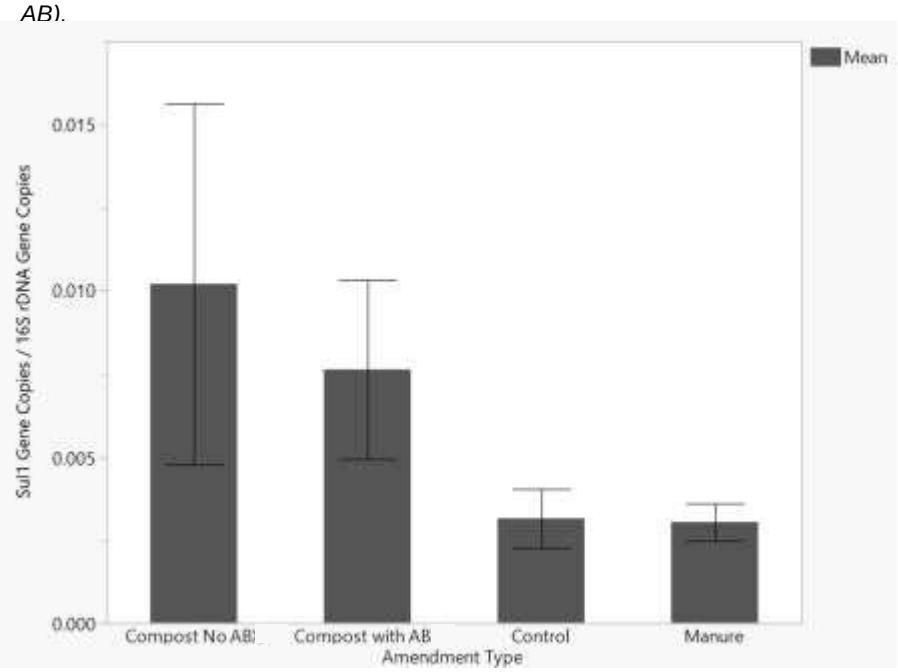

Figure 6: Effect of soil amendment on the proportion of sul1 gene copy numbers normalized by the 16s rDNA copy numbers recovered from radish surfaces. Wilcoxon and Steel-Dwass were used to determine significance (p<.05). Error bars represent 1 standard error from the mean (n=3, n=2 Compost AB).

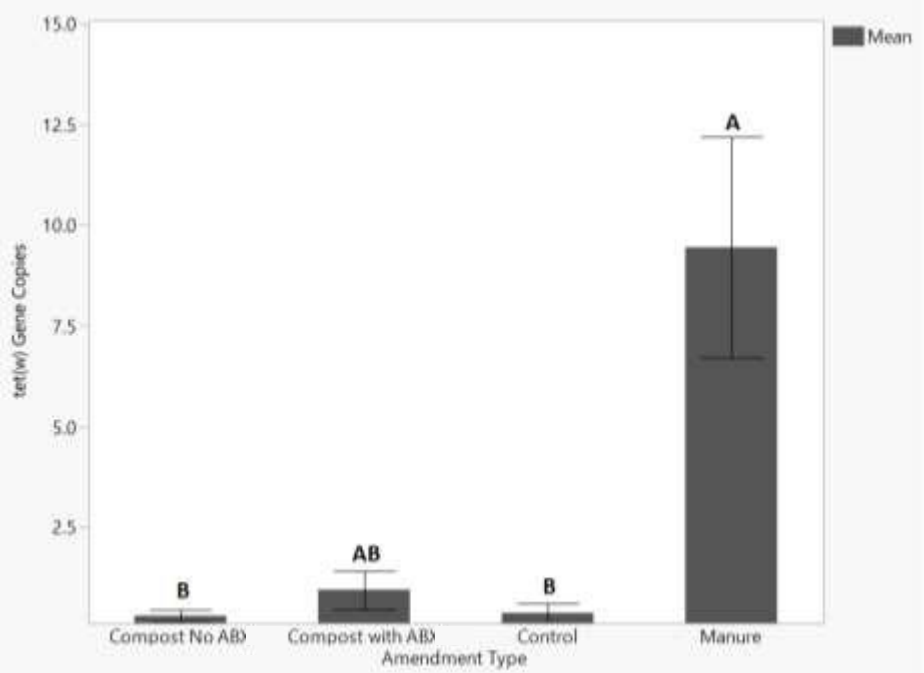

Figure 7: Effect of soil amendment type on the total tet(w) gene copy numbers recovered from radish surfaces. Wilcoxon and Steel-Dwass were used to determine significance (p<.05). Error bars represent 1 standard error from the mean. Letters denote significant differences between soil amendment type (n=3, n=2 Compost AB).

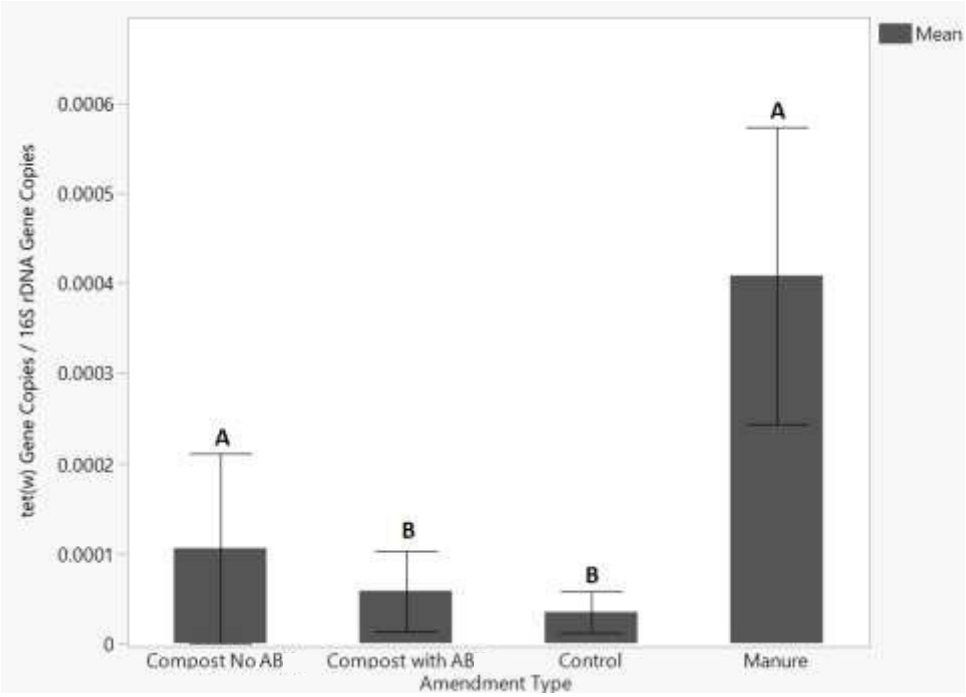

Figure 8: Effect of soil amendment type on the proportion of tet(w) gene copy numbers normalized by the 16s rDNA copy numbers recovered from radish surfaces. Wilcoxon and Steel-Dwass were used to determine significance (p<.05). Error bars represent 1 standard error from the mean. Letters denote significant differences between soil amendment type (n=3, n=2 Compost AB).

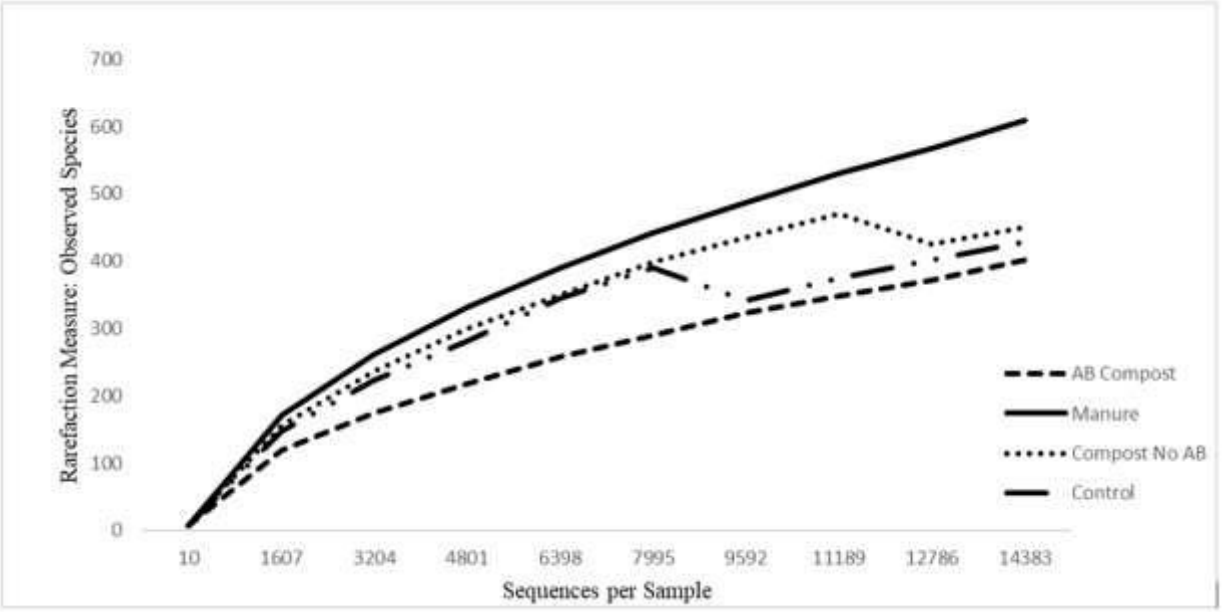

Figure 9: Rarefaction curves for lettuce grown in biological soil amendments indicating the observed number of operational taxonomic units within the 16S rDNA amplicons derived from lettuce samples.

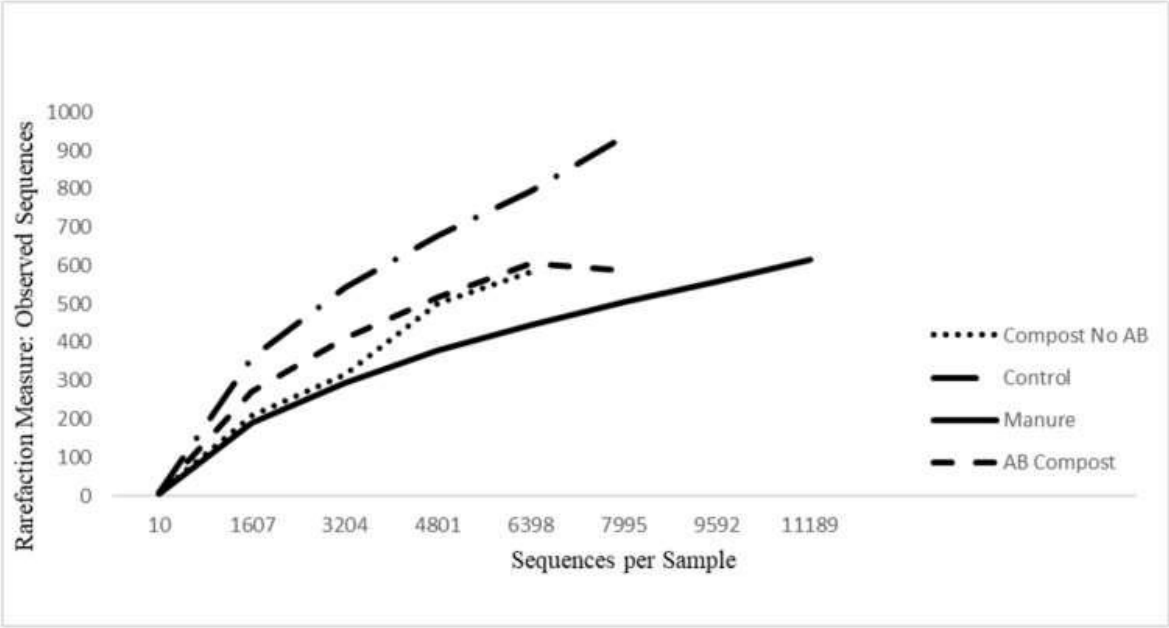

Figure 10: Rarefaction curves for radish grown in biological soil amendments indicating the observed number of operational taxonomic units within the 16S rDNA amplicons derived from lettuce samples.

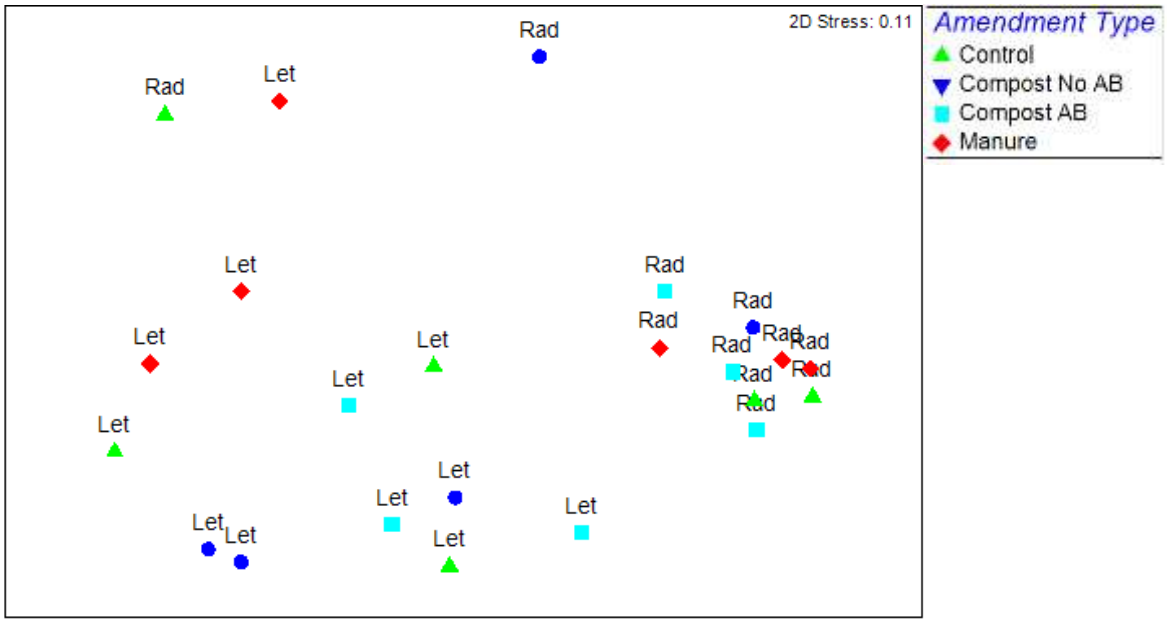

Figure 11: Unweighted Unifrac distances of the lettuce and radish samples grown in the field. ANOSIM concluded that samples were significantly different based on vegetable type (p<.001) and amendment type (p=.02). Samples were strongly separated based on vegetable type (Global R=0.77).

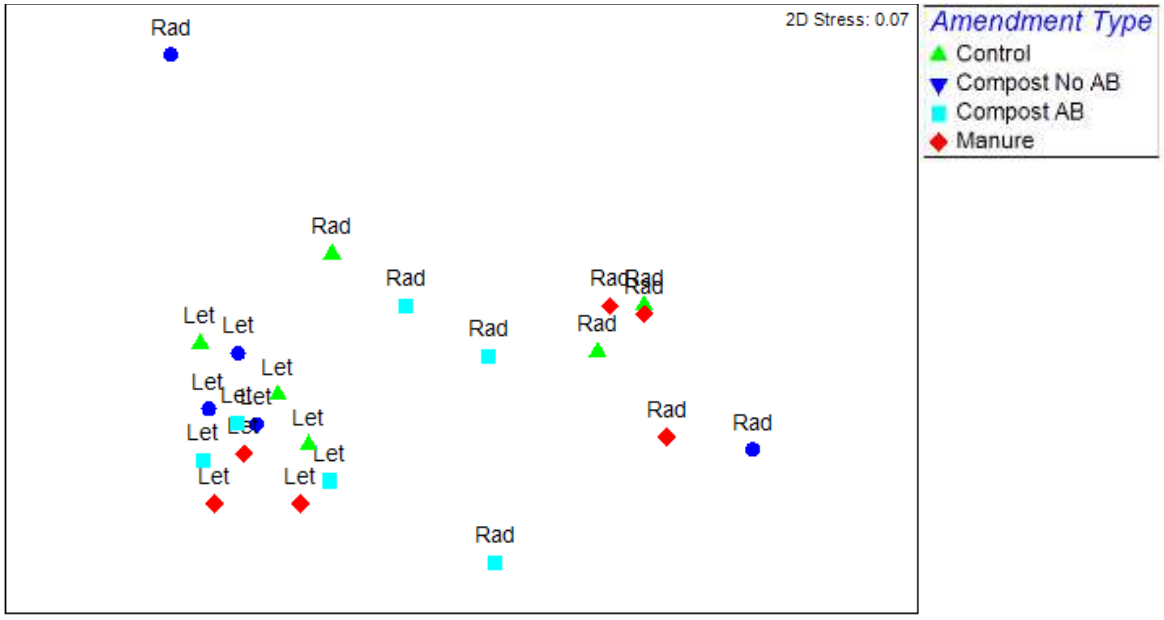

Figure 12: Weighted Unifrac distances of the lettuce and radish samples grown in the field. ANOSIM concluded that control samples were significantly different (p<.001) and strongly separated (Global R=0.76) based on vegetable type.

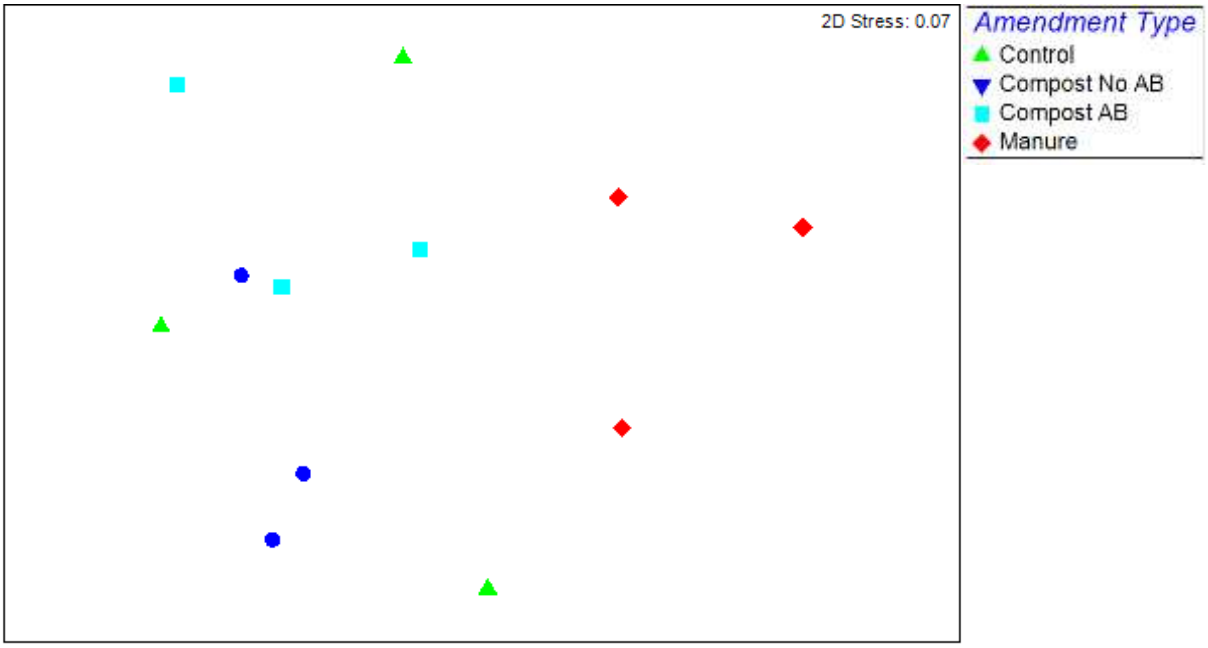

Figure 13: Unweighted Unifrac distances of the lettuce samples grown in the field. ANOSIM concluded that control lettuce was strongly separated from lettuce grown in antibiotic exposed compost (R= 0.96), separated from manure lettuce (R= 0.70), and compost without antibiotics (R= 0.37).

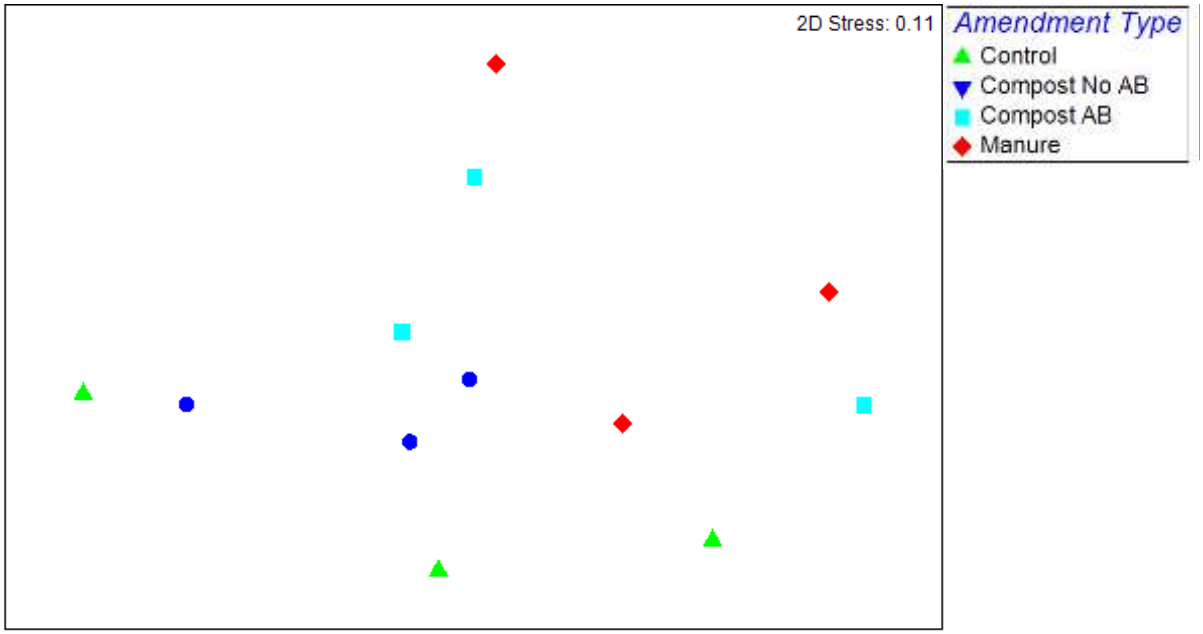

Figure 14: Weighted Unifrac distances from lettuce samples grown in the field. ANOSIM revealed that lettuce samples grown in control plots were slightly separated from lettuce grown in antibiotic exposed compost (R= 0.33).

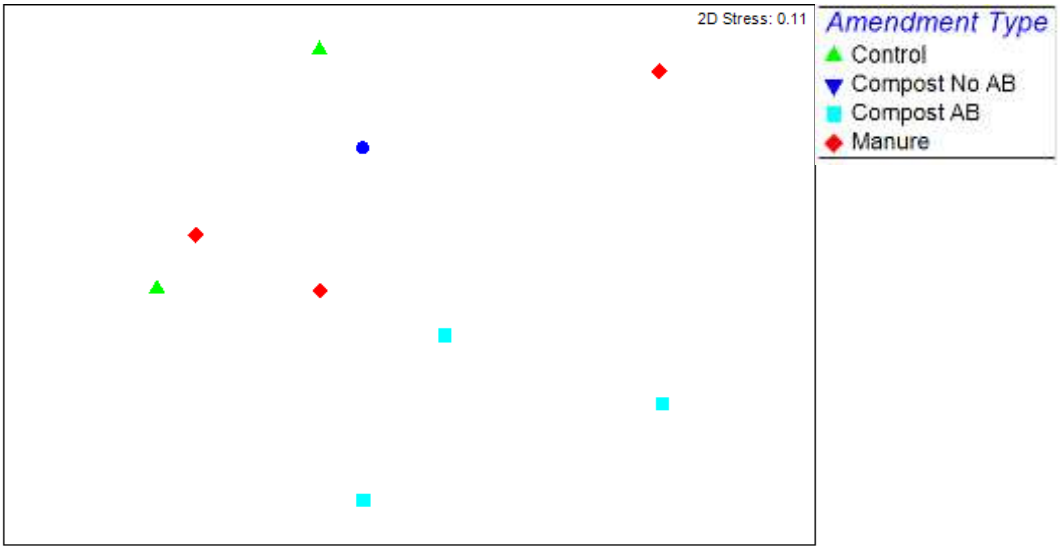

Figure 15: Unweighted Unifrac distances of the radish samples grown in the field. A Compost AB sample and Compost No AB sample is missing from the plot as they were outside of the 2D Stress Range. Control radishes were slightly separated from manure amended radishes (R=0.26) and antibiotic exposed radishes (R=0.25). Manure amended radishes were slightly separated from radishes grown in antibiotic exposed manure (R=0.33).

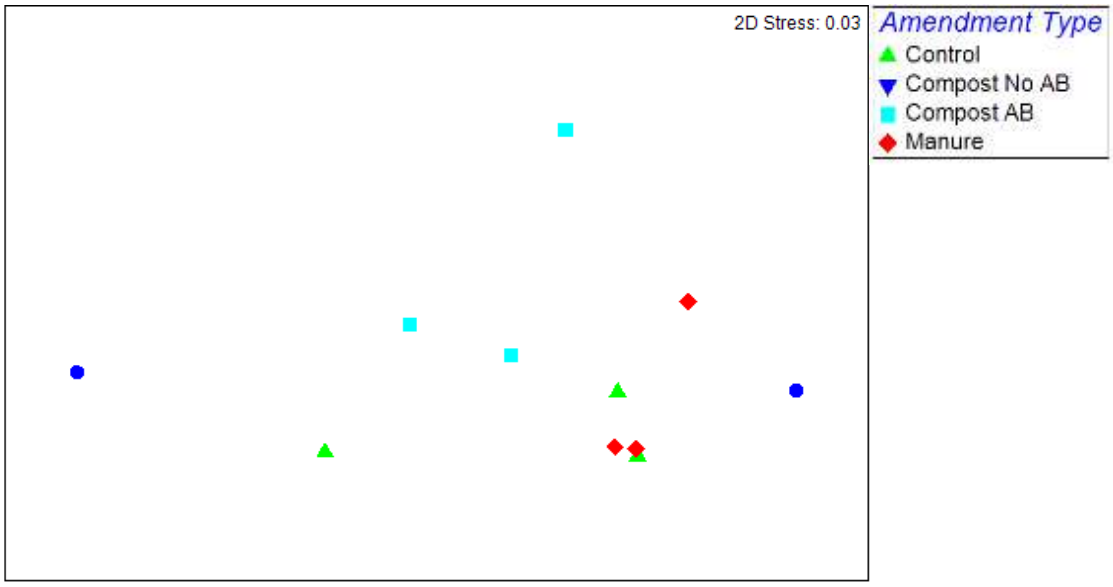

Figure 16: Weighted Unifrac distances from radish samples grown in the field. Radishes grown in manure were separated from control radishes (R=0.59) and antibiotic exposed compost samples (R=0.50). Control radishes were additionally separated from samples grown in antibiotic exposed compost (R=0.42).

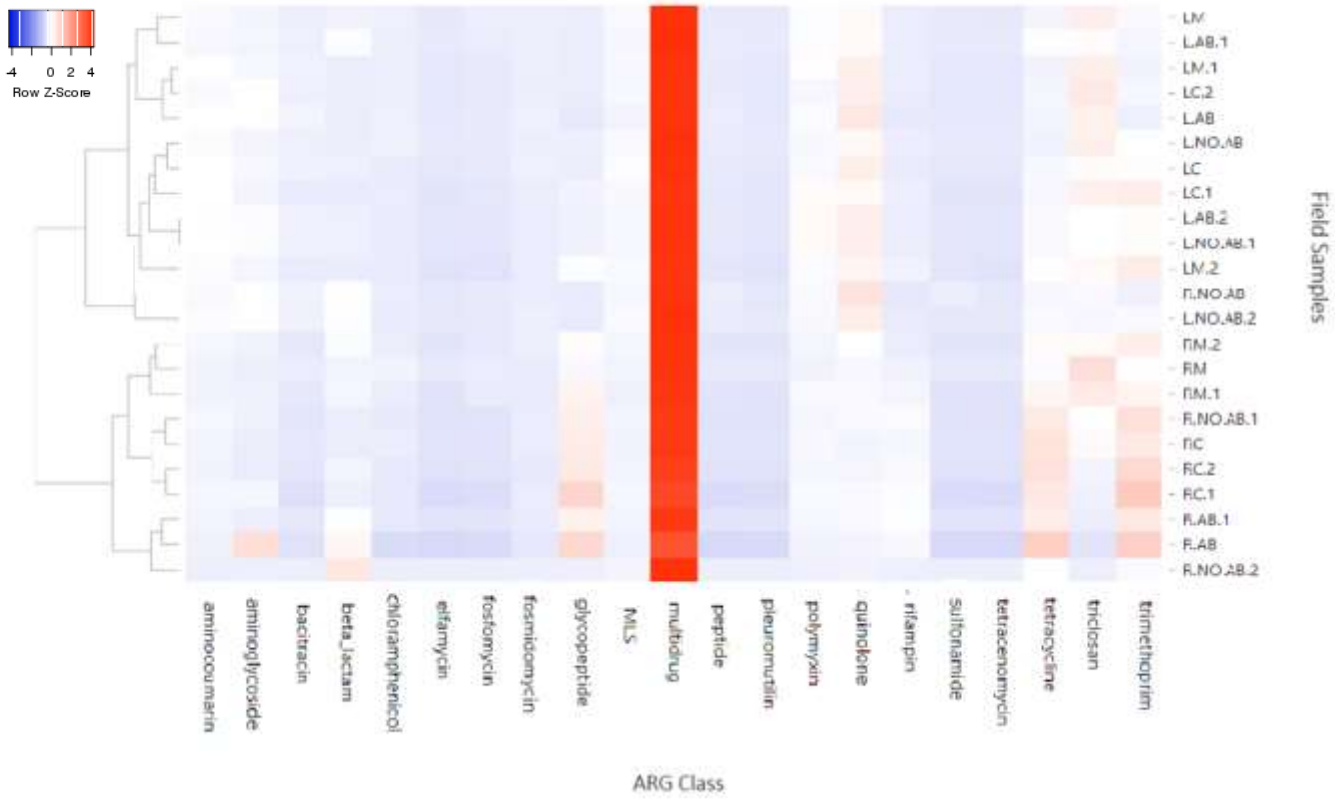

Figure 27: HeatMap displaying the Pearson Correlation values of the vegetable samples grown in the field. Red represents a positive association with the antibiotic class (y-axis) while blue represents a negative association. L = lettuce sample while R= radish sample. C= Control, NO AB= Antibiotic free compost, AB= Antibiotic exposed compost, M = manure samples.

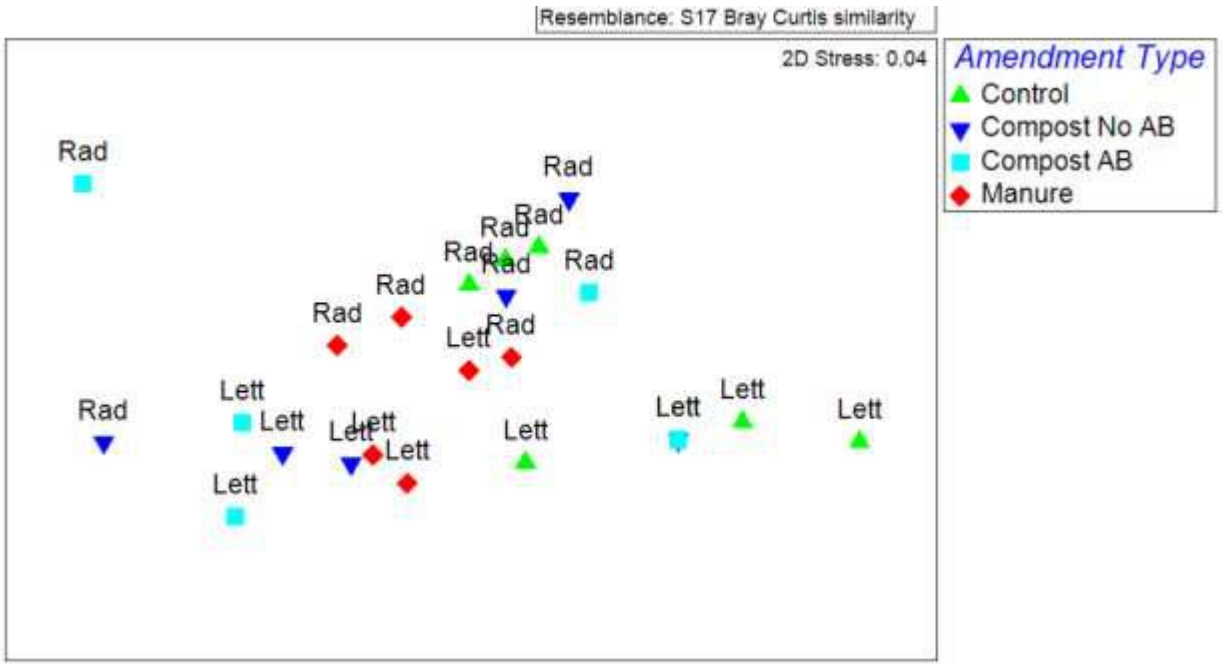

Figure 18: Bray Curtis similarities of the relative abundances of ARGs detected from the surfaces of the field samples. Vegetable type created a significant difference between samples (p=0.01) but amendment type did not (p>0.10). However, pairwise testing revealed that vegetables grown in manure were significantly different from vegetables grown in control plots and antibiotic exposed compost (p=0.02, p=0.03) and separated slightly (R=0.26, R=0.29), respectively (Fig 1).

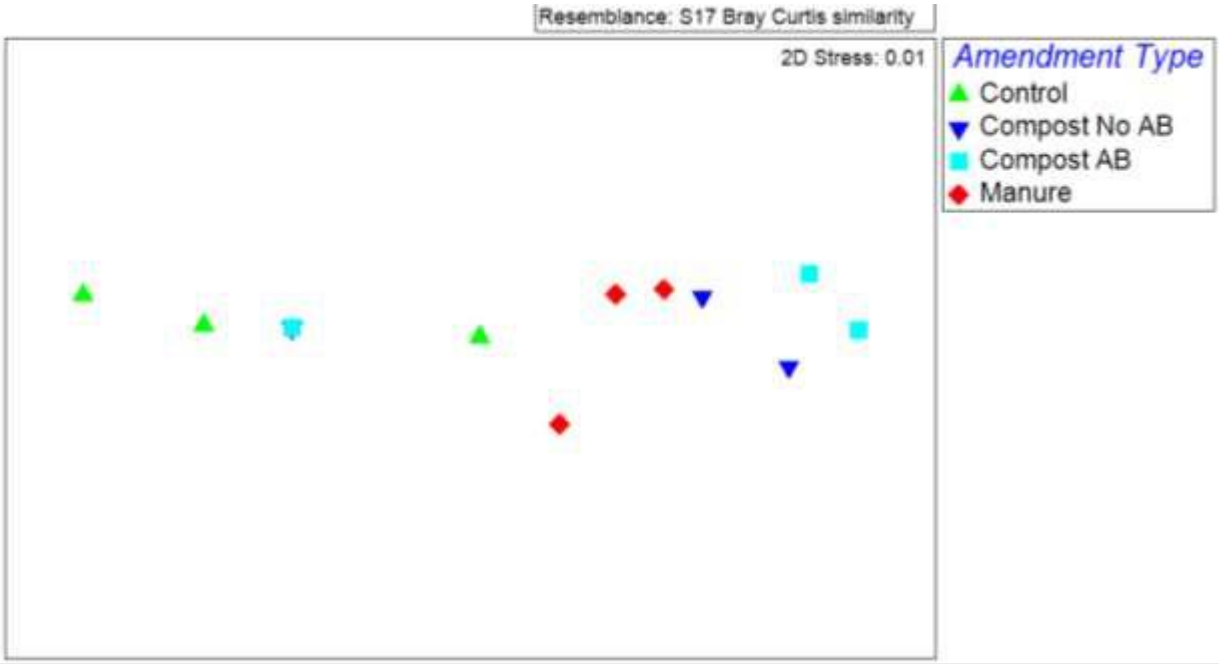

Figure 19: Bray Curtis similarities of the relative abundances of ARGs detected from the surfaces of the lettuce samples. ANOSIM determined that amendment type did not created a significant difference overall (Global R < 0.25, p>0.10) however lettuce grown in manure were significantly different from vegetables grown in control plots and antibiotic exposed compost (p=0.10) and were separate from one another (R=0.56).

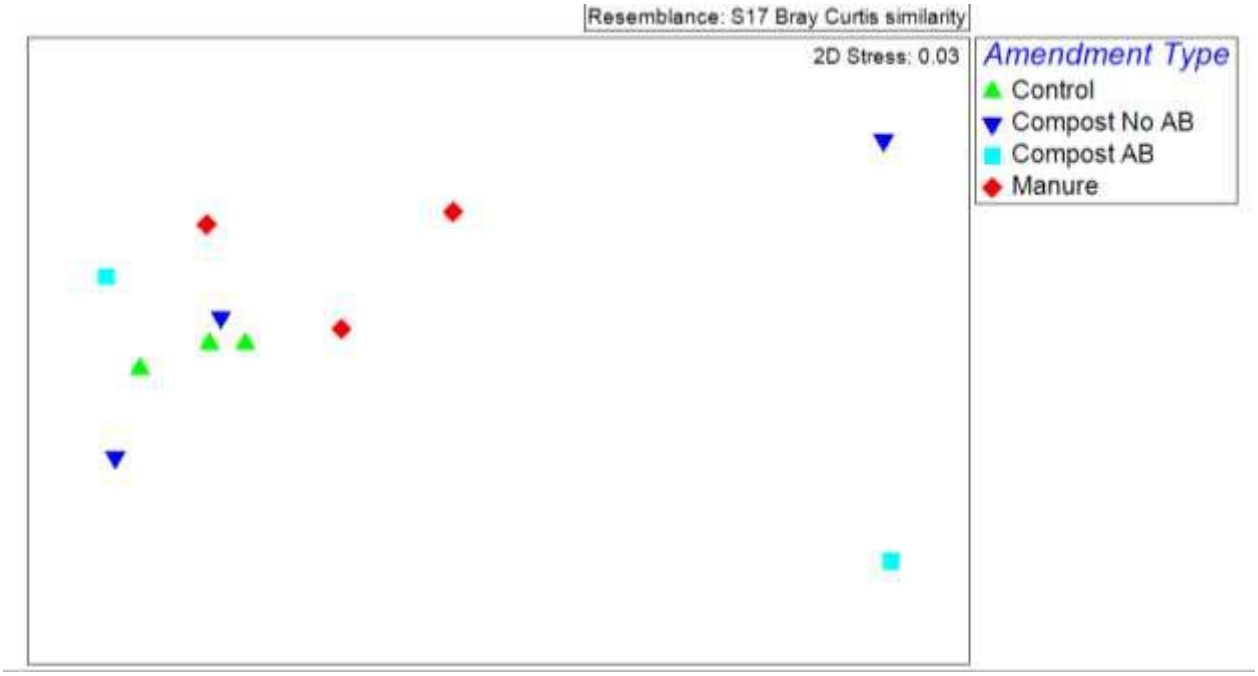

Figure 20: Bray Curtis similarities of the relative abundances of ARGs detected from the surfaces of the radish samples. ANOSIM did not define amendment type as an overall separation factor (p>0.10, Global R< 0.25) however radish samples grown in control plots were significantly different (p=0.10) and separated (R= 0.42) from radishes grown in antibiotic exposed compost and manure (R=0.41).

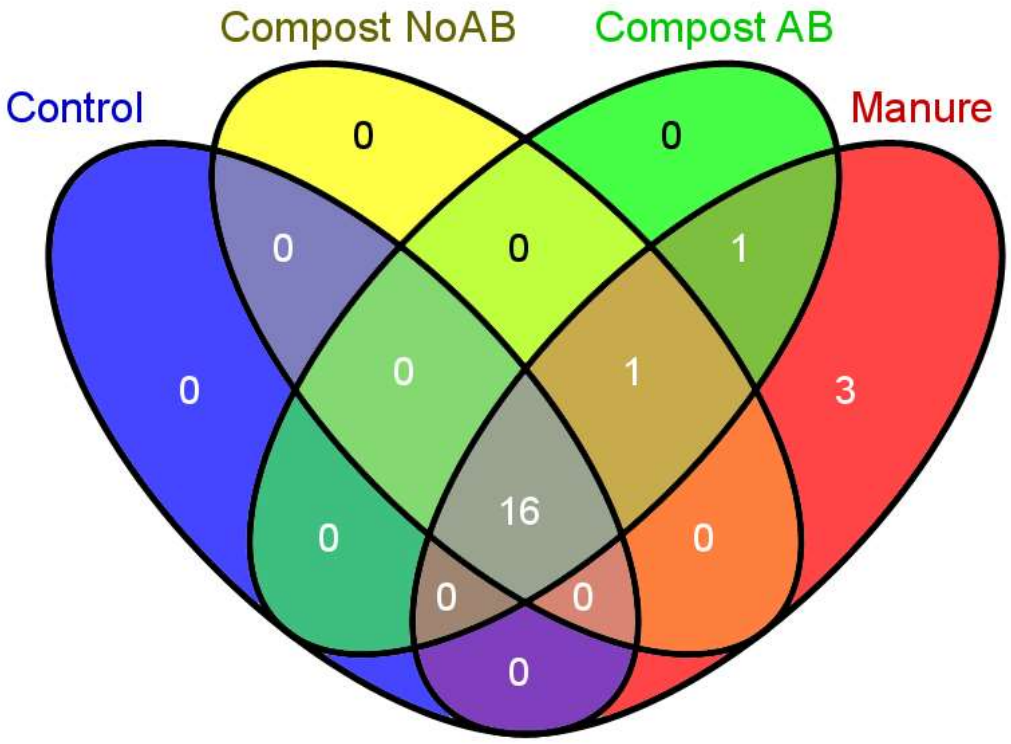

Figure 21: Venn Diagram of ARGs clustered by antibiotic classes identified from the 12 lettuce samples. Numbers represent the number of classes shared between samples, or unique to the samples, based on amendment type (n=3).

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Chapter 4: CONCLUSIONS AND FUTURE WORK

The aim of this study was to determine how agricultural practices affect the ARB and ARGs of vegetables commonly consumed raw. Raw manure created the most significant differences in the microbial communities and ARGs quantified from the vegetable surfaces. ARGs were more abundant on vegetables grown in raw manure; *Psedomonadaceae*, which are susceptible to ARG uptake because of their association with class 1 integrons, were also more abundant on radishes grown in raw manure. Static-composting tended to neutralize ARB and ARGs back to the same quantities as control vegetable samples, which were not exposed to BSAs. Vegetables grown in raw manure acquired resistance to three additional antibiotic classes. The microbial communities were also distinctly different based on ANOISM analysis, showing that *Pseudomonadaceae* were 40 times more abundant on radishes grown in raw manure. BSAs, both composted and raw, increased culturable bacteria tolerant to clindamycin from lettuce surfaces. Total *tet(w)* gene copies decreased on radishes grown in composted manure compared to the raw manure amendment. Normalized *sul1* gene copies decreased significantly for lettuce samples grown in composted manure, compared to raw manure samples. *Methylophilales* and *Methylophilaceae*, which are important microbes in composting, rhizosphere and gut microbiomes, were more abundant on compost-grown lettuce samples. This study concludes that that raw manure utilized as a soil amendment for crop production could act as an enhancer of antibiotic resistance in the farm-to-fork continuum.

The information derived from the present study helps answer several questions concerning ARGs in the farm-to-fork continuum, however there are still several knowledge gaps in existence. Studies with far greater sample size (>n=23) should be conducted in various locations to determine the influences of climate and geographical location on the ARB and

ARGs found on vegetables. The small sample size in this study greatly inhibited data interpretation, especially regarding the 16s rDNA Amplicon sequencing and Shot-gun metagenomic data which ideally requires a large samples size for data analysis. Additionally, clay loam was the soil of choice in this experiment; however, soil type should be further investigated as a factor in field studies as ARB and ARGs may not react the same in different soil matrices. Time is another important factor that was not fully investigated in this experiment. Plants were harvested before the recommended 120 days of raw soil amendment contact could be reached; a more detailed evaluation of the NOP guidelines, and FSMA composting guidelines should be investigated. Also, the age of a plant can affect the microbial communities and genes present on the surfaces of that plant, therefore different stages and seasons of harvest should studied in a large-scale field study to better understand. Studies testing the sensitivity of the detection of ARGs form vegetable surfaces via metagenomics should be conducted, as our study indicated that the results from metagenomics were not very sensitive. Lastly, the microbial communities associated with the radish taproots should investigated, as there is a gap in research on this subject.

Supplementary Data

Table S1: Analysis of field plot soil samples Conducted by Waypoint Analytical (Richmond, Virginia)

Organic Matter (%)	**Estimated Nitrogen Release (lbs/A)**	**Phosphorus: Mehlich 3 (ppm)**	**Potassium (ppm)**	**Magnesium (ppm)**
2.2	84	59	135	126
Calcium (ppm)	**Soil pH**	**Buffer Index**	**Acidity (meq/100g)**	**Cation Exchange Capacity (meq/100g)**
652	5.2	6.69	2.4	7.1
% Base Saturation: K	**% Base Saturation: Mg**	**% Base Saturation: Ca**	**% Base Saturation: H**	**Nitrate (NO_3N ppm)**
4.9	14.8	45.9	34	57

Supplementary Table 2: Number of reads generated and passed through quality filtering by MetaStorm; each sample and number of direct annotations identified from GreenGenes 2013 and CARD 1.1.8.

Vegetable Type	Amendment Type	# Reps Pooled	Input Read Pairs	Both Surviving	Forward Only Surviving	Reverse Only Surviving	Dropped	GreenGenes (2013)	CARD 1.1.8
Lettuce	Control	3	12916259	12705320	202394	7225	1320	20698	7783
Lettuce	Control	3	12262544	12077415	177927	6324	878	24970	7124
Lettuce	Control	3	1037000	1021244	15263	422	71	1685	1059
Lettuce	Compost No AB	3	12638396	12439634	190239	7381	1142	19471	8507
Lettuce	Compost No AB	3	12337620	12121516	205530	8954	1620	16533	20680
Lettuce	Compost No AB	3	15749048	15459798	279158	8388	1704	23953	28858
Lettuce	Compost AB	3	16079665	15784711	282749	10068	2137	24878	38092
Lettuce	Compost AB	3	1600000	1565180	33348	1176	296	2896	4503
Lettuce	Compost AB	3	14752653	14525025	215945	10121	1562	17213	21998
Lettuce	Manure	3	14815545	14568423	235101	10206	1815	21291	22665
Lettuce	Manure	3	17517012	17214530	286749	13266	2467	19306	16006
Lettuce	Manure	3	15810163	15568474	229322	10833	1534	21655	20510
Radish	Control	3	17663391	17358063	291951	11015	2362	11001	10150
Radish	Control	3	14162301	13922922	228613	8839	1927	8553	6407
Radish	Control	3	15293048	15028247	252510	10100	2191	11832	9461
Radish	Compost No AB	3	16131115	15867125	252061	9978	1951	9739	7931
Radish	Compost No AB	3	13829222	13558554	259637	8900	2131	25698	50782
Radish	Compost No AB	3	14593809	14373557	213377	5844	1031	30006	58670
Radish	Compost AB	3	15899871	15886345	0	0	13526	9224	5662
Radish	Compost AB	3	5264000	5166732	93212	3238	818	3419	2310
Radish	Manure	3	16806523	16488458	304766	10741	2558	16427	12994
Radish	Manure	3	15613728	15331959	267963	11482	2324	14118	19073
Radish	Manure	3	12061616	11861556	190612	7945	1503	8550	9380