Effect of Soil Type, Composting, and Antibiotic Use on Fate of Antibiotic Resistance Genes and Microbial Community Composition in Dairy and Beef Manure Applied Soils

By

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ACADEMIC ABSTRACT

Manure is a commonly used soil fertilizer, but there are concerns that this practice could affect the spread of antibiotic resistance genes (ARGs) from farm to fork. A microcosm-scale study evaluated the effect of prior antibiotic use (manure-based soil amendments generated from dairy and beef cattle with or without antibiotic administration), composting, and soil type on the quantity of ARGs and the microbial community composition of dairy and beef manure applied soil. ARGs were analyzed through novel metagenomic techniques and quantitative polymerase chain reaction of *sul1*, *tet*(W), and 16S rRNA gene, while the microbial community composition was determined via 16S rRNA amplicon sequencing. The results indicated that while prior antibiotic administration elevated the relative abundance of ARGs and changed the microbial community of raw manure applied soils, composting reduced this effect. However, compost applied soils still had a higher relative abundance of ARGs than the unamended soils and occasionally soil applied with raw manure of untreated cattle. Soil type may be a mediating factor as there were differences observed between the three soil types (sandy loam, silty clay loam, and silty loam) with sandy loam amended soils often having the least attenuation of ARGs. As the relative abundance of ARGs was still elevated and the microbial community composition still significantly different from the unamended soils after 120 days, these results suggest that 120 days is not a long enough waiting period between biological soil amendments and crop harvest for ARG dissipation.

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GENERAL AUDIENCE ABSTRACT

Antibiotics are lifesaving drugs that kill infection-causing bacteria. However, bacteria are living organisms and can adapt to stresses, such as antibiotics. When antibiotics are used, not all of the targeted bacteria are necessarily killed, and populations of resistant bacteria can survive. Resistant bacteria can not only continue to grow, but can also share their resistance capabilities with other unrelated bacteria through the transfer of antibiotic resistance genes (ARGs). ARGs are segments of DNA encoding mechanisms for the bacteria to survive antibiotic attack, such as pumping antibiotics outside of the cell or strengthening the cell wall so antibiotics cannot enter. The transfer of ARGs to human pathogens is of utmost concern, as it can cause once treatable diseases to turn deadly. Antibiotics are thus a double-edged sword because they can save lives on one hand, while their overuse or misuse can undermine their effectiveness by increasing antibiotic resistance. In the U.S. and many other countries, the biggest user of antibiotics is the livestock industry. Thus, there is growing interest in possible routes by which antibiotic resistance can spread from agriculture to humans. While some previous work has been done on direct contact with animals and meat products, less attention has been paid to the potential role raw produce grown in soils fertilized with manure-based amendments. This study thus sought to determine which factors impact ARG levels in soil. Questions of interest included: What is the effect of composting raw manure prior to soil application? Does prior treatment of cattle with antibiotics matter? Does the soil type influence the levels of ARGs? Do the ARG composition and microbial community composition respond similarly to such factors? These and other

questions were evaluated in a controlled environment by simulating amended field conditions in small glass jars (microcosms) containing mixtures of different soils and manure-based amendments. Three different soils were amended with one of the following manure-based amendments: raw manure from antibiotic administered cattle, composted manure of antibiotic administered cattle, raw manure from cattle not given antibiotics, composted manure of untreated cattle, and no amendment. This experimental setup was done in duplicate, one for treatments from dairy cows and one for the beef steer treatments. The experiment lasted 120 days, as that is a current standard for how long organic farmers must wait between manure application and crop harvest. Samples were taken throughout the 120-day experiment, and the quantity of targeted ARGs was determined by analyzing the DNA through qPCR, while the overall ARG profile was studied using a new tool, called metagenomics. To identify the kinds of bacteria present in the samples (microbial community composition), the 16S rRNA gene, which is a universal gene in organisms, was targeted and sequenced via amplicon sequencing. The results of these analyses indicated that administering antibiotics to cattle and then subsequently amending soil with their manure was associated with the highest levels of ARGs compared to the other treatments, but composting reduced the effect of prior antibiotic use. Depending on the ARG, composting decreased ARG levels relative to the other treatments, but in some instances, it increased ARGs compared to soils with raw manure of untreated cattle. Even after composting, there were still higher levels of ARGs in the soil than unamended soils. Different soil types did react differently to the amendments, but more research is needed. All of the treatments resulted in different changes to the microbial community composition and did not return to the unamended soil's community structure even after 120 days. Overall, based on these results, ARGs and the microbial community do not return to the initial condition within 120 days, which is a

recommended wait time between amendment and harvest, while composting and soil type appear to be mediating factors. Additional research is needed to further advance understanding of potential mitigation options and to benchmark them to defined and measureable risk endpoints.

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ANOSIM: Analysis of Similarities

ARB: Antibiotic Resistant Bacteria

ARG: Antibiotic Resistant Gene

CDC: Centers for Disease Control and Prevention

CEC: Cation Exchange Capacity

CFU: Colony Forming Unit

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

Compost without ABX: Soils amended with composted manure of cattle without antibiotics

FDA: Food and Drug Administration

FSMA: Food Safety and Modernization Act

HGT: Horizontal Gene Transfer

Manure with ABX: Soils amended with raw manure of antibiotic treated cattle

Manure without ABX: Soils amended with raw manure of cattle without antibiotics

MDS: Multidimensional Scaling

No Amendment: Soils without biological amendment

qPCR: Quantitative Polymerase Chain Reaction

USDA: United States Department of Agriculture

WHO: World Health Organization

Chapter 1—Introduction

Antibiotic Resistance Crisis

The discovery of antibiotics revolutionized medical treatment. These 'miracle drugs' have saved millions of lives [1], but due to excessive use antibiotic resistance has become a growing problem [2]. Antibiotic resistance is a worldwide, major threat to human health and international organizations, such as the World Health Organization, have taken measures to combat the threat [2]. If action is not taken, it is projected that the world will enter a post-antibiotic era where once commonly cured infections could become deadly [2]. Already, the U.S. Centers for Disease Control and Prevention (CDC) estimates that 2 million people a year in the U.S. contract a "serious infection" that is resistant to one or more of the normally prescribed antibiotics [3]. Worse, at least 23,000 people in the U.S. die annually because of antibiotic-resistant infections and more are estimated to have died from complications related to antibiotic-resistant infections [3]. Antibiotic resistance also adds significantly to healthcare costs, as it has been estimated that resistant infections have resulted in up to $20 billion excess healthcare expenditures a year [3]. The development rate of new antibiotics has been continually decreasing even though antibiotic resistance only continues to increase [3].

Antibiotic Resistance Genes

The development of antibiotic resistance takes place when bacteria adapt and are able to survive and grow even with antibiotics present [2]. Intrinsic resistance (natural antibiotic resistance) occurs among a wide variety of bacteria [4]. Even if bacteria are naturally resistant to one or more antibiotics, they can still develop resistance to others. Acquired resistance is when

bacteria develop resistance either through mutation [5], recombination of free DNA into their chromosomes [6], or horizontal gene transfer (HGT) [7]. The exact mechanisms bacteria can use to fight antibiotics varies. Bacteria can prevent antibiotics from reaching their target by altering the target's binding site, changing the permeability of their cell wall so the antibiotic cannot enter, using efflux pumps to pump the antibiotic out of the cell, and/or producing enzymes to degrade or inactivate the antibiotic. These mechanisms are all coded for by antibiotic resistance genes (ARGs). ARGs can be vertically transferred to daughter cells through cell replication and division, but they can also be transferred to other bacteria through HGT. Thus, in regards to the transfer of resistance from environmental to clinical settings, HGT is the main mechanism of concern, especially since genes from living or dead lysed cells can be transferred. HGT is done through transformation, transduction, or conjugation. Transformation is the incorporation of free DNA from the environment into the cell, transduction transfers DNA through bacteriophages, and conjugation is the direct transfer of DNA through cell-to-cell contact. When transferred, ARGs can be within a chromosomal segment or within mobile genetic elements (MGEs), such as plasmids or transposons. HGT and mutations are stimulated when the bacteria are under stress, such as low concentrations of antibiotics. Bacteria can also uptake and accumulate multiple ARGs resulting in multidrug resistance. With the majority of antibiotics in the U.S. used in the livestock industry (>78%) [8], it is critical to better understand the possible risks of ARGs transferring from the environment to human pathogens.

Antibiotic Resistance and Agriculture

Several links between antibiotic usage in agriculture and antibiotic resistance in human pathogens have been identified. For example, it was shown that avoparcin use in broiler chickens resulted in an increase in vancomycin-resistant *Enterococcus faecium* in humans [9]. There also

appears to be a link between methicillin-resistant *Staphylococcus aureus* (MRSA) in humans and livestock [10]. An older study saw an increase in tetracycline resistance amongst humans when the chickens they were in direct contact with were switched to a tetracycline medicated feed [11]. Despite the connection between agricultural antibiotic use and rise of antibiotic resistance, there has been little regulation in the U.S. addressing antibiotic usage in livestock. In fact, it was only as of January 1, 2017 that regulations within the Veterinary Feed Directive (VFD) became active and began limiting the use of antimicrobials in agriculture [12]. These regulations prevent animal producers from purchasing over-the-counter feed containing medically important antimicrobials [12]. Instead, they have to receive authorization from a veterinarian to use these medicated grains and only for disease treatment, as it is no longer allowable to use feed with medically important antimicrobials for growth promotion and/or feed efficiency [12]. Such antibiotics that are considered medically important are: sulfonamides, tetracyclines, macrolides, glycopeptides, penicillins, cephalosporins, quinolones, and fluoroquinolones [13, 14]. Decreased use of antimicrobials has shown to lead to a decrease in subsequent resistance levels. For example, a study in Denmark saw a decrease in *Enterococcus* spp. levels in pigs and broilers after reductions in antimicrobial usage [15]. In the U.S., one study demonstrated significantly lower levels of antibiotic resistant and multi-drug resistant *Enterococcus faecium* and *Enterococcus faecalis* in large-scale organically raised versus conventionally raised poultry [16]. However, the rate of decrease appears to differ among different antibiotic classes [15], the effects of antimicrobial usage may still last for many years [17], and there may be a reaction to the VFD with an increase in therapeutic antimicrobial use as was observed in the EU after a similar ban was established [18]. The new regulations in the U.S. are a step in the right direction for more judicious use of antibiotics in agriculture, but, as medically important antimicrobials are still allowable for

disease treatment and there could be a potential increase in prophylactic use of antibiotics, it is necessary to better understand possible control points for reducing the spread of antibiotic resistance in the environment.

Manure and Compost Soil Amendments

A potential and little explored vector for transfer of antibiotic resistance from the farm to consumers is through produce, especially produce eaten raw. Agricultural soil is typically fertilized to add nutrients to support and increase crop growth. It has been estimated that lactating dairy cattle produce up to 130 lbs of manure a day per 1,000 lb animal and that corn fed beef cattle produce around 65 lbs of manure a day per 1,000 lb cow [19]. Land application of manure and manure-based products provides a means to dispose of animal waste while also serving as an abundant, low-cost source of fertilizer to the soil and avoids the need for chemical fertilizers. However, the use of a biological amendment presents some known risks in terms of pathogens and may present additional unquantified risks in terms of contributing to the spread of antimicrobial resistance. In particular, fruits and vegetables that are not cooked prior to intake carry the risk of transferring fecal (enteric) diseases to the consumer [20]. There have been numerous foodborne pathogen outbreaks that have resulted in illnesses, deaths, and economic losses [20]. The U.S. Food and Drug Administration (FDA) has put in place guidelines to minimize the risks of pathogen transfer through the Food Safety and Modernization Act (FSMA) [21]. Currently, FSMA contains no standard for how many days farmers must wait between raw manure application and harvest, but raw manure cannot make contact with covered produce, produce typically consumed raw, when applied [22]. The National Organic Program regulates that there must be 120 days between amending fields with raw manure and the harvest of crops in contact with the soil [23]. The FDA is currently performing risk analyses so that an official

waiting period between amending soil with raw manure and harvest can be established [22]. However, there are currently no such guidelines in terms of preventing the risk of spread of antibiotic resistance or ARGs. Ongoing risk assessment efforts may or may not consider ARGs as an emerging contaminant [24] and risk to human health. Research provides evidence that the fate of antibiotics should be considered in these regulations as antibiotic administered livestock can excrete large quantities (up to 90%) of antibiotics or their metabolites in their waste (manure and/or urine) [25]. Also, the subsequent application of manure to soil has been shown to increase ARGs in soil [26-28] and on the surfaces of raw vegetables [28, 29].

Another form of biological soil amendment is composting raw manure prior to soil application. FSMA states two examples of composting methods, static or turned, which comply with their microbial standards regarding pathogen removal [21, 22]. These standards do not consider the effect of composting on ARGs and there are few studies that have actually quantified ARGs after composting [30-34]. A knowledge gap remains regarding composting's effect on antibiotic resistance [35], and there is a need for a study investigating ARGs in soil amended with compost generated following FSMA standards.

Soil Selection

Soil is incredibly important to crop production, but little is known about its potential role in attenuating ARGs and antibiotic resistant bacteria (ARBs) that may be present in manure and manure-based amendments. The limited studies that have examined more than one manure applied field site (different soils) were not designed to directly compare between soil types and thus had multiple differences between the samples [36, 37]. However, there is evidence to support that soil type may be a factor as controlled studies using different soil samples demonstrated that after irrigation from wastewater effluent [38] or reclaimed water [39] there

were differences in ARG quantities amongst the soils. Furthermore, studies have shown that the physical-chemical properties of soils, such as organic matter content, pH, and CEC, impact DNA [40] and antibiotic adsorption [41-43].

Goals and Objectives

The overall goal of this project was to identify critical control points for reducing the spread of ARGs from farm to fork via a controlled microcosm study. Specifically, this study was designed to consider critical control points of: use of antibiotics in livestock; livestock type; manure composting; and soil type to which manure-based amendments are land applied. This project specifically focused on dairy and beef cattle as the cattle industry is one of the top U.S. livestock industries [44] and cattle produce large quantities of waste [19]. Also, cattle, especially dairy, are administered antibiotics that are medically important for human health. To find and evaluate the above critical control points for antibiotic resistance, the following objectives were pursued:

1. Compare the quantity of ARGs and the bacterial community structure of three different soils types, representing the three soils with distinct dominant components (sand, silt, and clay), when subject to application with manure-based amendments.
2. Determine if prior antibiotic administration to cattle affects the levels of ARGs and the microbial community composition of subsequent manure or compost-amended soils.
3. Evaluate if composting, following FSMA standards, reduces the amount of ARGs in amended soils and how the microbial community is affected.
4. Assess if the 120-day National Organic Program standard is an adequate length of time for ARG levels and the microbial community composition to return to unamended soil conditions.

These goals and objectives were met by using quantitative polymerase chain reaction (qPCR), 16S rRNA gene amplicon sequencing, and new shotgun metagenomic DNA sequencing techniques. In particular, metagenomics provided novel and deeper insight into the above-mentioned objectives unlike many other studies have been able to do. With these objectives analyzed, we are able to provide guidance to farmers and regulators for controlling the spread of antibiotic resistance.

Thesis Outline and Attributions

This master's thesis effort was conducted as part of a large, interdisciplinary, collaborative Coordinated Agricultural Project funded by the U.S. Department of Agriculture's National Institute of Food and Agriculture Program (NIFA) award #2014-05280 (PI- Pruden). As a member of this team, I helped lead a large-scale soil microcosm study, where my thesis focuses specifically on the behavior of ARBs, ARGs, and the microbial community composition. A post-doctoral researcher, Chaoqi Chen, co-led the soil microcosm effort and is the co-author on the following manuscripts as well as the lead author on a companion study in preparation describing the fate of the antibiotics dosed to the cattle in these microcosms [45]. Co-author Giselle Guron, a post-doctoral researcher, provided significant technical assistance and guidance for all aspects of this project. Dr. Amy Pruden and Dr. Kang Xia are also co-authors on the following manuscripts as Dr. Pruden is the program director and Dr. Xia is a principal investigator of the project. Both provided guidance on experimental design and data analysis. Additional assistance in the lab was provided by Robert Williams (MS 2016), Kendall Fogler (MS candidate 2017), and Kimberly Hughes (graduate researcher).

The following three chapters detail the results and discussion of the microcosm-scale study. Chapter 2 is the results of the microcosms amended with manure or composted manure of

dairy cows and Chapter 3 is the parallel study using raw and composted beef steer manure. The data were analyzed and discussed with focus placed on the four objectives. Chapters 2 and 3 are written in manuscript style for future journal submission (Target Journal: PLOS ONE; Authors: C.A. Pankow[1], G.K.P. Guron[1], C. Chen[2], K. Xia[2], and A. Pruden[1] ; [1]Department of Civil & Environmental Engineering [2]Department of Crop and Soil Environmental Sciences). The final chapter, 4, discusses recommendations for the agricultural industry and the overall conclusions that can be drawn from this study.

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Chapter 2—Dairy Study

Effect of Soil Type, Composting, and Antibiotic Use on Fate of Antibiotic Resistance Genes and Microbial Community Composition in Dairy Manure Applied Soil

Abstract

Dairy manure is a commonly used fertilizer, but knowledge gaps remain regarding the risk and potential factors impacting the spread of antibiotic resistance genes (ARGs) from farm to fork. The objective of this study was to determine the effect of prior antibiotic use (cephapirin and pirlimycin), manure composting, and soil type on ARGs and the microbial community composition in amended soils. Microcosms representing three soil types (sandy loam, silty clay loam, and silty loam) were amended with dairy manure, with and without prior history of cephapirin and pirlimycin administration and with and without prior composting, and sacrificed with time in triplicate over 120 days. After 120-days of incubation, *sul1* and *tet*(W) were found to be elevated in all conditions receiving manure-based amendment, regardless of prior antibiotic use history or composting. On the first day, soils receiving manure from antibiotic-treated cows contained the highest relative abundance of total ARGs based on metagenomic shotgun sequencing, but composting removed the effect of prior antibiotic use. The three soil types reacted differently to the amendments and had different levels of ARGs, with ARGs tending to persist the most in sandy loam applied soils. Application of any of the manure-based amendments changed the microbial community composition of all soils, relative to unamended controls, even after 120 days. These results indicate that soil type may be a mediating factor influencing ARG levels and while antibiotic use does impact ARGs and the microbial community, composting reduces this effect. However, the compost treated soil did not always

have the lowest relative abundance of total ARGs amongst the treatments. These results provide insight for on-farm practices that can help mitigate the spread of antibiotic resistance.

Introduction

Antibiotic resistance is a growing concern and, with the majority of antibiotics being administered to livestock, it is imperative to understand the effects of antibiotic use on the spread of antibiotic resistance via impacted environments. In 2013, it was estimated that over 13.5 million kg of antibiotics of the 17.2 million kg used annually in the US was for livestock (>78%) [1]. This number only continued to increase as by 2015 the US Food and Drug Administration (FDA) reported that around 15.58 million kilograms of antimicrobials were sold for use in food-producing animals in the US [2]. Antibiotics in agriculture are used as growth promoters as well as to treat and prevent disease [3]. In dairy cows, antibiotics for disease treatment are most commonly given to treat mastitis, an inflammation of the mammary gland [4]. In 2007, Eighty percent of dairy operations in the U.S. treated all of their cows with antibiotics during dry-off (lactation is ended) [4]. The top three antibiotic classes used in the U.S. for mastitis treatment in 2007 were cephalosporins, lincosamides, and noncephalosporin beta-lactams [4]. Pirlimycin is of particular interest as a lincosamide primarily used to treat mastitis through an intramammary infusion. In 2007, greater than 19% of dairy cows that had mastitis were given pirlimycin as the primary antibiotic treatment [4]. Lincosamides prevent protein synthesis by binding to the 50 S ribosomal subunit [5]. The World Health Organization [6] classifies lincosamides as a “highly important antimicrobial” in their published list of critically important antimicrobials for human health [6]. Cephapirin is also of special interest, as a first-generation cephalosporin also widely used to treat mastitis. In 2007, over 50% of U.S. dairy cows were administered a cephalosporin as the primary treatment for mastitis [4]. Cephapirin binds to penicillin binding proteins,

preventing cell wall synthesis and resulting in cell lysis [7]. First generation cephalosporins are also classified by WHO as a "highly important antimicrobial", but 3rd, 4th, and 5th generation cephalosporins, such as the commonly used veterinarian antibiotic ceftiofur (3rd generation cephalosporin), are "critically important" to human health [6].

Depending on the antibiotic and species type, antibiotics can be excreted in waste products unchanged or as a metabolite at rates as high as 90% [8]. These high excretion rates can result in a pathway for antibiotics to enter the environment when manure-based amendments are applied to soil as a fertilizer. Studies have shown that the application of animal manure to soil increases antibiotic resistance levels and can persist for varying lengths of time [9-16]. With raw manure being an abundant, economic, and relatively environmentally-friendly choice of fertilizer, it is important to seek optimization of management practices for balancing the benefits of manure-based soil amendments with the goal of reducing the potential to contribute to the spread of antibiotic resistance in the environment.

A proposed management strategy to reduce the spread of antibiotic resistance genes (ARGs) is to compost raw manure prior to field application [17]. The U.S. FDA Food Safety and Modernization Act (FSMA) recommends two composting methods, static and turned composting [18] in order to achieve standards for pathogen reduction. However, it is unknown the extent to which such practices could provide benefits with respect to reducing the potential for spread of antibiotic resistance. Besides composting, another possible control point for reducing ARGs is the type of soil to which the manure-based amendments are applied. Studies have shown that different soil characteristics affect antibiotic attenuation and sorption [19-22]. However, there are limited studies looking at the impact soil type has on ARGs following manure application [23, 24] and none, to the authors' knowledge, in a controlled environment with different soil types

applied with the same manure or compost amendment. A third possible management strategy is reducing antibiotic administration to cattle. A knowledge gap exists regarding the possible impact that antibiotic usage in cattle has on ARGs in soil influenced by manure-based amendments. While there have been many studies looking at the effects of manure-applied soil, there have been few studies that have specifically examined the effect antibiotic use has on ARGs and antibiotic resistant bacteria (ARBs) [25-27]. The studies that have been done have compared the effect of antibiotics by comparing two different herds (conventionally versus organically raised), which may be misleading as it has been suggested that the antibiotic resistome may naturally differ between herds [28]. Also, less work has been done looking specifically at the effects of antibiotics as administered, especially intramammary administration opposed to injected and/or orally administered antibiotics [29], rather than spiking antibiotics exogenously to the system.

The objective of this study was to determine the effect of prior antibiotic use in dairy cows, composting, and soil type on the fate of ARGs following application of manure-based amendments. Soil microcosms representing three soil types (sandy loam, silty clay loam, and silty loam) were amended with dairy manure with and without prior history of cephapirin and pirlimycin administration and with and without prior composting and sacrificed with time in triplicate. In addition to tracking ARGs via quantitative polymerase chain reaction (qPCR) and shotgun metagenomic sequencing, bacterial community composition was also profiled to characterize the microbiological stability with time. The results can help identify critical control points for informing agricultural management practices that minimize potential to spread antibiotic resistance.

Materials and Methods

Microcosm Setup

The compost and manure used in the microcosms were generated in a prior experiment [30]. Two manure types (dairy with or without prior intramammary administration of pirlimycin and cephapirin) were collected over a period of three days at peak antibiotic excretion (excretion rate determined in Ray et al. [30]). Half of the two manure types were composted according to subpart F of FSMA static composting guidelines (aerobically at 55°C for 3 days, cured to 42 days) [18], while the other half was stored at 4°C to preserve the manure during this composting period. Three locally-available soils were collected from different Virginia farms in order to represent the three main components of soil (sand, silt, and clay). Sandy loam (Eunola loamy fine sand), silt loam (Guernsey silt loam), and silty clay loam (Carbo and Chilhowie silty clay loam) were collected and identified with Web Soil Survey [31]. Prior to placement in the microcosms, the soil was air-dried, grinded, and screened by a 2 mm sieve. In addition, the soils were analyzed in order to determine several properties, such as field moisture capacity (S1 Table).

The three soil types were mixed with the following five treatments: raw manure from dairy cows administered antibiotics, raw manure from dairy cows not administered antibiotics, composted manure from dairy cows administered antibiotics, composted manure from dairy cows not administered antibiotics, and no amendment. Each soil and treatment combination was made in triplicate and for each time point throughout the 120-day experimental period, as a destructive sampling technique was used. The different amendments were hand mixed with 600 g of soil. Amendment amounts were determined using a normal application rate of 203 ± 25 pounds of nitrogen per acre, and the assumption that 10% of the specific amendment's total

nitrogen content was bioavailable. Each amendment underwent prior analysis to determine nitrogen and moisture content (S1 Table). After homogeneously mixing the amendment and soil together, it was distributed to 100 mL glass jars (Fisher Scientific, Waltham, MA) for a total mass between 15-18.9 grams. The aerobic microcosms were kept in the dark at 24°C, and the moisture was maintained at 50% field moisture capacity by weekly weighing each jar and watering with deionized water to compensate the weight loss due to evaporation.

Sample Collection and Preparation

At each time point, the microcosms were mixed to homogenize prior to sampling. The sample was placed in a 2.0 mL cryogenic screw cap vial (Corning Incorporated, Corning, NY) and stored at -20°C for later DNA extraction. The soil samples were extracted using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH). Five hundred milligrams of the sample was weighed into the Lysing Matric E tubes. Kit instructions were followed using the FastPrep-24™ 5G Instrument (MP Biomedicals, Solon, OH) to homogenize and lyse the samples. An optional incubation period following bead-beating was included, and the samples were maintained at room temperature for 2-hours to allow for maximum lysing of cells. Following DNA extraction, the DNA was cleaned using the OneStep PCR Inhibitor Removal Kit (Zymo Research Corporation, Irvine, CA). The final DNA product was stored at -80°C.

Molecular Analysis

For qPCR, SsoFast Evagreen Supermix (BioRad Laboratories, Hercules, CA) and CFX96™ Touch Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA) were used following manufacturer instruction to quantify the concentrations of the target genes. Triplicate technical replicates of each sample were run along with triplicate standard curves and

a negative control. The standard curve was a serial dilution, which 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 molecular grade water (Sigma-Aldrich, St. Louis, MO). For *tet*(W) and *sul1*, the lowest standard (10^1 gene copies/µl) in the linear range was considered the quantification limit. In order to obtain the relative abundances of *tet*(W) and *sul1*, the gene copies were normalized to the gene copies of the 16S rRNA gene quantified from qPCR. DNA was diluted to 1:1000 for 16S rRNA quantification and 1:100 for *sul1* and *tet*(W) quantification. The DNA dilutions were stored at -20°C and the primer sequences used are described in Ma et al. [32].

Metagenomic analysis was performed on a cross-section of samples from day 1 and 120. Five lanes of 12 samples each (total of 60 undiluted DNA samples) were submitted to the Genomics Research Laboratory of the Biocomplexity Institute (BI) of Virginia Tech (formerly Virginia Bioinformatics Institute), Blacksburg, VA. BI performed the library preparation (Trueseq) and sequenced the samples on an 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 [33]. MetaStorm is an online platform that allows metagenomics data to be analyzed using specific databases. The Comprehensive Antibiotic Resistance Database (CARD v1.0.6) was used as the ARG functional annotation reference database for the read matched samples [34]. The relative abundances of the total detected ARGs from shotgun metagenomics was determined in MetaStorm by normalizing the gene counts to the abundance of 16S rRNA gene as described in Li et al [35]. The plasmid associated genes were identified through the ACLAME database [36] and also normalized to the 16S rRNA gene copies through MetaStorm.

All samples from day 1 and 120 underwent PCR amplification using barcoded primers (515F/926R), which target the V3 region of the 16S rRNA gene. Due to soil's vast biodiversity and in order to reduce amplification bias, samples were normalized based on 16S rRNA gene copy numbers quantified by qPCR prior to barcode PCR. Dilution tests also showed that a 1:1000 DNA dilution resulted in a strong amplification of PCR product, as examined on an agarose gel. Triplicate PCR products were pooled on an equal mass basis of 200 ng and cleaned using QIAquick PCR Purification Kit (QIAGEN, Valencia, CA). The final pooled product was submitted to BI for paired-end 300 cycle sequencing on the Illumina Miseq. PANDAseq [37] 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 [38] was used to annotate the reads to the Greengenes 16S rRNA gene database [39], after which mitochondrial and chlorophyll sequences were filtered out of the OTU table. The samples had a read length of 3940-21572, and all samples were rarefied to 3940.

Statistical Analysis

For all analyses, the significance cutoff value was set at $p \leq 0.05$. Depending on the data distributions (Shapiro-Wilk test), parametric or non-parametric tests were selected. For qPCR, the target gene copy numbers normalized to the 16S rRNA gene copy numbers were analyzed with nonparametric tests. Comparisons of the relative abundances of various bacterial phyla were also done using the same nonparametric tests as qPCR. These tests were run on JMP (SAS Institute Inc., Cary, NC) using the Wilcoxon/Kruskal-Wallis Test (Rank Sums) and, for multiple comparisons, the Steel-Dwass All Pairs test. The relative abundance of total plasmid associated genes had a normal distribution ($p=0.7371$; Shapiro-Wilk test), and thus the parametric tests Oneway ANOVA and Tukey-Kramer HSD were run in JMP. The relative abundances of total

ARGs from shotgun metagenomics and the overall rarefied bacterial compositions were compared in PRIMER-E (version 6.1.13) using analysis of similarities (ANOSIM). Multidimensional scaling (MDS) plots were created in the PRIMER-E software.

Results

Soil microcosms were sacrificed in triplicate on day 1 and day 120 and compared with respect to relative abundance of target ARGs *sul1* and *tet*(W) determined by qPCR, relative abundance of total ARGs determined by shotgun metagenomic sequencing, and microbial community composition determined by 16S rRNA amplicon sequencing. For the 16S rRNA amplicon sequencing data, the statistics and graphs compared the nine most abundant bacterial classes in the samples with the remaining classes combined into an "other" category. The data were analyzed to determine the effect of prior antibiotic administration, static composting, and soil type on ARGs and the microbiota of manure and compost-applied soils relative to unamended background soils.

Effect of Prior Antibiotic Administration on ARGs and Microbiota

Results indicated that the prior administration of antibiotics to dairy cows was associated with higher relative abundance of *sul1* in soil amended with raw manure ($p<0.05$; Steel-Dwass All Pairs). Soil amended with manure from cows that received antibiotics contained the highest levels of normalized *sul1* at both days 1 and 120 (Fig 1). This trend was not observed with respect to *tet*(W) (Fig 2), as there was no significant difference between soil with manure from cows with or without antibiotic administration at either time point ($p >0.05$; Steel-Dwass All Pairs). Soil receiving composted manure showed no significant difference in relative abundance of *sul1* or *tet*(W) between cows with or without antibiotic treatments ($p >0.05$; Steel-Dwass All

Pairs) (Figs 1 and 2). The one exception was *tet*(W) on day 1 (Fig 2A), where compost applied soil from cows not administered antibiotics was significantly higher than soil with compost from cows dosed with antibiotics (p=0.035; Steel-Dwass All Pairs).

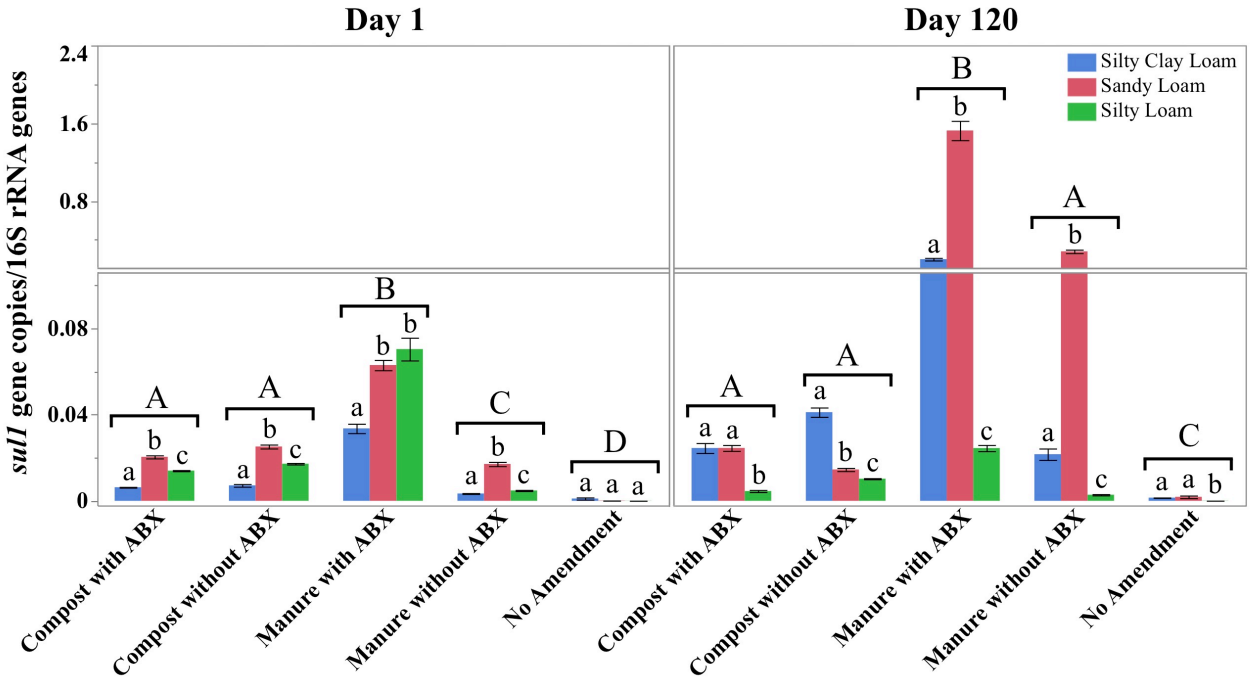

Fig 1. *sul1* qPCR Results. Relative abundance of *sul1*, normalized to 16S rRNA gene copy numbers, for the five different amendment conditions and specific soil types. Per timepoint, the capital letters indicate significance between the five amendment conditions, and the lowercase letters indicate significance among the soil types within each specific treatment (p<0.05; Steel-Dwass All Pairs). ABX = prior antibiotic administration of corresponding manure.

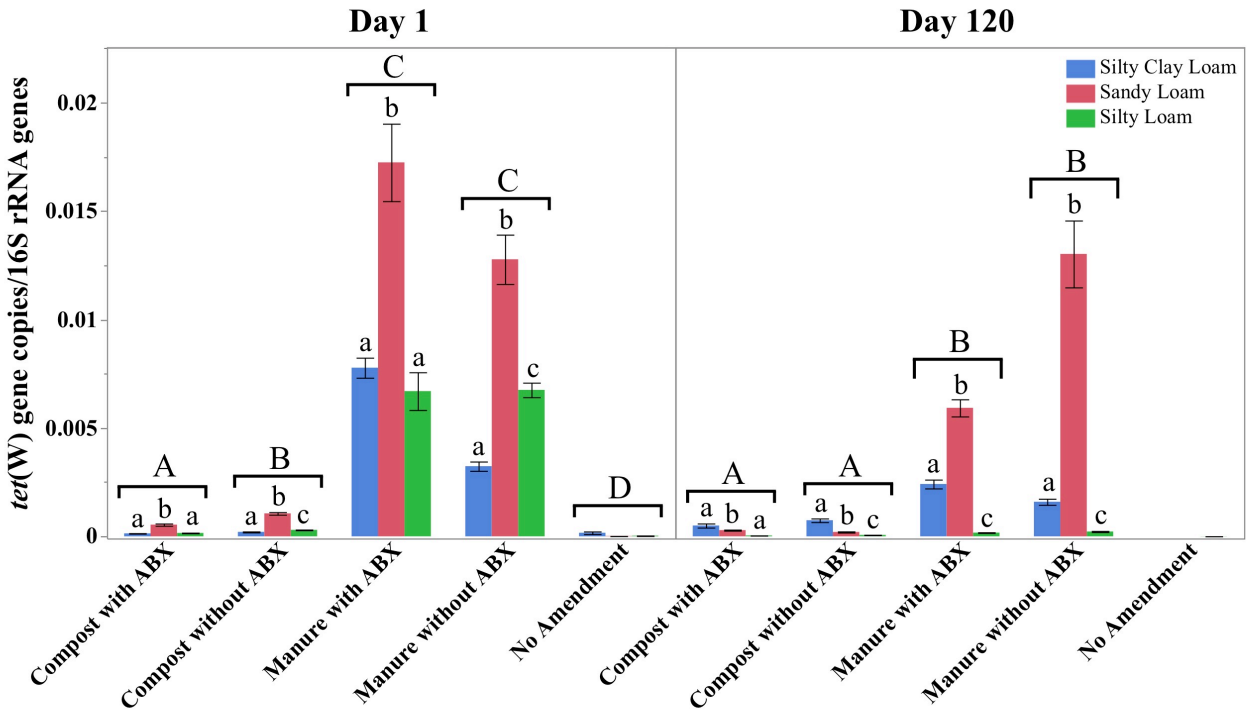

Fig 2. *tet*(W) qPCR Results. Relative abundance of *tet*(W), normalized to 16S rRNA gene copy numbers, for the five different amendment conditions and specific soil types. Per timepoint, the capital letters indicate significance between the five amendment conditions, and the lowercase letters indicate significance among the soil types within each specific treatment ($p<0.05$; Steel-Dwass All Pairs). Statistical comparisons of the non-amended soils (No Amendment) were limited as most were below the detection limit, particularly the DNA of samples from day 120. ABX = prior antibiotic administration of corresponding manure.

Shotgun metagenomic sequencing similarly indicated that, at day 1, there were significant differences in the relative abundance of total ARGs between the soils applied with raw manure from cows administered versus not administered antibiotics ($p=0.008$; ANOSIM) (Fig 3A). However, in contrast to the trend observed for *sul1*, this difference was no longer significant at day 120 ($p=0.063$; ANOSIM) (Fig 3B). When manure was composted, there was no significant effect of prior administration of antibiotics on the total ARG relative abundance profiles, either at day 1 or 120 ($p=0.659$ and $p=0.857$ respectively; ANOSIM) (Fig 3).

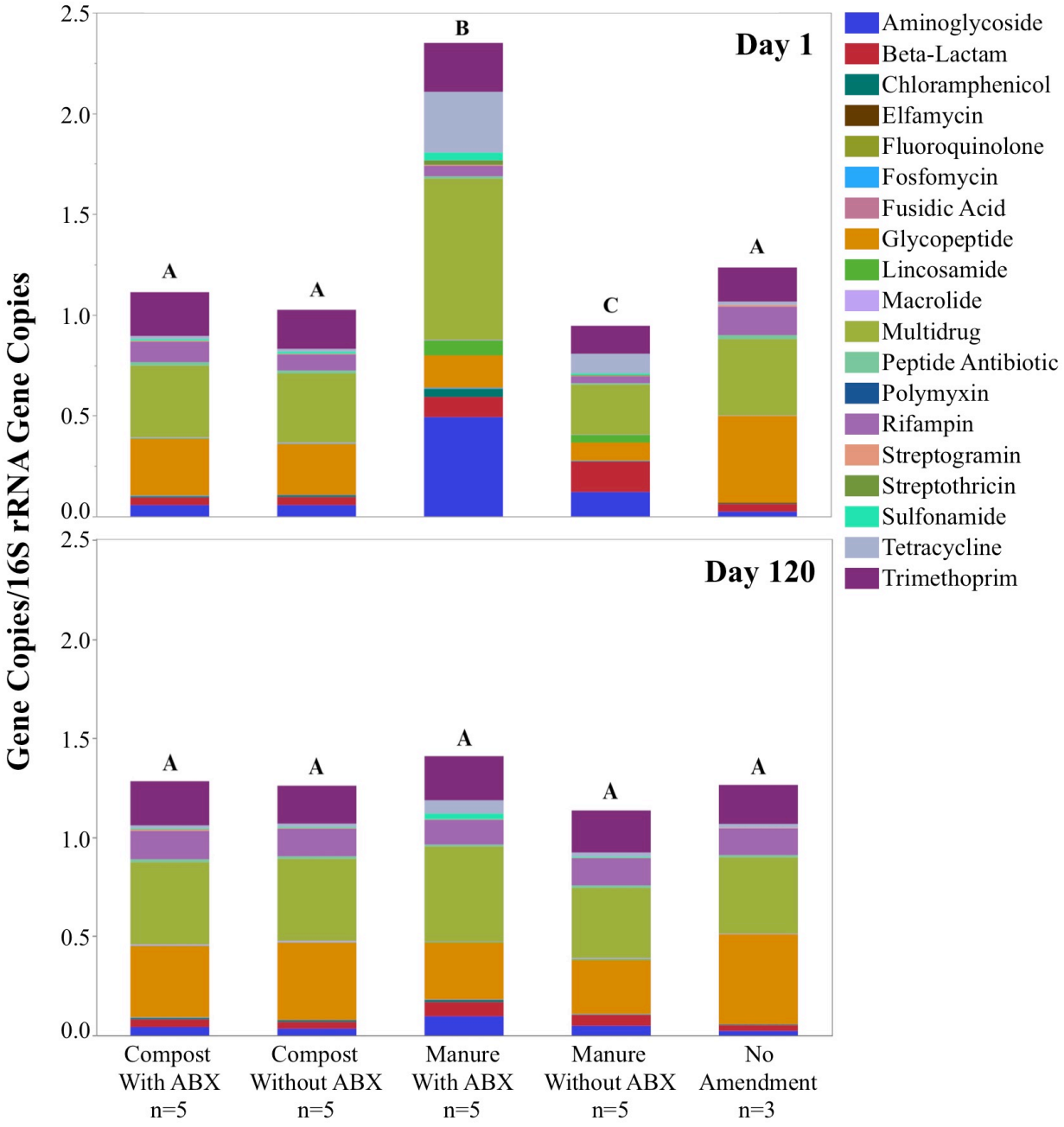

Fig 3. Metagenomic Sequencing: Effect of Amendment on Relative Abundances of ARG Classes. Effect of soil amendment type on relative abundance (normalized to 16S rRNA gene reads) of total ARGs and individual classes of ARGs. Per timepoint, the capital letters indicate significance between soil amended with the different treatment types ($p<0.05$; ANOSIM).

Consistent with the patterns observed for *sul1* and relative abundances of total ARGs on day 1, 16S rRNA amplicon sequencing also indicated that prior administration of antibiotics had a significant effect on the microbial community composition of soils applied with raw manure at

both time points ($p<0.05$; ANOSIM) (Fig 4). Likewise, the bacterial community profile of the compost-amended soils was not affected by antibiotic usage ($p>0.05$; ANOSIM) (Fig 4).

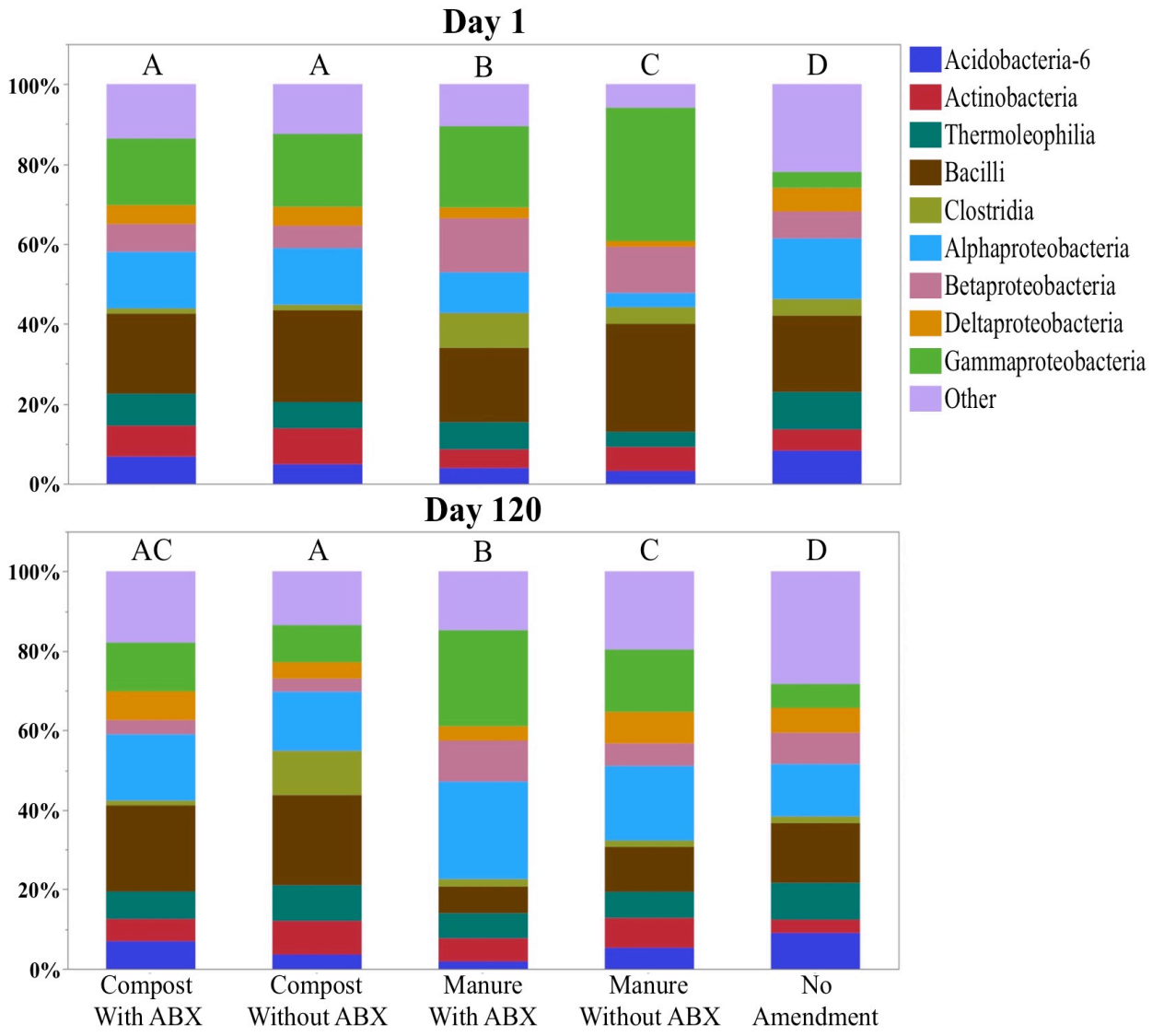

Fig 4. Microbial Community Composition Determined by 16S rRNA Amplicon Sequencing. Comparison of the relative abundance top nine bacterial class compositions of the different treatments applied to soil. Per timepoint, the capital letters indicate significance between soils applied with the different amendment types ($p<0.05$; ANOSIM).

Effect of Static Composting on ARGs and Microbiota

Composting was associated with significantly lower relative abundance of *sul*1 and *tet*(W) compared to the raw manure treated soil ($p<0.05$; Steel-Dwass All Pairs; Figs 1 and 2). An exception was where the relative abundances of *sul*1 in the compost conditions were either greater than or equivalent to the raw manure condition without prior administration of antibiotics (Fig 1). When comparing to no-amendment controls, soils receiving a biological amendment never returned to the background *sul1* or *tet*(W) relative abundance levels, regardless of whether the manure was composted and even after 120 days (Figs 1 and 2).

On day 1, shotgun metagenomic sequencing similarly indicated that soil applied with compost was significantly different from the raw manure applied soils ($p=0.008$; ANOSIM) (Fig 3A). Compared to the raw manure from cows treated with antibiotics, the compost treatments had a lower relative abundance of total ARGs. However, like the qPCR *sul*1 results, the compost treatments had a higher relative abundance of total ARGs than raw manure applied soil from cows that were not administered antibiotics. After 120 days, the metagenomics results indicated that all of the treatments, including the non-amended control, were not significantly different from each other ($p>0.05$; ANOSIM) (Fig 3B).

Using shotgun metagenomic data, resistance classes that are considered the most "critically important" to human health by WHO [6] (glycopeptide, macrolide, and beta-lactam) were compared among the five treatments (Fig 5). On both days, there was an overall significant difference between the different treated soils in regards to the relative abundance of beta-lactam and macrolide resistance categories ($p<0.05$; Kruskal-Wallis). The relative abundance of glycopeptide was not significantly different across the treatments at either time point (day 1: $p=0.0615$, day 120: $p=0.5287$; Kruskal-Wallis). It is interesting to note that while the group of

antibiotic treated cows received a cephalosporin (a beta-lactam), this did not appear to select for greater levels of beta-lactam genes. However, screening for the specific CTX-M beta-lactamases, which have become a major resistance mechanism of concern [40], there were no significant differences among the samples with CTX-M ($p<0.05$; Kruskal-Wallis), but the number of samples that had the CTX-M gene increased from day 1 to 120 and soil samples amended with dairy manure from antibiotic treated cows had the highest occurrence of the gene (S1 fig). Metagenomics indicated that extended-spectrum beta-lactamases (ESBL) were not detectable in any of the samples.

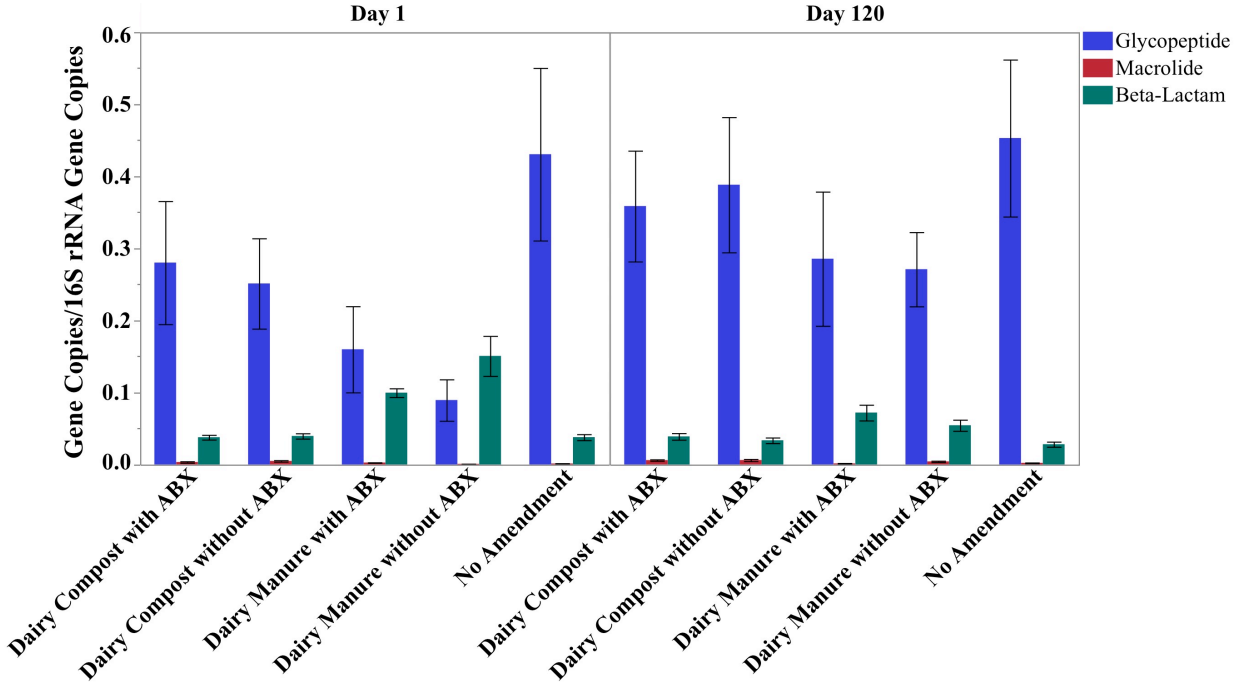

Fig 5. Metagenomic Sequencing: ARGs of Highest Priority for Human Health. Effect of soil amendment condition on the relative abundance of ARGs belonging to classes of resistance corresponding to the most "critically important" antibiotics classified by the World Health Organization [6] and identified by mining the shotgun metagenomic data. Error bars indicate the standard error of the number of reads corresponding to the resistance class, normalized to the number of 16S rRNA genes (relative abundance) (amendments n=5; no amendment n=3). The macrolide and beta-lactam ARG classes have an overall significant difference amongst the treatments at day 1 and 120 ($p<0.05$; Kruskal-Wallis), but there was no significant difference with respect to the glycopeptide ARG levels ($p>0.05$; Kruskal-Wallis).

In addition to further analyzing the "critically important" resistance classes, the relative abundance of total plasmid associated genes was compared across treatments (Fig 6). On day 1, soils amended with dairy manure from untreated cows had significantly lower relative abundances of total plasmid associated genes compared to soils amended with composted manure of antibiotic treated cows (p=0.0126; Tukey-Kramer HSD), composted manure of untreated cows (p=0.0464; Tukey-Kramer HSD), and unamended soils (p=0.0021; Tukey-Kramer HSD). However, after 120 days, there were no significant differences amongst the treatments (p=0.9242; Oneway ANOVA).

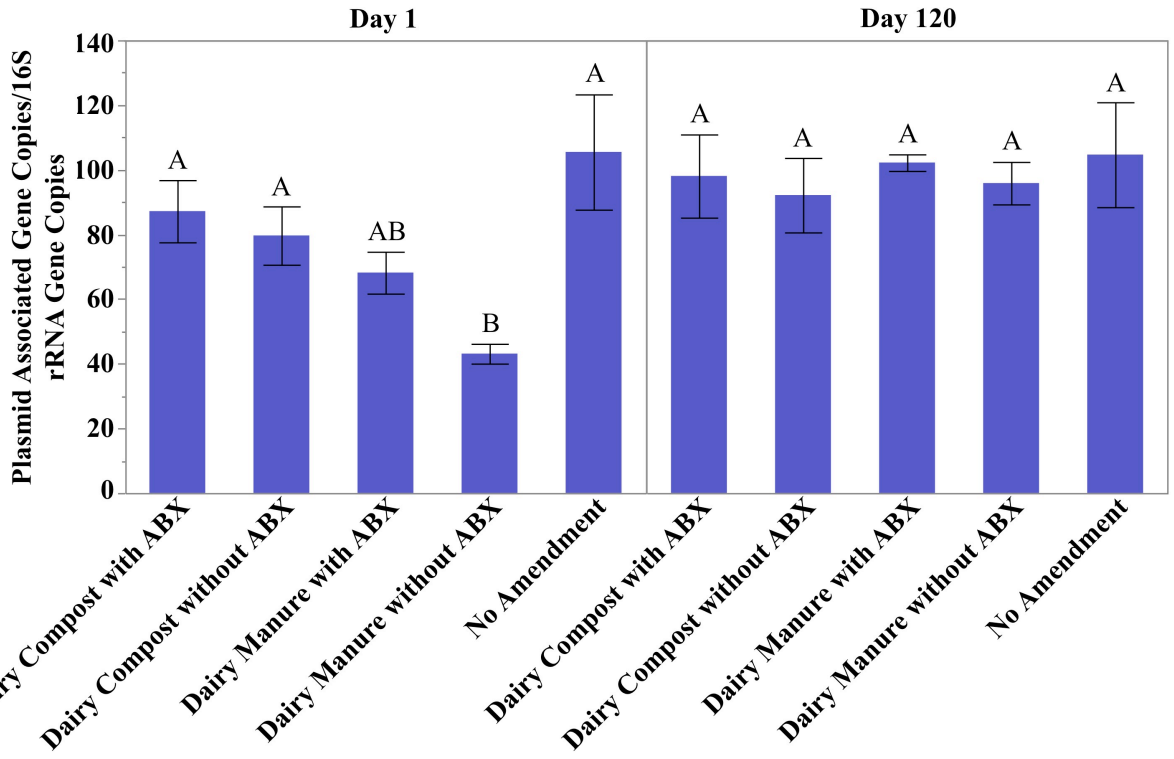

Fig 6. Metagenomic Sequencing: Effect of Amendment on Relative Abundances of Plasmid Associated Genes. Effect of soil amendment type on relative abundance (normalized to 16S rRNA gene reads) of total plasmid associated genes. Per timepoint, the capital letters indicate significance between soils applied with the different amendments ($p<0.05$; Tukey-Kramer HSD).

The 16S rRNA amplicon sequencing results indicated that the overall class-level taxonomic profiles between compost and raw manure applied soils were statistically different at both time points ($p<0.05$; ANOSIM), except for amended soils with composted manure of antibiotic treated cows and amended soils with raw manure of non-administered cows on day 120 ($p=0.258$; ANOSIM) (Fig 4).

Effect of Soil Type on ARGs and Microbiota

Based on qPCR, *sul1* and *tet*(W) data indicated a soil type effect among the treatments at both day 1 and 120 ($p<0.05$; Kruskal-Wallis) (Figs 1 and 2). Most often, treatments applied to sandy loam soils showed the highest amounts of normalized *sul*1 and *tet*(W) compared to silty clay loam and silty loam ($p<0.05$; Steel-Dwass All Pairs) (Figs 1 and 2). However, this trend was not universal amongst all treatments and time points. At day 120, all of the compost and manure amendments had the lowest relative abundances of *sul1* and *tet*(W) on the silty loam soil ($p<0.05$; Steel-Dwass All Pairs) (Figs 1 and 2).

Due to the inability to include triplicate samples for all the soil types, only limited statistical comparison of the effect of soil type is possible based on shotgun metagenomic sequencing data. On day 1 and 120, since the two compost types were not statistically different, they were combined in order to compare among soil types for a general compost treatment. Silty clay loam and sandy loam were found to be statistically different from each other for compost on both days ($p=0.029$; ANOSIM) (S2 and S3 figs), with compost amended silty clay loam having the higher total relative abundance of ARGs. On day 120, since the manure types were also no longer significantly different, a general manure type was statistically tested for differences among soil types. As seen in the compost-amended soils, manure-applied silty clay loam also had significantly greater total relative abundance of ARGs than manure-applied sandy loam on

day 120 (p=0.029; ANOSIM) (S4 fig). Soils amended with manure of antibiotic treated and untreated cows were also qulaitatively compared seperately among soil types (S5 and S6 figs), with the sandy loam applied soils appearing to have maintained the effects of the manure treatments the strongest.

MDS plots enabled more comprehensive comparison of the ARG profiles based on soil type (Fig 7). At day 1, the two raw manure types applied to sandy loam ("sand") clustered separately from the silty clay loam ("clay") and silty loam ("silt") (Fig 7). At 80% similarity, the sandy loam did not cluster with the silty clay loam and silty loam soils for the raw manures at day 1. Also at day 120, the sandy loam applied with raw manure from dairy cows that were administered antibiotics clustered separately from the other two soil types at both 75% and 80% similarity. Observed in the expanded view of the outlined MDS cluster, the sandy loam soil generally tended to cluster separately (80%) from the amended silty clay loam and silty loam. From the MDS plot (Fig 7), the same groupings as previously described were observed. The two manure types were significantly different from each other on day 1 (p=0.008; ANOSIM), and all of the treatments at day 120 were not significantly different from each other and clustered together, except for the raw manure from cows administered antibiotics and applied to sandy loam, at 75% similarity.

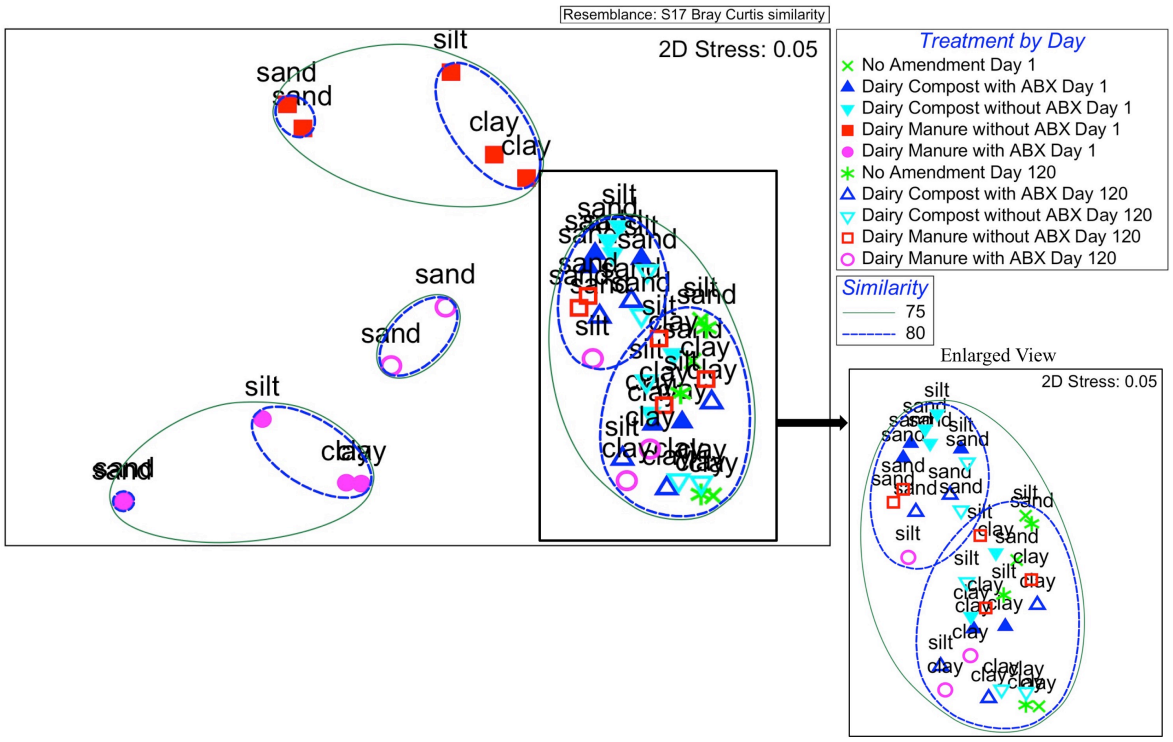

Fig 7. MDS Plot of Shotgun Metagenomic Data. MDS plot of relative abundance of total ARG class similarity amongst all treatments, both time points (day 1 and 120), and soil types. Similarity groupings are based off of 75% (green line) and 80% (blue dashed line) similarity. The enlarged view shows more clearly the samples and groupings within the outlined cluster.

Similar to the shotgun metagenomics data, in the 16S rRNA amplicon sequencing dataset the compost treatments were again combined into a general compost treatment since they were not statistically different and would yield a more powerful statistical soil type comparison. The 16S rRNA class-level taxonomic profiles of the three soil types amended with compost were significantly different from each other at both time points (p=0.002; ANOSIM) (S7 and S8 figs). The manure treatments could not be compared statistically, but visually it also appeared that sandy loam applied soil had the largest differences in taxonomy composition relative to the other two amended soil types (S9 and S10 figs). The differences between soil types were also observed on an MDS plot (Fig 8) where the different amendments tended to separate out by soil type. The raw manures applied to sandy loam on day 1 also were clearly separated from the rest of the samples (Fig 8).

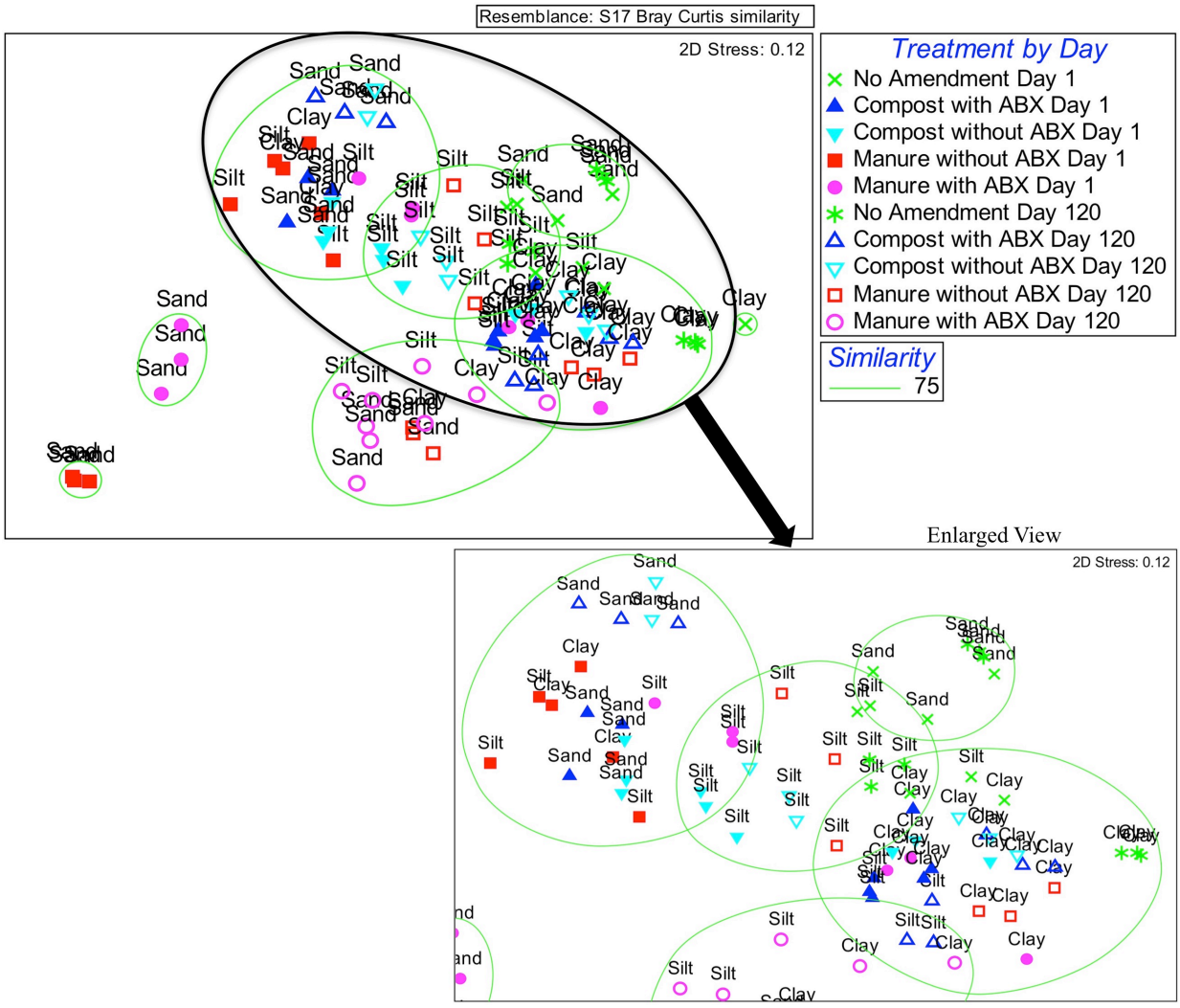

Fig 8. MDS Plot of Microbial Community Composition Determined by 16S rRNA Amplicon Sequencing. MDS plot of the class-level taxonomic composition similarity amongst all treatments, both time points (day 1 and 120), and soil types. Similarity groupings are based off of 75% (green line) similarity. The enlarged view shows more clearly the treatments and groupings within the black outlined cluster.

Discussion and Conclusions

The *sul1* and shotgun metagenomics results of this study indicated that prior use of antibiotics had an effect on the relative abundances of ARGs in soils receiving manure-based amendments. The relative abundance of *sul1* was significantly higher in soils with raw manure of antibiotic treated dairy cows than the other amendments on day 1 and 120. Shotgun

metagenomics likewise revealed that soil amended with antibiotic administered dairy cows' manure had significantly greater relative abundance of total ARGs on day 1 compared to the soil with dairy manure from untreated cows, but there was no longer any significant differences after 120 days. Despite the increase in relative abundance of *sul1*on both days and total ARGs on day 1, the use of antibiotics did not appear to selectively enhance the relative abundance of ARGs within "critically important" resistance classes [6], including beta-lactam. This result contradicts past studies of fecal samples from cattle raised with or without antibiotics that found significantly higher tetracycline genes in beef steers fed antibiotics [27] and higher beta-lactam ARGs in dairy cows administered ceftiofur [28]. The difference seen between the dairy fecal study [28] and the lack of increase in beta-lactam genes in this study may be due to the differences in antibiotics used (ceftiofur [28] versus cephapirin) and that this experiment applied the manure to soil. However, it was observed that soils applied with manure from dairy cows that received cephapirin and pirlimycin had an increased occurrence of CTX-M genes compared to the other treatments and that the number of samples with CTX-M increased for all treatments from day 1 to 120. The relative abundance of *tet*(W) was not significantly different between the two raw manure treated soils, but this trend correlates with another study's metagenomic analysis that noted that tetracycline ARGs were abundant in dairy fecal samples and not affected by antibiotic administration [28].

While the relative abundances of *sul1* and *tet*(W) were still higher in soils applied with biological amendments after 120 days, this was not the observed trend in the shotgun metagenomics data. These contradicting results are inline with prior studies that have found either that the impact of biological amendments on ARGs in the soil is only a transient effect or that they persist for long periods of time. For example, a field study that applied a dairy manure

slurry and dry stacked manure to soil found that *ermF*, *sul1*, and *sul2* returned to background soil levels after 2 months [11]. A study applying swine manure to soil also concluded that an increase in macrolide, lincosamide, and streptogramin B resistance in soil did not last longer than 20 days [41]. Other studies have found manure amendments to increase soil resistance levels for varying lengths of time [9, 10]. The 16S rRNA amplicon sequencing data indicated that the microbial community composition of the amended soils was not the same as the control soils even after 120 days. However, the non-amended soils maintained a relatively stable microbial community composition throughout 120 days, which indicates that the changes to the microbiota were due to the compost or manure-based amendments. This is noteworthy as shifts in the microbiota have been observed to be the main driver of ARG compositions in soil samples [42, 43]. One hundred twenty days is also an important timeframe to study as it is a current standard set by the United States Department of Agriculture's National Organic Program. The standard states that farmers must wait 120 days between raw manure application for crops that are "in contact with the soil" [44]. It is recommended in FSMA, but not regulated, that farmers follow these standards until further data is analyzed from ongoing risk assessment research [44].

Eventhough the relative abundances of *sul1* and *tet*(W) did not return to unamneded quantities, the absolute abundances of *sul1*, *tet*(W), and 16S rRNA genes decreased from day 1 to day 120 (S11-S13 figs). The dissipation of ARGs could be the result of several different mechanisms. As there was a decrease in 16S rRNA genes, bacterial cell death is likely a primary mechansim causing the observed decrease in ARGs, which has been noted in other studies [11, 45]. Another study observed that DNA degradation occurs more readily in unsatruated soils (the soils in this experiment were not fully saturated), and ARG dissipation is observed to occur with this DNA degradation [46]. Since qPCR quantifies both intracellular and extracellular DNA, the

degradation of extracellular DNA over time could also cause the decrease in ARG abundances [47]. This is a probable mechanism causing the observed results since extracellular DNA is a food source for bacteria [48], and the microcosms received no additional nutrients following the initial biological amendment. The ARG dissipation was not a result of bacterial or extracellular DNA transport, as all mass, except water loss due to evaportaion, was contained within the microcosms.

The results of this study suggest that composting reduces the effect of prior administration of antibiotics, but may temporarily increase plasmid associated genes in soils compared to raw manure of untreated cows and does not necessarily reduce the total ARGs in the amended soil. On day 1, compost-amended soils had significantly greater relative abundance of plasmid associated genes than soils amended with raw manure of untreated cows ($p<0.05$; Tukey-Kramer HSD). This is noteworthy as plasmids are mobile genetic elements. These results are contradictory to a prior microcosm study using chicken manure with or without composting that observed within *Escherichia coli* a decrease in plasmids that encode ARGs post composting [49] and another study that saw an increase in plasmid transfer frequency in pig manure amended soils [10]. The differences between the studies could be due to the different analytical techniques (measurement of plasmid associated genes rather than targeted plasmids), composting procedures, animal species studied, and that this study applied the compost to soil.

The shotgun metagenomic results on day 1 indicated that the prior use of antibiotics did not affect the relative abundance of total ARGs in compost-amended soils. Notably, the compost-amended soils actually had more total resistance gene copies per 16S rRNA than the soil with raw manure from cows not given antibiotics. The *sul1* results at both time points also showed that there was no effect from the prior use of antibiotics on the relative abundance of

sul1 in the compost-amended soils. However, like the metagenomics data, there were higher levels of normalized *sul1* in the compost treated soils compared to the soil with manure of untreated cows. Contrary, the qPCR results for *tet*(W), indicated that there was an effect of antibiotic administration on the relative abundance of *tet*(W) in compost-amended soils on day 1, as the composted manure of cows that were given antibiotics had higher levels of *tet*(W) than the composted manure of untreated cows. However, there was no longer a statistically significant difference in the relative abundance of *tet*(W) between the two compost types after 120 days. In addition, the two compost-amended soils were significantly lower in relative abundance of *tet*(W) compared to the two manure-applied soils at both time points. The loss of effect from antibiotic administration post-composting is possibly due to the degradation of the excreted antibiotics during the composting process.

The manures and composts used in this experiment were also chemically analyzed to determine the antibiotic concentrations (results in draft for publication [50]). It was found that cephapirin rapidly degrades and was below the detection limit (1.31 μg/kg) in the manure and compost stored at 4°C at the start of the experiment [50]. After static composting, pirlimycin was also below the detection limit (0.54 μg/kg), and the raw manure in soil decreased to low concentrations of pirlimycin after 120 days (<0.80 μg/g) [50]. The three soils used in this study also were analyzed, and the concentrations of sulfamethazine, tylosin, chlortetracycline, cephapirin, and pirlimycin were below the detection limit [50]. These chemical results agree with previous research that has shown that composting manure reduces antibiotic concentrations [30, 51, 52]. This is also possibly why there was no significant difference between the bacterial populations of the two compost-applied soils.

In terms of the impact composting has on ARGs, there is still a knowledge gap [53]. The few studies that have been done on the topic have resulted in conflicting conclusions. One study found that thermophilic composting of swine manure increased efflux pump, enzymatic inactivation proteins, and sulfonamide resistance genes [54]. In contrast, another experiment that subjected swine manure to thermophilic composting found a decrease in sulfonamide, tetracycline, and fluoroquinolone resistance genes [55]. Research specific to cows manure found that composting decreased sulfonamide, tetracycline, and erythromycin resistance genes after 90 days [56]. Another research project saw a decrease of *ete*(W) and *tet*(O) genes after 6 months of composting for beef manure and 4 months for dairy manure [57]. This study's results and prior research indicate that composting treatment is a complex process and interacts differently with different ARGs.

The molecular data indicates that soil type may play a role in ARG attenuation. The sandy loam soils appear to often have the highest relative abundances of *sul1* and *tet*(W), and, after 120 days, silty loam had the lowest relative abundances of *sul1* and *tet*(W) among all the amended soils. This is noteworthy as the sandy loam had the lowest cation exchange capacity (4.6 milliequivalents per 100 grams) (S2 Table) of the three soil types, and a higher abundance of cations has been associated with an increase in DNA adsorption [58]. While increasing pH has shown to decrease adsorption of DNA to soil [58], there was not a wide range in the pH of the three soils (6.17-6.79) (S1 Table) and sandy loam had the lowest pH. In regards to antibiotic sorption, one study concluded that higher cation exchange capacity tightly binds oxytetracycline to the soil particle and does not allow desorption as readily [22]. However, different antibiotics adsorb differently to soils with unique interactions [19-21]. The 16S rRNA amplicon sequencing data also indicates that there is a difference between the soil types, which agrees with a study by

Fernandez et al. that concluded that differences in bacterial compositions in different soils were explained largely by soil physiochemical properties [59]. This study further verifies that soil properties and ARG interactions are highly complex and particular to each antibiotic.

The tendency for higher relative abundances of *sul1* and *tet*(W) in sandy loam amended soils may be due to the increase in nutrients, as studies have speculated that the input of nutrients from soil fertilization may be the mechanism behind the enhancement of ARGs in soil [10, 12]. The sandy loam soil may have shown the strongest response (increased 16S rRNA gene and relative abundance of *sul1* and *tet*(W)) to the nutrient inputs, as it had the lowest initial organic matter content and (S2 Table) and 16S rRNA gene copies (S13 fig). There may also be DNA extraction bias among the soil types [60] possibly due to the adsorption of DNA particularly to clay particles [58].

It has been shown in prior research that adding biological amendments to soil will change the bacterial community [12, 61]. In order to compare to a similar soil microcosm study amended with sulfadiazine spiked manure by Ding et al. [61], the 16S rRNA amplicon sequencing data were also grouped by the top five phyla with the remaining being combined into an "other" category (S14 fig). As was found in the Ding et al. [61] study, *Acidobacteria* was higher in the untreated soils than amended. Specifically, both manure-amended soils (on day 1 and 120) and soil with composted manure of untreated cows (on day 120) had significantly lower *Acidobacteria* levels than the non-amended soils ($p<0.05$; Steel-Dwass All Pairs). This is important as *Acidobacteria* are widely found in soil and thus most likely have an important ecosystem function [61]. Ding et al. also found that *Proteobacteria* was dominant and increased with soil treatment [61]. This study similarly saw that soil amended with either compost treatment (on day 1), manure from cows treated with antibiotics (on day 1 and 120), or manure

from cows not administered antibiotics (on day 120) had significantly greater *Proteobacteria* than the unamended soils ($p<0.05$; Steel-Dwass All Pairs). These results agree with prior research that amending soils results in a change in the microbiota that does not rapidly return to non-amended soil conditions.

In summary, application of compost or manure does impact the ARGs and affects the microbiota of soil for at least 120 days. This result is also supported by culturing data of this study's samples on antibiotic infused R2A media (heterotrophic bacteria) (S3 Table), which showed that both manure and compost applied soils almost always had the highest log CFU per gram in antibiotic infused media compared to the unamended samples (S15 and S16 figs). Prior use of antibiotics does increase the relative abundance of *sul*1 and affect the taxonomic composition in raw manure applied soils compared to soils amended with raw manure of untreated cows. This difference was still present after 120 days but not for the relative abundance of total ARGs from shotgun metagenomics. However, this difference may be because shotgun metagenomics does not quantify as precisely as the more targeted qPCR and 16S rRNA amplicon sequencing analyses do. The prior antibiotic administration affect can be neutralized by static-composting, but composting does not necessarily decrease ARGs in the soil and further research is needed in this area. The different physiochemical properties of soil do have an impact on ARGs and the bacterial community. From this study, sandy loam had the least attenuation of ARGs following biological amendment, while silty loam had the best. However, further study is needed as to why this may be occurring.

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Chapter 3—Beef Study

Effect of In-Feed Antibiotics, Composting, and Time on Antibiotic Resistance Genes and Microbiota in Different Soils Receiving Beef Manure-Based Amendments

Abstract

While land application of manure-based amendments provides an economical fertilizer and means of disposing of livestock waste, there is concern that the practice could elevate antibiotic resistance in affected soils and the corresponding microbiota of crops. The objective of this study was to determine the effect of soil type (sandy loam, silty clay loam, or silty loam), manure composting, and prior antibiotic use in beef steers on the microbial composition and fate of antibiotic resistance genes (ARGs) in soils via a controlled microcosm study. The relative abundance of *sul1* and *tet*(W) was elevated in all biologically-amended soils and persisted for the entire 120-day study. Prior antibiotic use was noted to elevate the relative abundance of *sul1* and total ARGs identified by shotgun metagenomic sequencing when raw manure was applied, but composting appeared to override the effect of antibiotics while still resulting in elevated ARGs relative to background soil. Further, soil type was noted to be a mediating factor and could be an additional factor to be taken into consideration in developing best management practices for reducing the spread of antibiotic resistance. In particular, sandy loam appeared most susceptible to elevated ARGs, while silty clay loam indicated some promise for buffering the effects of manure-based amendments. While some attenuation did occur, 120 days was not sufficient for ARG levels or the microbial community composition to return to the background condition. Given that different ARGs responded differently to the various conditions, particularly with

respect to composting, future efforts should be aimed at aligning research towards ARGs of concern.

Introduction

Antibiotics are most frequently used in the U.S. for livestock [1], where steers represents the largest segment of the livestock industry [2]. Given that rates of excretion, via feces and/or urine, are as high as 90% [3], this could be a possible source of antibiotic release into the environment. Animal manure has benefits as an abundant, low-cost soil amendment and fertilizer and thus is commonly land applied. Land-application of manure and manure-based products thus serve as an input of antibiotics to soils [4-6]. However, more of a concern is the potential for manure and manure-based amendments to influence antibiotic resistance in soils. This could occur either as a direct result of antibiotic inputs exerting selective pressure, through shifting microbial communities native to the soils, and through direct inputs of antibiotic resistant bacteria (ARBs) and antibiotic resistance genes (ARGs). In fact, several studies have shown that soil amended with or in contact with animals, such as cows, swine, or poultry, results in an increase in various measures of antibiotic resistance, including sulfonamide, tetracycline, beta-lactam, and erythromycin ARGs measured via quantitative polymerase chain reaction (qPCR), PCR detection assays, and culturing based methods [7-15]. This is a concern because not only does this increase the overall abundance of ARBs and ARGs, but it could also potentially transfer ARGs to human relevant pathogens through the sharing of mobile genetic elements via horizontal gene transfer. Prior studies have found that the addition of manure increased horizontal gene transfer of ARGs in soil [16, 17].

The agroecosystem is complex and there are many possible factors that could potentially enhance or reduce the spread of antibiotic resistance. Soil is of particular interest given that it is

the direct recipient of manure and manure-based amendments while also serving as a precious resource for crop production. Soil is an extremely complex environment that is formed and dynamically influenced over time by its parent material, climate, organisms, and the landscape. It consists of a wide array of textures from the combinations of three particle groups (sand, silt, and clay) and many types of structures (granular, blocky, platy, etc.). The biochemistry of soil is diverse as well with differing levels of organic matter, cation exchange capacity (CEC), pH, and mineral content. Soil is also home to complex microbial ecosystems, with each teaspoon containing between 100 million to 1 billion microbes [18] and tens of thousands of operational taxonomic units (OTUs) [19] that varies amongst ecosystems [20]. Thus, there is a need to understand how such complexities encountered in the soil may or may not mediate the spread of antibiotic resistance associated with various livestock management practices.

Antibiotic usage in agriculture appears to play a role in the increase of antibiotic resistance, but there have been limited controlled studies directly linking it to an increase in environmental antibiotic resistance [21]. The benefits of composting versus raw manure application in terms of controlling antibiotic resistance spread has had mixed results with at least one study observing that composting increases ARGs [22] and others finding that it reduces them [23]. However, there have not been studies using composting methods specifically following the new composting standards of the U.S. Food and Drug Administration's (FDA) Food Safety and Modernization Act (FSMA) [24]. There have also been few studies that have examined the downstream role that soil type may play in the attenuation of antibiotic resistance genes (ARGs) in manure or compost-applied soil [25, 26]. These two field-scale studies [25, 26] were limited in that the samples were collected from different locations (different soil types: loam and sandy loam[26] and paddy soil [25]), which may not have had the same manure amendment. It is well

known that different soil characteristics affect DNA [27] and antibiotic adsorption [28-30], so it is important to better determine the effects soil type may have on the fate of ARGs when receiving the same amendment.

Using a soil microcosm approach, we previously reported a systematic examination of the interactive effects of prior administration of dairy cows with cephapirin and pirlimycin, composting, and soil type on the fate of ARGs in amended soils (Ch.2). It was found that the application of compost or manure to soil shifted the microbial community composition and resulted in increased relative abundance of *tet*(W) and *sul1* over a 120-day period (National Organic Program's regulated timespan between manure application and harvest of crops in contact with soil [31]). While amendment of manure from dairy cows that had previously received antibiotics resulted in the highest total relative abundance of ARG classes, composting eliminated the effect of prior antibiotic use and the three soil types exhibited different ARG attenuation trends. Though this study provided new insight into potential critical control points for limiting the spread of antibiotic resistance associated with manure-derived amendments (i.e., antibiotic use, composting, and soil type), it is important to verify the trends with other livestock types. In particular, comparing beef and dairy is of interest because of the contrasting antibiotic management practices applied within the same livestock species. Specifically, dairy cows are typically dosed antibiotics only when ill (e.g., with a mastitis infection), but often with antibiotics considered "critically important" to human health by the World Health Organization [32] [33], whereas beef steers are often prophylactically administered antibiotics in the feed to prevent illness, but with antibiotics that are "highly important" rather than critical to human health. While the recent Veterinary Feed Directive implemented in the U.S. [34] bans the use of antibiotics for the purpose of promoting weight gain, based on the trend that occurred when a

similar ban was emplaced in the EU 11 years ago [35], there will likely be increased prophylactic use of antibiotics for some time to come. Also, with beef steers making up 76.5% of all US cattle, as of January 2016 [2], it is critical to understand how this agricultural sector impacts environmental reservoirs of antibiotic resistance and the potential to transfer to humans.

Here we examined a controlled herd of beef steers (n=9) that were or were not administered antibiotics. Three steers were fed chlortetracycline and sulfamethazine, three steers were fed tylosin, and the final three steers were fed nonmedicated grain. The manure (feces and urine) was collected from the two medicated steers groups and mixed together to create the raw manure treatment from antibiotic administered steers. None of the steers used in the experiment had previous antibiotic treatment. Tylosin is a macrolide antibiotic frequently added to feed to help control liver abscesses [33], with 31% of U.S. feedlots using tylosin and 71.2% of all steers in U.S. feedlots receiving it in their feed and/or water [33]. Macrolides primarily are affective against Gram-positive bacteria, with their method of action being to prevent protein synthesis via binding to the 50S ribosomal subunit [36]. Chlortetracycline is a tetracycline, a broad spectrum class of antibiotics that works against both Gram-positive and Gram-negative bacteria [37]. Tetracyclines act by binding to the 30S subunit of the ribosome and inhibiting ribosomal translation [37]. Greater than 71% of all U.S. feedlots (greater than 1,000 head) add chlortetracycline to the feed/water, with 18.4% of steers within those feedlots administered chlortetracycline [33]. Sulfamethazine/sulfadimethoxine are sulfonamides administered less often, and is estimated to be administered on 5.4% of U.S. feedlots to 0.2% of their steers [33]. Sulfonamides tend to be widely used in veterinarian medicine as they are economical and effective at treating common diseases [38]. Sulfonamides are affective against both Gram-positive and negative bacteria [38]. Their method of action is disrupting metabolic functions by

outcompeting the dihydropterate synthetase enzyme, which prevents the role of para-aminobenzoic acid (PABA) in synthesizing folic acid [38], an essential vitamin required for RNA formation [38]. The WHO [32] classifies tetracyclines and sulfonamides as "highly important antimicrobials", while macrolides have the highest priority for risk management and are classified as "critically important antimicrobials" for human medicine [32].

The purpose of this study was to determine the effect of prior antibiotic use, composting, and soil type on the fate of ARGs and the bacterial community composition in soils receiving beef manure derived amendments. A controlled, replicated soil microcosm study was employed to examine three local Virginia soil types (sandy loam, silty clay loam, and silty loam), manure with versus without prior history of sulfamethazine, chlortetracycline, and tylosin administration, and static composts of these two manure types. The microcosms were sacrificially sampled in triplicate up to 120 days, with subsequent characterization of ARGs by qPCR and shotgun metagenomic sequencing, along with 16S rRNA gene amplicon sequencing to profile the microbial community structure. These results can help identify critical control points for reducing the potential for antibiotic resistance to spread from farm to fork.

Materials and Methods

Microcosm

The materials and methods used in this experiment are identical to the previously described parallel study with dairy cows (Ch.2). In short, a herd of beef steers (n=9), without history of antibiotic usage, was split into groups that were fed antibiotic medicated feed (3 steers fed chlortetracycline and sulfamethazine; 3 steers fed tylosin) or non-medicated grain [39]. The collection and composting processes for the manure (feces and urine) and compost used in the

microcosms are described in further detail in Ray et al. [39]. Composting procedure followed the standards set by FSMA for static-composting (55°C for 3 days in aerobic conditions and cured to 42 days) [24]. The soils used for the microcosms were sandy loam (Eunola loamy fine sand), silty loam (Guernsey silt loam), and silty clay loam (Carbo and Chilhowie silty clay loam) in order to study the three main soil particles (silt, sand, and clay). A 2 mm sieve was used to screen the air-dried and ground soils. The physical properties of the soils are found in the supplementary tables of the dairy cow study (Ch.2).

The five treatments amended to the soils are as follows: raw manure from beef steers fed antibiotic feed (manure with ABX), raw manure from beef steers not fed medicated grain (manure without ABX), composted manure from beef steers fed antibiotic feed (compost with ABX), composted manure from beef steers not fed medicated grain (compost without ABX), and no amendment. These treatments were added to the soil at a representative application rate of 203 ± 25 pounds of nitrogen per acre. Triplicate microcosms were made for each sampling time point through the 120-day experiment. The 100 mL glass jars containing a final mass of between 15-18.9 grams, depending on the treatment, were incubated at 24°C in dark, aerobic conditions. Moisture content was maintained at 50% field capacity via weekly watering.

Molecular Analysis

The microcosms were destructively sampled at each time point and stored in 2.0 mL cryogenic screw cap vials at -20°C until DNA extraction. The instructions for the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH) were followed using a FastPrep-24^{TM} 5G Instrument (MP Biomedicals, Solon, OH) and, for maximum cell lysis, an optional 2-hour room temperature incubation after bead-beating was included. Following extraction, the DNA was cleaned with the

OneStep PCR Inhibitor Removal Kit (Zymo Research Corporation, Irvine, CA). The final DNA was stored at -80°C.

Quantifications of 16S rRNA, *sul1*, and *tet*(W) were done through qPCR using SsoFast Evagreen Supermix (BioRad Laboratories, Hercules, CA). Based off of dilution curves, the DNA was diluted with molecular grade water to 1:1000 for 16S rRNA and 1:100 for *sul1* and *tet*(W) analyses. The primer sequences are described in Ma et al [40] and diluted DNA was stored at -20°C.

Due to cost, metagenomic analysis could only be done on a small portion of the samples (manure with ABX, compost with ABX, and no amendment on sandy loam and silty clay loam) and were submitted in the same pool as the parallel dairy study (Ch.2). The submitted samples were sequenced by the Genomics Research Laboratory of the Biocomplexity Institute (BI) of Virginia Tech (formerly Virginia Bioinformatics Institute), Blacksburg, VA using the Illumina HiSeq 2500. As described in the microcosm study with dairy cows (Ch.2), the online metagenomic data analysis tool, MetaStorm [41], was used to match the data with the Comprehensive Antibiotic Resistance Database (CARD v1.0.6) [42] and the ACLAME database [43].

16S rRNA amplicon sequencing was performed on the samples using the Illumina Miseq. Barcoded primers (515F/926R) were used for PCR amplification, and the final pooled product was cleaned with the QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) prior to sequencing submission. Data was analyzed through the QIIME pipeline [44] and referenced to the Greengenes 16S rRNA gene database [45]. The read length of the samples was 5230-30104, and the samples were rarefied to 5230.

Statistical Analysis

All statistical tests were analyzed with a significant cutoff of $p \leq 0.05$. The *sul1* and *tet*(W) data from qPCR were normalized to 16S rRNA gene copy numbers and then analyzed in JMP (SAS Institute Inc., Cary, NC) using the nonparametric Wilcoxon/Kruskal-Wallis Test (Rank Sums) and Steel-Dwass All Pairs test. The bacterial phyla from 16S rRNA amplicon sequencing were individually compared amongst treatments using the same nonparametric tests as qPCR. As the relative abundance of total plasmid associated genes had a normal distribution ($p=0.6960$; Shapiro-Wilk test), the parametric tests Oneway ANOVA and Tukey-Kramer HSD were run in JMP. The relative abundance of total ARGs from shotgun metagenomics and class-level taxonomic compositions from 16S rRNA amplicon sequencing were compared in PRIMER-E (version 6.1.13) using analysis of similarities (ANOSIM) and multidimensional scaling (MDS) plots.

Results

DNA extracts obtained from the microcosms were analyzed via qPCR, shotgun metagenomics, and 16S rRNA amplicon sequencing. Specifically, the *sul1* and *tet*(W) genes were quantified by qPCR as sulfonamide and tetracycline antibiotics were given to the beef steers, and these two genes have been found to be the top mobile ARGs that are widely transferred amongst bacteria at the genus level [46]. A cross-section of samples was selected for shotgun metagenomic sequencing to enable comparisons of soils amended with manure or composted-manure of antibiotic treated steers and unamended soils. Thus, the effect of static-composting can only be statistically compared within antibiotic treated steers, and the effect of prior antibiotic administration cannot be evaluated. Also, due to limited samples submitted per soil type (sandy clay loam and sandy loam), the effect of soil type can only be compared

qualitatively. 16S rRNA amplicon gene sequencing data was grouped by the top nine most abundant bacterial classes found in the samples with the remaining classes combined into an "other" category. These three molecular analyses were used to evaluate the effect of antibiotic usage, static-composting, and soil type on ARG levels and the bacterial communities in raw manure and compost amended soils.

Effect of Prior Antibiotic Administration on ARGs and Microbiota

The administration or non-administration of antibiotics to beef steers had a significant effect on the relative abundance of *sul1* on day 1 and 120 and *tet*(W) on day 1 in raw manure applied soil ($p<0.05$; Steel-Dwass All Pairs). At both day 1 and 120, soils with raw manure from steers administered antibiotics had significantly higher levels of normalized *sul1* gene than soils applied with raw manure from steers that did not receive antibiotics ($p<0.0001$; Steel-Dwass All Pairs) (Fig 1). On day 1, the result was the opposite for *tet*(W), where soils amended with raw manure of steers not administered antibiotics had significantly greater relative abundance of *tet*(W) than the soils with raw manure of steers given antibiotics ($p=0.0008$; Steel-Dwass All Pairs) (Fig 2A). At day 1 for *sul1* and day 120 for *tet*(W), there was no difference caused by antibiotic use when the raw manures were composted before soil incorporation (*sul1*, $p=0.4805$ and *tet*(W), $p=0.0864$; Steel-Dwass All Pairs) (Figs 1A and 2B). However, at day 120 for *sul1* and day 1 for *tet*(W), soils applied with composted manure of antibiotic administered steers were significantly higher than the non-antibiotic counterpart (*sul1*, $p=0.0368$ and *tet*(W), $p=0.0014$; Steel-Dwass All Pairs) (Figs 1B and 2A).

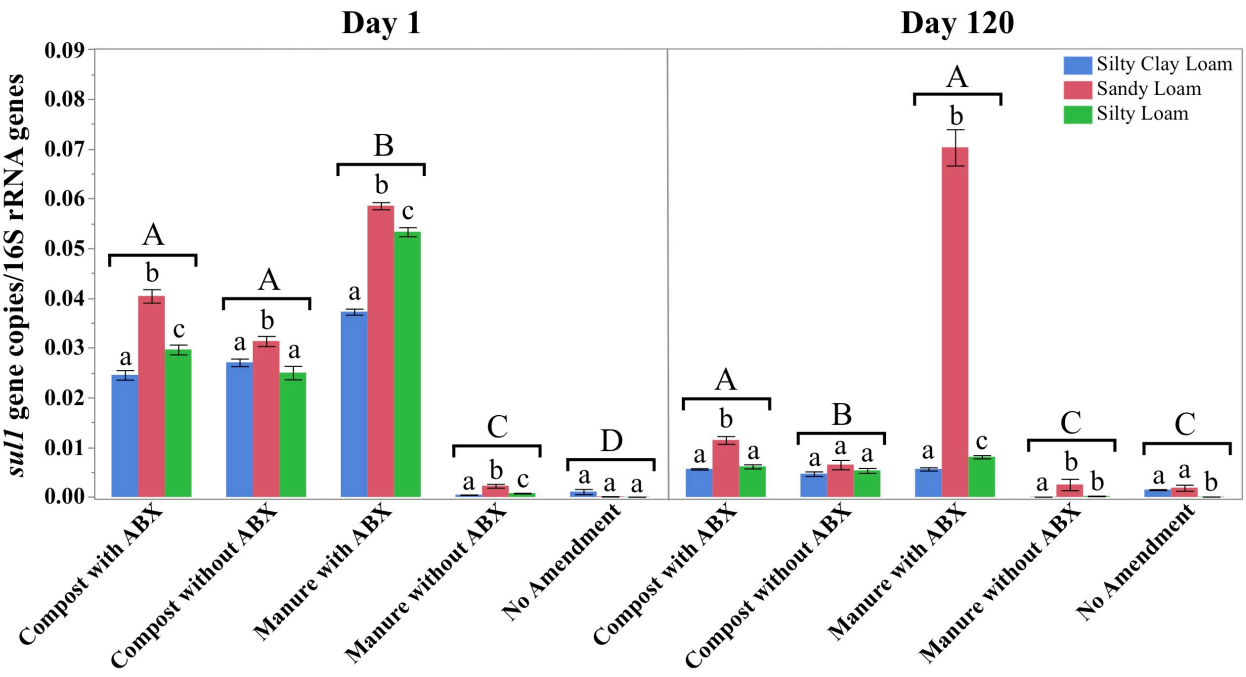

Fig 1. *sul1* qPCR Results. Relative abundance of *sul1* (normalized to 16S rRNA gene copies) for the five different amendment conditions and three soil types. Per timepoint, the capital letters represent significance between the five amendment conditions, and the lowercase letters indicate significant differences among soil types within a specific treatment ($p<0.05$; Steel-Dwass All Pairs). ABX= prior antibiotic administration of corresponding manure.

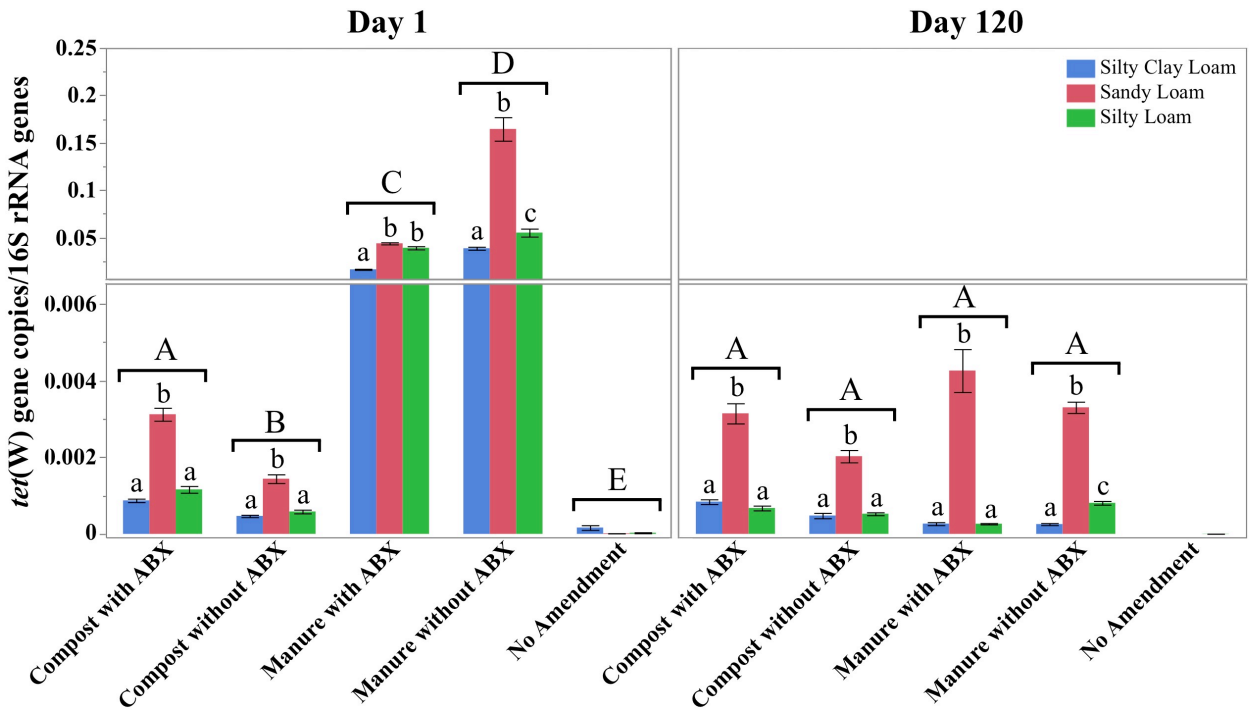

Fig 2. *tet*(W) qPCR Results. Relative abundance of *tet*(W) (normalized to 16S rRNA gene copies) for the five different amendment conditions and three soil types. Per timepoint, the capital letters indicate significance between soils applied with the different amendment conditions, and the lowercase letters indicate significance among soil types within each specific treatment ($p<0.05$; Steel-Dwass All Pairs). Almost all of the non-amended soils (No Amendment) were below the detection limit, particularly DNA of samples taken after 120 days. ABX = prior antibiotic administration of corresponding manure.

An antibiotic administration effect was seen on the microbial community structure of the raw manure applied soils (Fig 3). At both day 1 and 120, the class-level taxonomic compositions of the soils with raw manures from steers with or without antibiotics were significantly different (day 1, $p=0.001$ and day 120, $p=0.029$; ANOSIM). There were no significant differences seen at either sampling day between the soils with composted manure relative to prior antibiotic usage (day 1, $p=0.115$ and day 120, $p=0.185$; ANOSIM) (Fig 3).

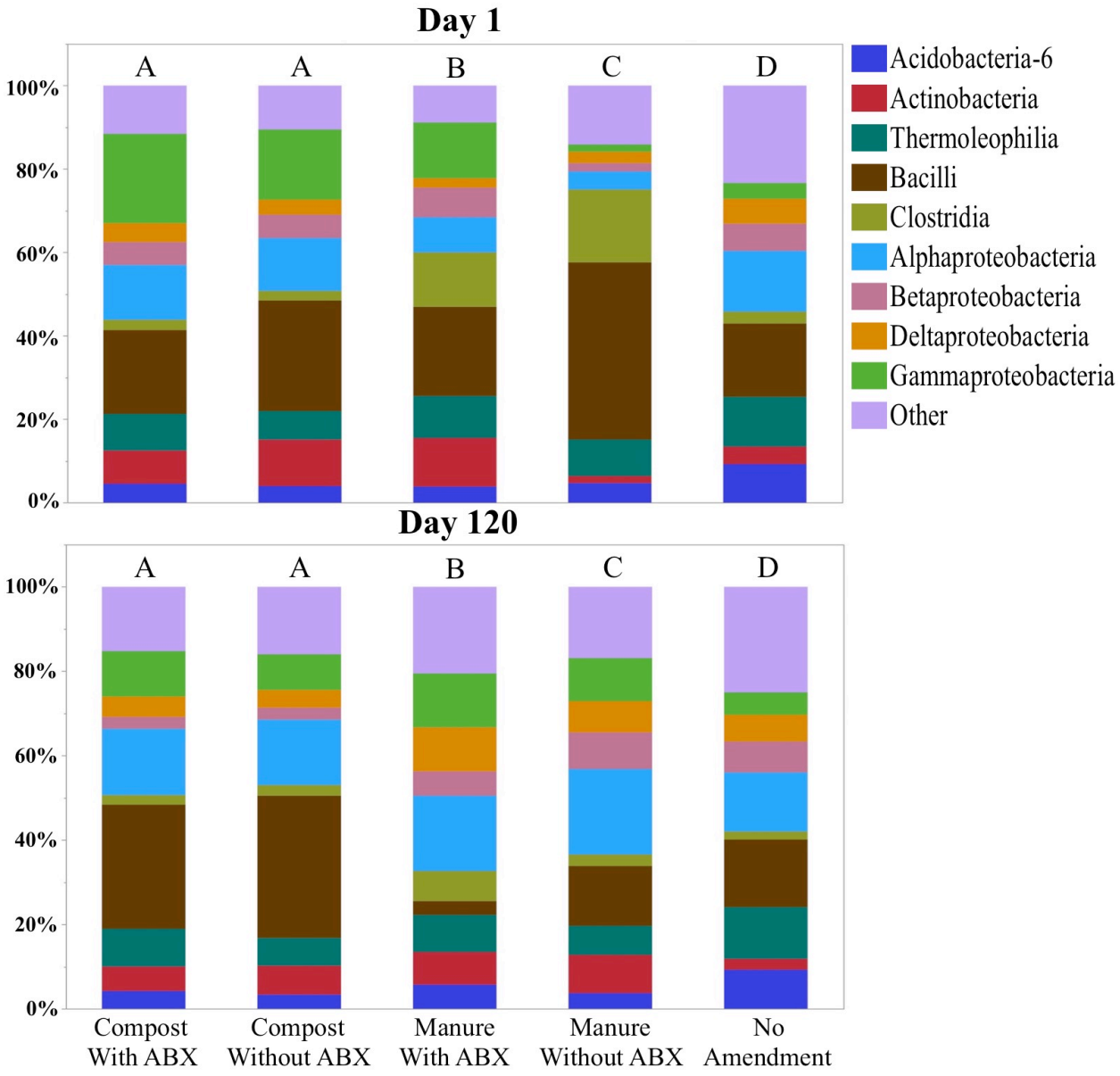

Fig 3. Microbial Community Composition via 16S rRNA Amplicon Sequencing. Comparison of the relative abundances of the top nine bacterial classes of the different amended soils. The capital letters indicate significance among treatment types per timepoint ($p<0.05$; ANOSIM).

Effect of Static Composting on ARGs and Microbiota

Compared to soils applied with raw manure from antibiotic treated steers, composting reduced the relative abundance of *sul1* on day 1 ($p<0.0001$; Steel-Dwass All Pairs) (Fig 1A). At day 120, there was no longer a significant difference in the relative abundance of *sul1* between

soils amended with raw or composted manure of antibiotic treated steers (p=0.4260; Steel-Dwass All Pairs) (Fig 1B). However, the soils amended with composted manure of steers not treated with antibiotics was still lower in normalized *sul1* gene copies than the soils receiving raw manure of antibiotic administered steers at day 120 (p=0.0009; Steel-Dwass All Pairs) (Fig 1B). Interestingly, the relative abundance of *sul1* was higher in soils receiving either types of compost (with and without antibiotics) than in soils that received raw manure from antibiotic-free steers, both on day 1 and day 120 (p<0.0001; Steel-Dwass All Pairs) (Fig 1). For *tet*(W), soils applied with either compost type contained significantly lower levels of normalized *tet*(W) than soils treated with either of the two raw manures on day 1 (p <0.0001; Steel-Dwass All Pairs) (Fig 2A).

In terms of relative abundance of *sul1*, only soils applied with manure from steers not treated with antibiotics reached the background levels measured in non-amended control soils on day 120 (p=0.30; Steel-Dwass All Pairs) (Fig 1B). At day 120, the relative abundance of *tet*(W) in the non-amended control soils was below the detection limit, so while it cannot be statistically compared, all compost and manure treatments were still above the detection limit at day 120 and, thus, did not reach the background levels (Fig 2B).

The shotgun metagenomics data also indicated that at day 1 soils amended with raw manure from steers that received antibiotics had significantly greater relative abundance of total ARGs than compost applied and the non-amended soils (p=0.029; ANOSIM) (Fig 4A). Unlike the qPCR results, the soils with composted raw manure of steers administered antibiotics were not significantly different from the non-amended soils at day 1 (p>0.05; ANOSIM) (Fig 4A). Also, at day 120, there were no longer any significant differences amongst the treatments (p>0.05; ANOSIM) (Fig 4B). These results are also observed in the MDS plot of the metagenomic data. On day 1, the soils amended with beef manure from antibiotic treated steers

cluster together and away from the other treatments (p=0.029; ANOSIM) (Fig 5). On day 120, all of the treatments cluster together at 75% similarity (Fig 5). The exception is a sample from a sandy soil applied with compost from antibiotic treated steers, and the beef manure from antibiotic treated steers on sandy soil (Fig 5).

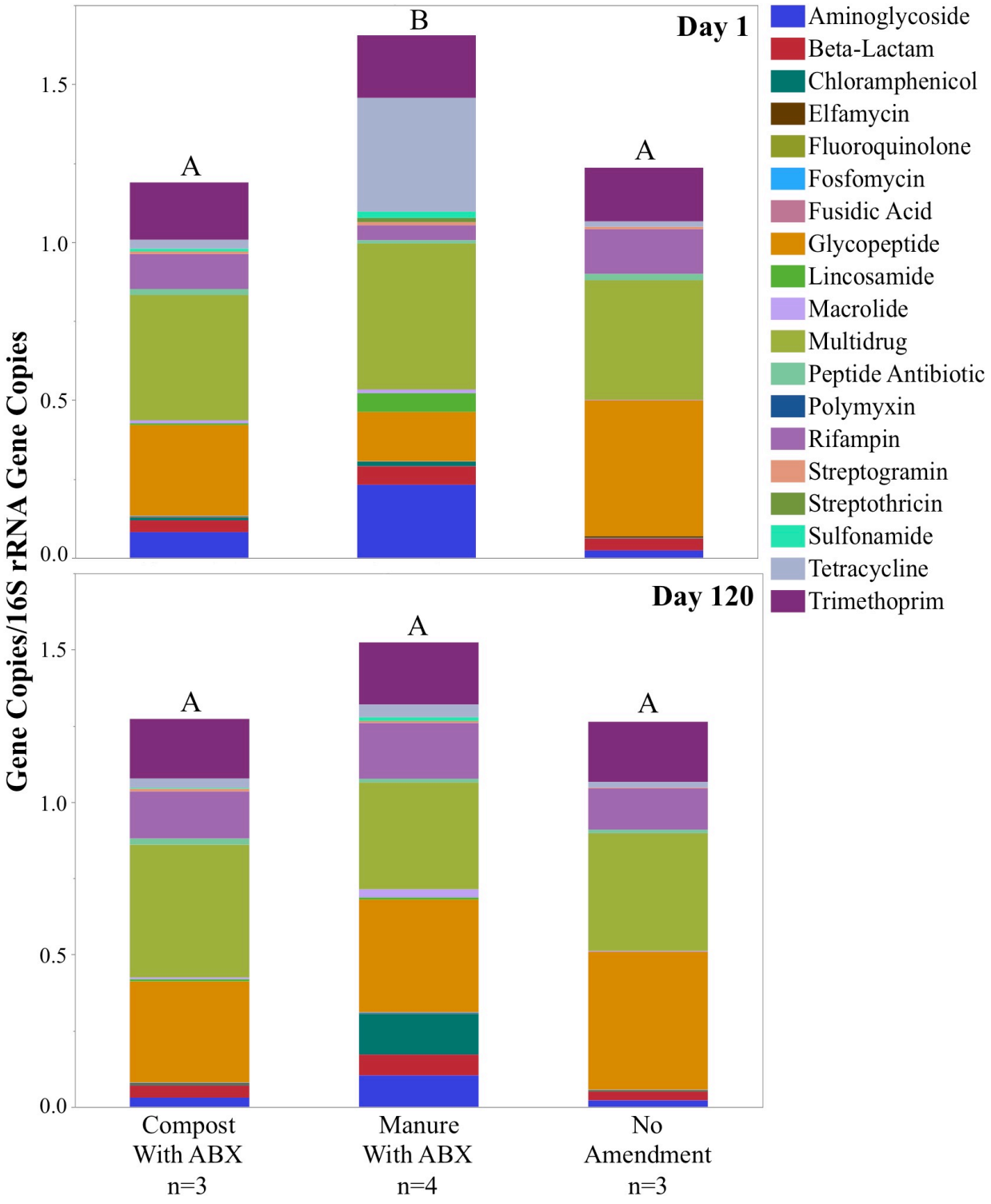

Fig 4. Shotgun Metagenomic Sequencing: Effect of Amendment on Relative Abundances of ARG Classes. Comparison of the relative abundances of total ARGs among the raw manure or composted manure of treated beef steers applied soils and unamended soils. The capital letters indicate significance among treatment types per timepoint ($p<0.05$; ANOSIM).

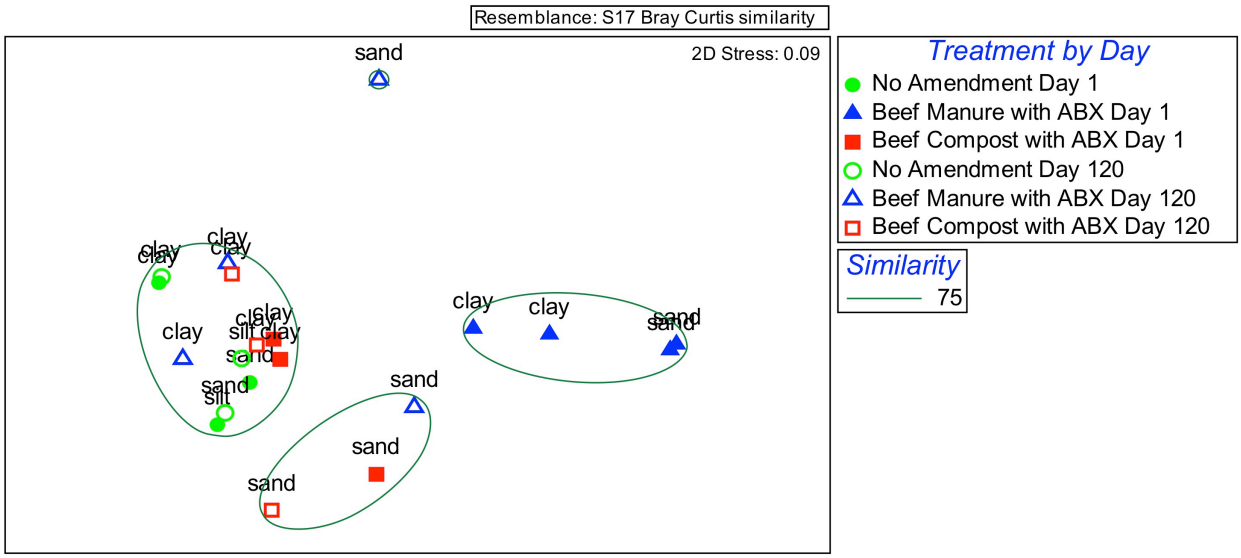

Fig 5. Shotgun Metagenomic Sequencing: MDS Plot. MDS plot of relative abundances of total ARG class similarity among the three treatments, both time points (day 1 and 120), and two soil types, sandy loam ("sand") and silty clay loam ("clay"). The clusters are based off of 75% similarity (green line).

In addition to comparing the overall ARG profiles, the "critically important" resistance classes of highest priority for human medicine, classified by WHO [32], were compared among the treatments (Fig 6). None of the resistance classes (glycopeptide, macrolide, and beta-lactam) were significantly different amongst the three different treatments at either time point ($p>0.05$; Kruskal-Wallis). While not significantly different, the non-amended soils trended towards the highest amounts of relative glycopeptide gene copies. Also, despite being fed a macrolide (tylosin), this did not significantly enhance the quantity of the macrolide class in the soils with raw manure or composted manure of antibiotic administered steers compared to the non-amended control soils. However, it is should be noted that these results are limited in statistical power due to the low number of replicates.

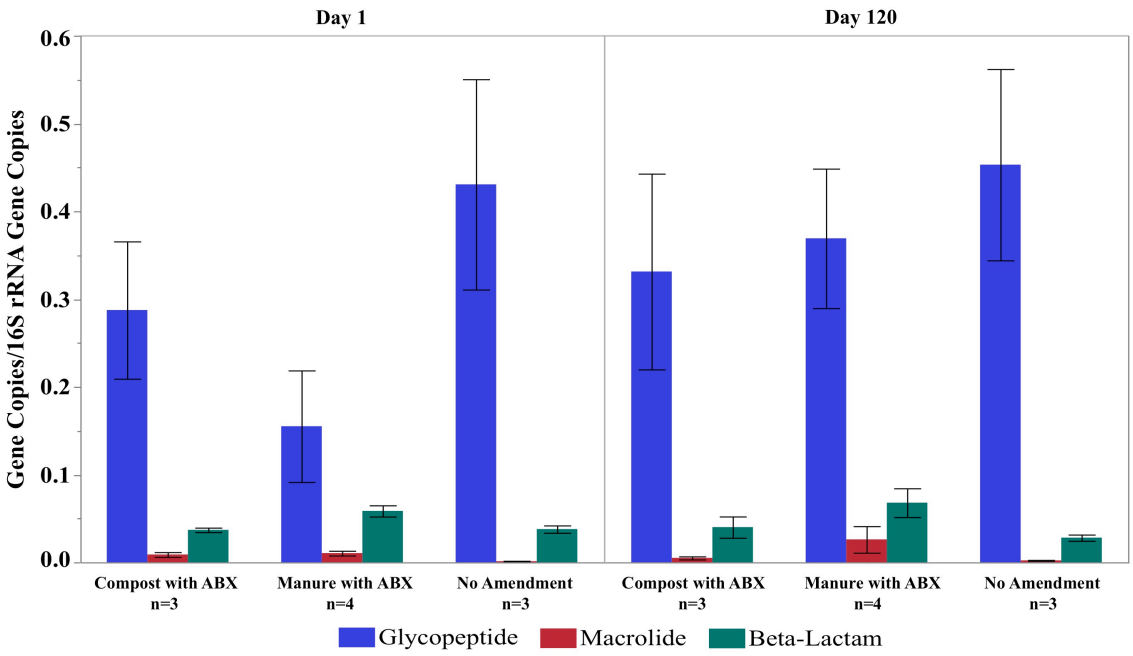

Fig 6. Shotgun Metagenomic Sequencing: ARGs of highest Priority for Human Health. Effect of soil amendment on the relative abundance of ARGs belonging to the "highest-priority critically important" resistance classes identified by WHO [32]. Error bars indicate the standard error of the number of reads corresponding to the resistance class, normalized to the number of 16S rRNA genes (relative abundance). The relative abundance of the three resistance classes were not significantly different between the three treatments at either time point ($p>0.05$; Kruskal-Wallis).

While examining resistance classes is important, it is also valuable to look closer at ARGs associated with clinical concern. The CTX-M beta-lactamases have become a major concern [47] and were examined in more detail from the metagenomics data (S1 fig). While there was no significant difference between treatments at either time point ($p>0.05$; Kruskal-Wallis), it is worthwhile to note that there was an increase in the occurrence of the gene family from day 1 to day 120. Specifically, more samples from the soil amended with raw manure of steers with antibiotics had CTX-M genes at day 120 than the soil with compost or no amendment (S1 fig).

In order to assess the potential risk of gene transfer, the shotgun metagenomic sequencing data was annotated to the ACLAME database to evaluate the levels of plasmid associated genes (Fig 7). On day 1, the soils with manure of treated steers had significantly lower relative

abundance of total plasmid associated genes than the unamended soils (p=0.0404; Tukey-Kramer HSD). After 120 days, there were no significant differences in relative abundance of plasmid associated genes among the treatments (p=0.9651; Oneway ANOVA).

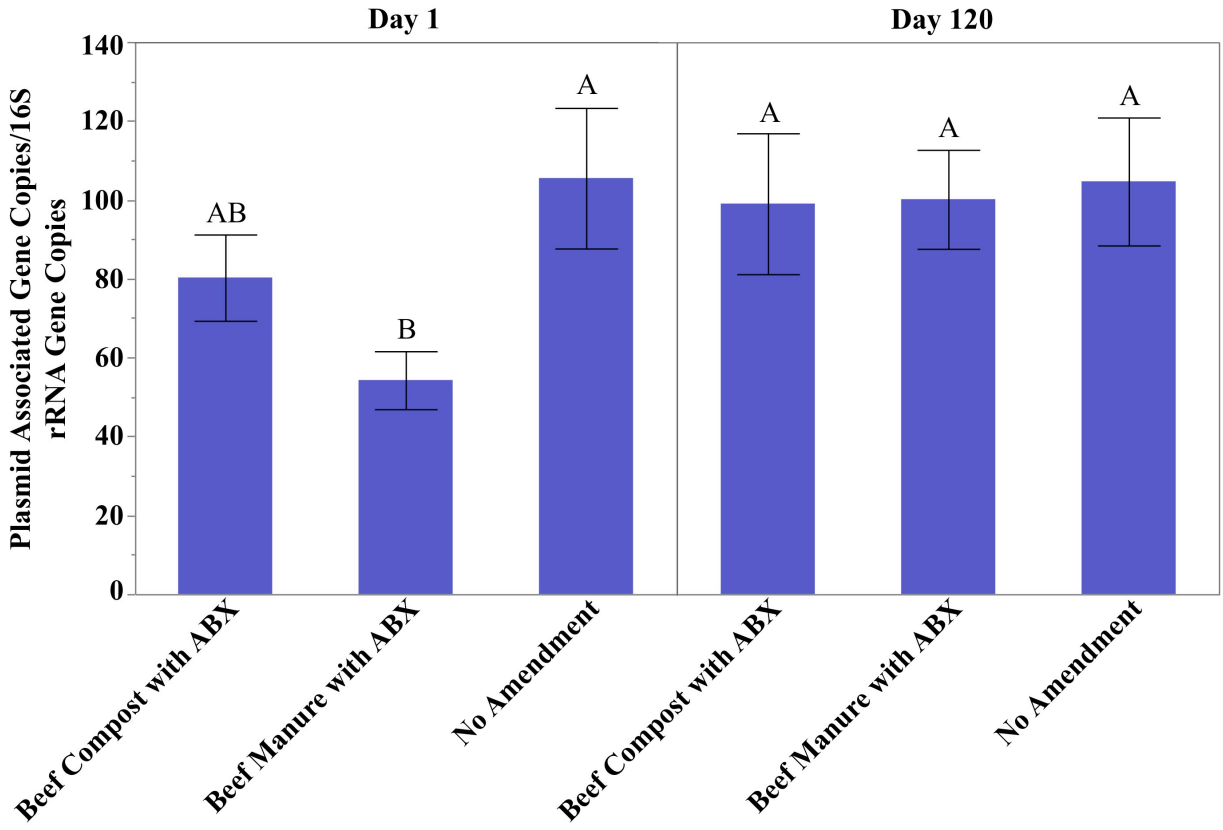

Fig 7. Shotgun Metagenomic Sequencing: Plasmid Associated Genes. Comparison of the relative abundances of plasmid associated genes among the raw manure or composted manure of treated beef steers applied soils and unamended soils. Per timepoint, the capital letters indicate significance between soils applied with the different amendments (p<0.05; Tukey-Kramer HSD).

Based on 16S rRNA amplicon sequencing, it is apparent that the compost application uniquely shapes the soil microbial community structure, relative to raw manure application, at both time points (p<0.05; ANOSIM) (Fig 3).

Effect of Soil Type on ARGs and Microbiota

Soil type had a significant effect on the relative abundance of *sul1* and *tet*(W) at both day 1 and 120 ($p<0.05$; Kruskal-Wallis). Amended sandy loam soil frequently contained greater relative abundance (normalized to 16S rRNA gene copy numbers) of *sul1* and *tet*(W) than amended silty clay loam and silty loam ($p<0.05$; Steel-Dwass All Pairs) (Figs 1 and 2). This trend was observed for all treatments at both time points, except for *sul1* relative abundance in soil with composted manure or raw manure from non-antibiotic administered steers on day 120 and normalized *tet*(W) in soil with raw manure from antibiotic administered steers on day 1 ($p>0.05$; Steel-Dwass All Pairs) (Figs 1B and 2A). These trends were not observed in the non-amended control soils, where the relative abundance of *sul1* was not significantly different among the three soil types at day 1, and at day 120 only silty loam was significantly different ($p<0.05$; Steel-Dwass All Pairs) (Fig 1). *tet*(W) was at or below the detection limit for a substantial portion of the non-amended control soils. Generally, this indicates that observed differences in *sul*1 and *tet*(W) are related to the amendment condition, rather than background levels.

Based on available shotgun metagenomic sequence data, it is possible to qualitatively examine the effect of soil type (silty clay loam and sandy loam) (S2 and S3 figs). Similar to the qPCR results, it was again observed that the sandy loam amended soils tended to have the strongest difference in terms of the total ARG profile (S2 and S3 figs). The ARG profile of the raw manure of antibiotic administered steers on sandy loam soil diverged from the non-amended sandy loam at day 1 and particularly on day 120 (S2 and S3 figs). On day 120, the raw manure treatment contained a higher relative abundance of macrolide, sulfonamide, tetracycline, chloramphenicol, beta-lactam, and aminoglycoside ARGs than the non-amended sandy loam (S3

fig). The profile makeup of the compost treatment on sandy loam at day 120 appeared to be more similar to the non-amended sandy loam (S3 fig). At day 120, the ARG profiles of all the treatments on silty clay loam were similarly structured (S3 fig).

Consistent with the qPCR and metagenomic results, the microbial community composition from 16S rRNA amplicon sequencing data also indicated that the greatest difference amongst soil types was with the sandy loam applied soil. Given that the compost-amended soils were not statistically different from each other, the soil types were compared within a combined compost treatment (S4 and S5 figs). On day 1 and 120, the microbial composition of compost treated sandy loam was significantly different from the compost-amended silty clay loam and silty loam ($p=0.002$; ANOSIM), while the microbiota of amended silty clay loam and silty loam were not significantly different from each other ($p>0.05$; ANOSIM). While the soils with raw manure cannot be compared statistically, there were apparent qualitative differences among the three soil types (S6 and S7 figs). The MDS plot of the 16S rRNA amplicon sequencing data also clearly indicated a soil type effect (Fig 8). All treatments applied to sandy loam ("sand"), except for the non-amended control, clustered away from the other two soil types at 70% similarity (Fig 8 and S8 fig).

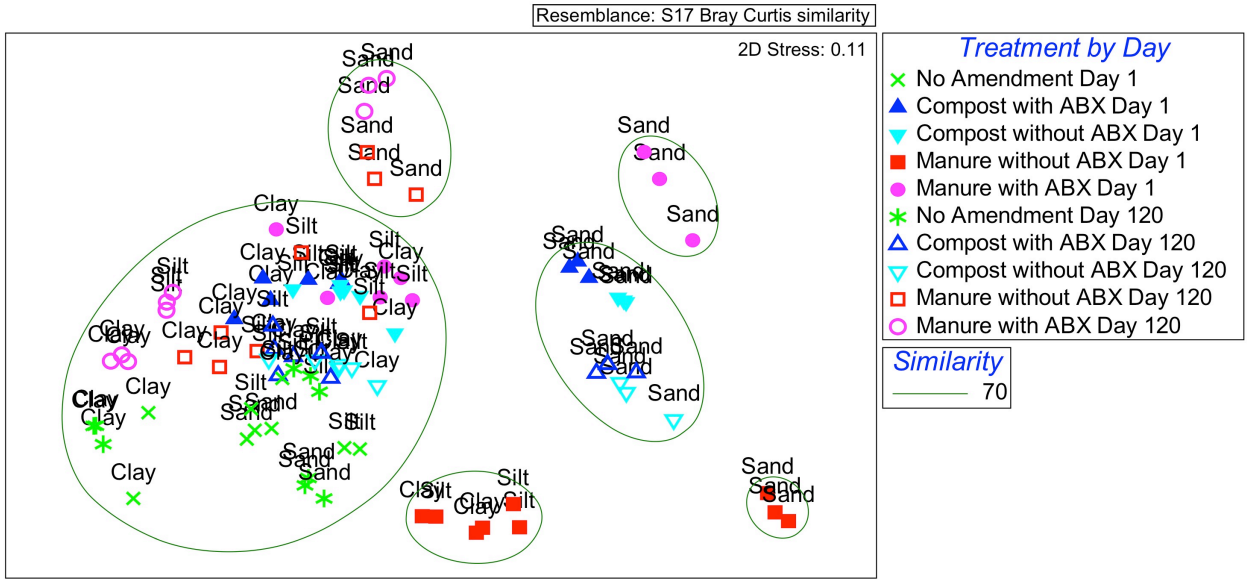

Fig 8. MDS Plot of Microbial Community Composition from 16S rRNA Amplicon Sequencing. MDS plot of the class-level taxonomic composition similarity amongst all treatments, both time points (day 1 and 120), and three soil types (sandy loam "sand", silty clay loam "clay", and silty loam "silt"). Clusters are based on 70% (green line) similarity.

Discussion and Conclusions

Consistent with our parallel investigation of dairy cow manure and compost (Ch.2), it was found that the prior administration of antibiotics also affected the relative abundance of ARGs and the microbial community composition in amended soil microcosms. Interestingly, this was in spite of the fact that different antibiotics were used in beef steers and that they were administered via oral route, rather than intramammary. Soils amended with raw manure from beef steers that received antibiotics contained significantly higher relative abundances of *sul1* than soils amended with raw manure of antibiotic-free steers. This supports prior studies that also indicated an increase in the abundance of *sul1* and *sul2* in soil amended with manure from pigs that received sulfadiazine compared to soil with manure from pigs without antibiotic treatment [48, 49]. For *tet*(W) on day 1, there was a higher relative abundance in soils amended with raw manure from steers without versus with prior administration of antibiotics. While this is an

interesting result, it was similarly found in a study feeding antibiotic dosed milk replacers to calves that *ermF* decreased in feces of calves fed antibiotics relative to the untreated calves [50]. It was theorized that the use of antibiotics decreased the particular bacteria carrying *ermF* [50], which could be the reason for the initial decrease in relative abundance of *tet*(W) genes seen here in the soil with manure of antibiotic administered steer. For *tet*(W), the effect of prior antibiotic use was no longer significant in the manure-amended microcosms after 120 days. A similar result was also noted in the identical dairy manure amended soil microcosms (Ch.2), where there was no significant difference in the relative abundance of *tet*(W) in soils amended with manure from cows with or without antibiotics at day 1 or 120. The overall increase but lack of difference in the quantity of tetracycline ARGs between treatments was also observed in another microcosm study amending soil with manure from dairy cows with or without oxytetracycline [51]. One explanation could be the prevalence of tetracycline ARGs in cattle's systems that was noted in a recent metagenomic analysis of dairy fecal samples [52]. Analysis of the microbial community composition also indicated that antibiotic usage had an impact, as there was a significant difference between the class-level taxonomic compositions of soils with raw manures from steers with or without antibiotics.

Applying compost or manure to soils generally was observed to increase the relative abundance of ARGs and impact the microbial community composition relative to the non-amended control soils up through the 120-day experimental period. The fact that the non-amended control soils remained relatively stable in microbial community composition over the 120 days supports the conclusion that observed shifts in the experimental microcosms were in fact due to the biological amendments. The shift of the microbial community is important to study in conjunction with ARGs, as metagenomic studies have shown that different microbial

community compositions are the main factors driving the ARGs present in soil samples [53, 54]. It was also observed that the frequency of HGT of ARGs was limited since the ARG content was primarily a function of the microbial community [54], which would perpetuate ARGs primarily through vertical gene transfer. The only conditions that were equivalent to the background after 120 days incubation were *sul1* in soils amended with antibiotic-free raw manure and, from shotgun metagenomic sequencing, the relative abundance of total ARGs of soils applied with raw manure or composted manure of antibiotic-administered steers.

One hundred twenty days is a key timespan of interest because, while FSMA does not yet have a specified number of days between raw manure application and crop harvest [55], it is a standard set by the United States Department of Agriculture's National Organic Program for the use of untreated animal waste as fertilizer for crops that have contact with the soil [31]. In the current literature, there is no discernable trend with respect to the duration that ARGs persist in the environment following manure or compost application. For example, a soil microcosm study using pig manure and soil injected sulfadiazine saw effects on sulfonamide resistance genes lasting greater than 2 months [56]. Another microcosm experiment using manure of dairy cows not treated with antibiotics found soils to have increased levels of beta-lactam genes persisting through the end of the 140-day study [57]. A field scale study saw an increase in *ermF*, *sul1* and *sul2* in soil following dairy manure slurry and dry stacked manure application, but the gene levels returned to background conditions by 2 months [9]. There remains a knowledge gap regarding the length of time manure and compost amendments impact the ARGs in the soil environment, particularly in representative, full-scale field studies and representing the wide diversity of soil types.

As observed in the parallel dairy microcosm study (Ch. 2), while the relative abundances of *sul1*, *tet*(W), and 16S rRNA genes did not return to the unamended soil conditions, the absolute abundances of the three genes did decrease from day 1 to day 120 (S9-11 figs). The dissipation of ARGs could be from bacterial cell death [9, 58], which is supported by the reduction in absolute abundance of 16S rRNA gene. Also, the degradation of extracellular DNA [59, 60], possibly a result of it being a food source for bacteria [61], could be an additional mechanism behind the observed decreases in absolute abundances of *sul1*, *tet*(W), and 16S rRNA genes.

Due to the cross-section of samples submitted to shotgun metagenomics and the inconsistent qPCR results, there is not as strong of evidence as there was in the dairy microcosm study (Ch.2) supporting the conclusion that composting neutralized the effect of antibiotic usage. A possible explanation for the differing qPCR results seen between our composted dairy and beef manures is that from the previously published results of these samples' chemical analyses, it is seen that sulfamethazine and chlortetracycline did not completely dissipate during the composting process [62], and, in our dairy experiment, the dairy cows did not receive a sulfonamide or tetracycline antibiotic. At day 120 there was 1.2-1.5 $\mu g\ kg^{-1}$ of sulfamethazine in the static compost-amended soils, opposed to 12-18 $\mu g\ kg^{-1}$ of sulfamethazine in the raw manure applied soils. The final (day 120) concentration of chlortetracycline in the static compost-amended soils was 1.8-3.1 $\mu g\ kg^{-1}$ and 9.3-19 $\mu g\ kg^{-1}$ in the soils with raw manure [62]. Tylosin was below detection limit in the static compost-amended soils by day 120, although it was still present in the raw manures at low concentrations (0.51-0.87 $\mu g\ kg^{-1}$) [62]. These results are similar to other studies that have also shown that composting is effective at reducing the antibiotic concentrations in raw manure [39, 63, 64]. Supporting the conclusion that composting

reduces the effect of prior antibiotic usage, the 16S rRNA amplicon sequencing data showed that at both time points there was no significant difference between class-level taxonomic compositions of soils applied with either compost types, while there was a significant difference between soils amended with the two raw manure types.

While composting may reduce the effect of prior antibiotic usage in beef steers, its effect on ARGs is not as clear. The qPCR results indicate that composting did not provide benefit with respect to reducing the relative abundance of *sul1* in soil compared to applying raw manure of steers without antibiotic treatment. While the relative abundance of *tet*(W) at day 1 was less in the compost-treated soils than the raw manure amended soils, there was no significant differences amongst the biologically-amended soils at day 120. Due to the cross-section of samples submitted for metagenomics, it is unknown if the total ARGs of the soils amended with raw manure of steers without antibiotics would have been greater or lesser than the compost treated soils. Supported by both the *sul1* qPCR and metagenomics results, composting does reduce the relative abundance of ARGs relative to soil amended with raw manure from beef steers administered antibiotics. The results seen in our parallel dairy study (Ch.2) also indicated that composting decreased ARGs compared to soils amended with raw manure of antibiotic-treated dairy cows, but not always in comparison to soils applied with manure of untreated dairy cows. The limited studies on ARGs and composting have generally found that composting decreases ARGs [23, 65, 66], but not always, as one study with swine manure found that thermophilic composting increased efflux pumps and sulfonamide resistance genes [22] and another saw an increase in *tet*(W) following composting of horse manure [66]. Also, compost applied soils temporarily had a higher relative abundance of plasmid associated genes than the corresponding soils with raw manure of antibiotic treated steers. These results follow the same

trend observed in the parallel dairy manure study (Ch.2), but contradict a prior composting study with chicken manure that more specifically measured plasmid transfer frequency and saw decreases after composting [67]. The results of the dairy (Ch.2) and beef microcosm studies and published literature indicate that there are likely numerous factors that influence the effectiveness of composting for reducing ARGs. As observed in this study, examining the interaction of composting with soil application is important for determining the downstream impacts of composting further along the farm-to-fork continuum.

The qPCR and metagenomics data both supported the conclusion that soil type influences ARG levels. Similar to the trend observed in our prior study of dairy manure (Ch. 2), sandy loam was associated with the least attenuation of *sul1* and *tet*(W). Silty loam had the greatest attenuation of *sul1* and *tet*(W) after raw manure application rather than the silty clay loam as observed in the dairy manure study (Ch. 2). This suggests promise of consideration of soil type as an additional factor in developing best management practices for minimizing the spread of antibiotic resistance. While unable to be statistically compared, there is also a visual difference between the ARG profiles of the silty clay loam and sandy loam from metagenomics (S2 and S3 figs). As proposed in the prior dairy micrcocosm study (Ch. 2), the observed tendency for the sandy loam ammneded soils to have the highest relative abundances of *sul1* and *tet*(W) may be due to a microbial reaction to the added nutrients [13, 14] by the sandy loam's natural bacterial community. Sandy loam soils may have had the strongest response to the nutrients, since the sandy loam soils initially had the lowest organic matter content (S1 Table) and 16S rRNA gene copies (S11 fig). The beef and dairy study (Ch.2) are the first studies, to the authors' knowledge, specifically to address the knowledge gap [68] of soil type as a factor influencing ARG levels of biologically-amended soils. Soil type also appears to be a factor influencing the bacterial

taxonomy (S4-S7 figs), which has been reported in other studies as well [69, 70]. The impact of soil type should continued to be explored as different pyhsico-chemical properties have been shown to affect DNA [27] and antibiotic adsorption [28-30, 71].

The 16S rRNA amplicon sequencing data indicate that just one application of manure or compost to soil impacted the bacterial community for at least 120 days. Other studies have also seen that the application of manure influences the taxonomic composition of soils [13, 72, 73]. As shown previously (Fig 3) and in the supplementary figures (S12 and S13 figs), *Proteobacteria* was in enriched in soil with compost from antibiotic treated steers on day 1 and both manure soil treatments at day 120 ($p<0.05$; Steel-Dwass All-Pairs). This is important to analyze as it was found in a comprehensive bacterial genome study that horizontal gene transfer of mobile ARGs are predominately found in *Proteobacteria* [46]. The parallel study with dairy cows also saw an increase in *Proteobacteria* in the biologically amended soils relative to the unamended soils (Ch. 2). *Acidobacteria* was also notably lower in the amended soils rather than the non-amended soils ($p<0.05$; Steel-Dwass All Pairs), except for soil with raw manure from steers receiving antibiotics on day 120. This could be important as this phylum is believed to play an important role in ecosystem function due to its abundance in soil [73]. The same trend was also observed in the dairy study (Ch.2) and another soil microcosm using spiked sulfadiazine manure [73].

In conclusion, the application of raw manure or compost in a microcosm environment impacts the ARGs and microbiota of soils for at least 120 days. Prior use of antibiotics also seems to have an effect, as soils amended with manure from steers that were given antibiotics had greater relative abundance of *sul1* relative to the amendment of untreated steers' manure. However, the magnitude of that effect is dependent on the ARG of interest, as there were clear

differences between the trends exhibited by *sul1* and *tet*(W). Prior antibiotic usage also did not appear to significantly select for "critically important" resistance gene classes, but the occurrence of CTX-M did increase over time. There was a significant difference in the bacterial communities of soils applied with the raw manures of steers with or without antibiotic treatment. Composting has the potential to reduce the relative abundance of ARGs compared to soil applied with raw manure of antibiotic administered steers. However, applying compost to soils still increased ARGs compared to the non-amended soils and, sometimes, relative to soils amended with raw manure of untreated steers. Composting did appear to reduce the effect of prior antibiotic usage on the microbiota, as there was no significant difference between the soils amended with the two compost types. Regardless of composting, amending the soil saw an increase in *Proteobacteria* and decrease in *Acidobacteria* relative to the non-amended soils. The results of this study also support soil type as a factor affecting ARG levels and the microbial community composition. Amended sandy loam soil had the largest differences in ARGs and taxonomy relative to the silty clay loam and silty loam. These conclusions are further verified by the similar results seen in the parallel dairy microcosm study (Ch.2).

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Chapter 4—Conclusions

Antibiotic Resistant Bacteria

In addition to the molecular work done, the samples were also cultured on media infused with antibiotics of interest (for methods see Appendix: Ch.4), but due to the lack of trends, the data was not included in Ch. 2 and 3 manuscripts. The samples were spread plated onto R2A (heterotrophic bacteria) and MacConkey (enteric bacteria) media that contained empirically determined concentrations of clindamycin, erythromycin, ceftazidime, cefotaxime, tetracycline, and vancomycin (concentrations listed in methods). The resulting colony forming units (CFUs) were log transformed and plotted against time (Ch.2 S10-S11 Figs and Ch.4 S1-S6 Figs). Soils amended with dairy or beef manure from cattle administered antibiotics typically had the lowest log CFU/g amongst all the treatments, excluding the non-amended soils, on the R2A and MacConkey plates. The non-amended control soils predominately had the lowest log CFU/g. There was no overall trend regarding the changes in log CFU/g over time on the R2A media, and the treatments tended to decline or remain relatively constant in log CFU/g on the MacConkey media.

Comparisons of treatment type (manure with ABX, compost with ABX, etc.), time, and soil type revealed that for the dairy and beef derived samples on both media types (R2A or MacConkey), treatment type and time were the primary factors causing the different log CFU/g. Treatment type as a single factor and as a part of a two-way interaction with soil type or time were significant factors for almost every antibiotic infused plate type (S2 and S3 Table). Interestingly, for both dairy and beef samples on each of the media types, soil type was rarely a significant factor in log CFU/g differences (S1 and S2 Table).

Project Conclusions and Summary

The two parallel beef and dairy microcosm studies had the same conclusions. Both studies showed that applying an amendment (compost or manure) to soils affected the microbial community composition and elevated the relative abundances of *sul1* and *tet*(W) up through the 120-day study. Importantly, the microbial community composition of the unamended soils stayed relatively constant while the amended soils' did not, indicating that the amendment was the main factor affecting the microbial community composition. There was also a significant impact caused by prior antibiotic usage with the relative abundances of *sul1* elevated in the soils amended with raw manure of both antibiotic treated cattle types. The relative abundance of *tet*(W) was not affected by prior antibiotic usage though, as the soils amended with manure of untreated cattle had higher levels or were not significantly different from soils with manure of antibiotic treated cattle. This trend was observed in both the dairy and beef microcosms despite dairy cows not receiving tetracycline antibiotics.

The shotgun metagenomic data also indicated on day 1 an effect of prior antibiotic usage in the dairy samples, as the relative abundance of total ARGs in soils with manure of antibiotic cows were significantly higher than the untreated manure amended soils. This effect of prior antibiotic use appears to diminish after composting in both studies, although there is more supportive evidence in the dairy study than the beef. However, compost-based amendments still resulted in an increase in ARGs relative to the control soils and occasionally had similar or greater relative abundances of ARGs compared to soils amended with antibiotic-free cattle manure. Soil type is a possible mediating factor, as there were significant differences between the soil types. Trends indicate that there were higher relative abundances of ARGs in amended

sandy loam soils. On the other hand, amended silty clay loam (beef study) and silty loam (dairy study) tended to have the lowest levels of ARGs amongst manure-based amendments.

Recommendations

The overall goal of this project is to transfer the results of this study to best management practices for the agricultural community. Following are recommendations resulting from the beef and dairy microcosm data, but prior to public recommendations this data should be compared to the results from the full-scale field and vegetable experiments funded by the same grant:

1. A time period greater than 120 days should be considered between the application of biologically based amendments and harvest of crops.
2. To reduce the use of antibiotics, farm conditions should be maintained to prevent disease and infections in livestock.
3. Antibiotic treated and untreated cattle should be in different pens/enclosures so that their manure can be stored separately. Based on this study and concerning ARG levels, manure of untreated cattle can be applied to soil raw, but prior to field application, manure of antibiotic treated cattle should be composted following FSMA standards.
4. Synthetic fertilizers instead of biologically based amendments should be considered for farms with sandy soils.

Future Work

While this project contributed to helping answer several questions concerning ARGs in the agroecosystem, there are still many knowledge gaps that exist and additional questions that were raised from this study. A main question I feel that this study prompted is the usefulness of composting. Composting appears to have both advantages and disadvantages when it comes to

ARG levels in soils. I think future studies should try amending soil with multiple different composting procedures (forced aeration, static, turned, enclosed, windrow, etc.) generated, if possible, at full-scale as differences in results has been observed between bench-scale and full-scale composting studies [1]. Also, amending soils with different manure treatments/techniques, such as dehydration or direct injection methods at multiple depths, would be interesting to study in conjunction with composting.

From the microcosm study, running shotgun metagenomic analysis on the remaining soils samples amended with beef compost and manure of untreated cattle would enable a better comparison of the effects of prior antibiotic usage and composting. Comparison of the field scale study's results to the microcosms' will also be interesting to see if the results seen in this study are replicated in a more real-world setting.

Soil type appears to have an effect on ARGs, so I think there should be more research into this factor. The types of soils across the nation are extremely heterogeneous. In order to have recommendations that can be applied to more of the agricultural community, I think a study should be done using one soil type and biologically based amendment but adjusting different physico-chemical properties of the soil, such as the pH by adding lime or maintaining different moisture contents. This will allow a more controlled observation of specific properties within soils that may be affecting the differing ARG levels seen in this study. Also, it will enable the ability to develop better recommendations for farmers, as farmers cannot change the soil type of their fields, but they do have the ability to adjust the properties of their soils within conditions still suitable for crop growth.

Finally, I think more research is needed for determining the optimum length of time between manure or compost amendment and crop harvest. It would also be interesting to do a

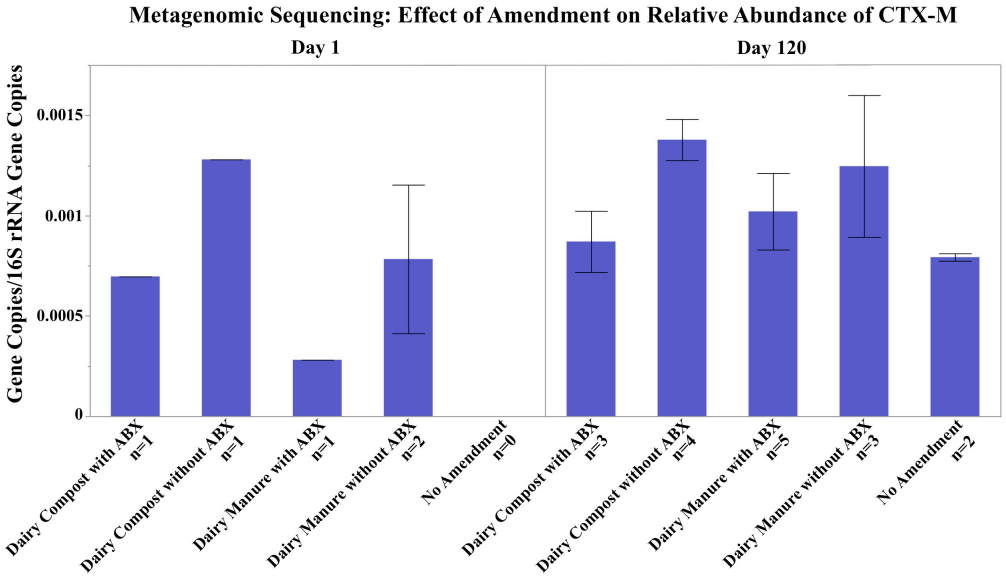
study in multiple different climate conditions, as there may be different lengths of time necessary for ARGs to return to baseline conditions depending on the climate.

Reference

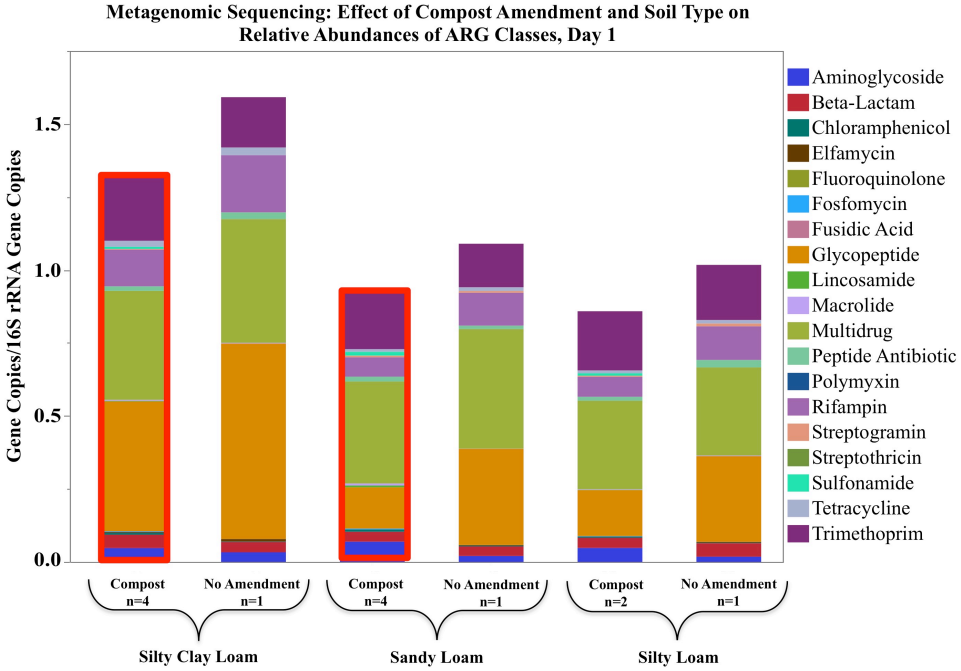
1. Williams RK. Effect of Composting on the Prevalence of Antibiotic Resistant Bacteria and Resistance Genes in Cattle Manure: Virginia Tech 2017.

Appendix—Supplementary Figures

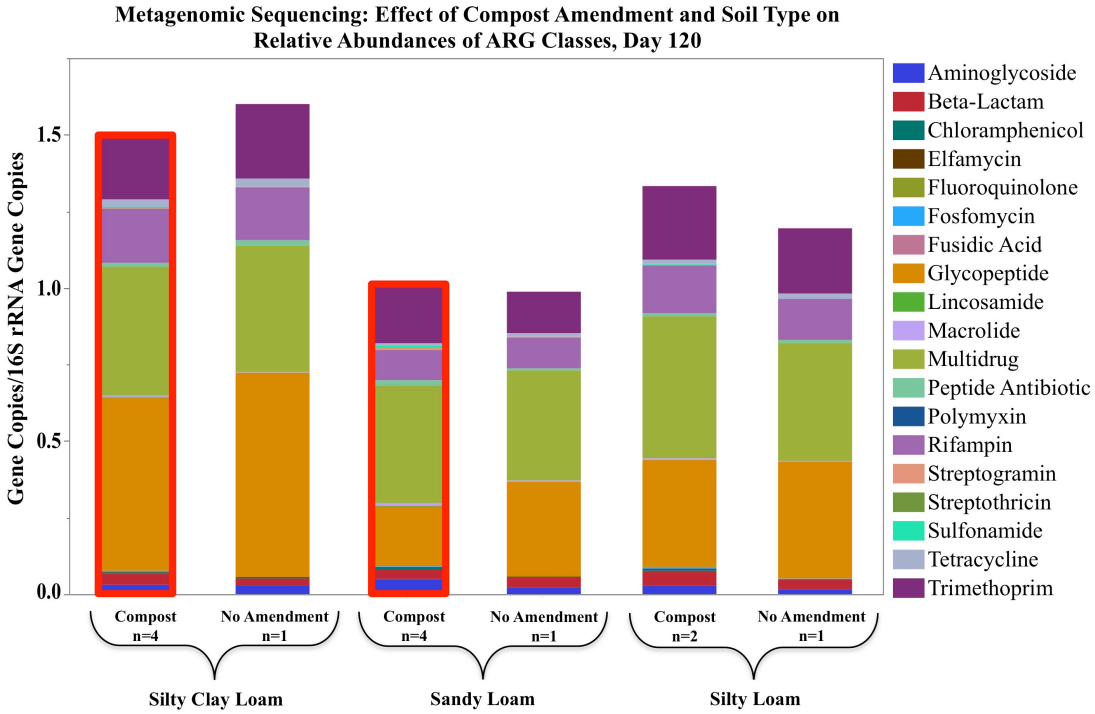
Chapter 2—Dairy Supplementary Figures



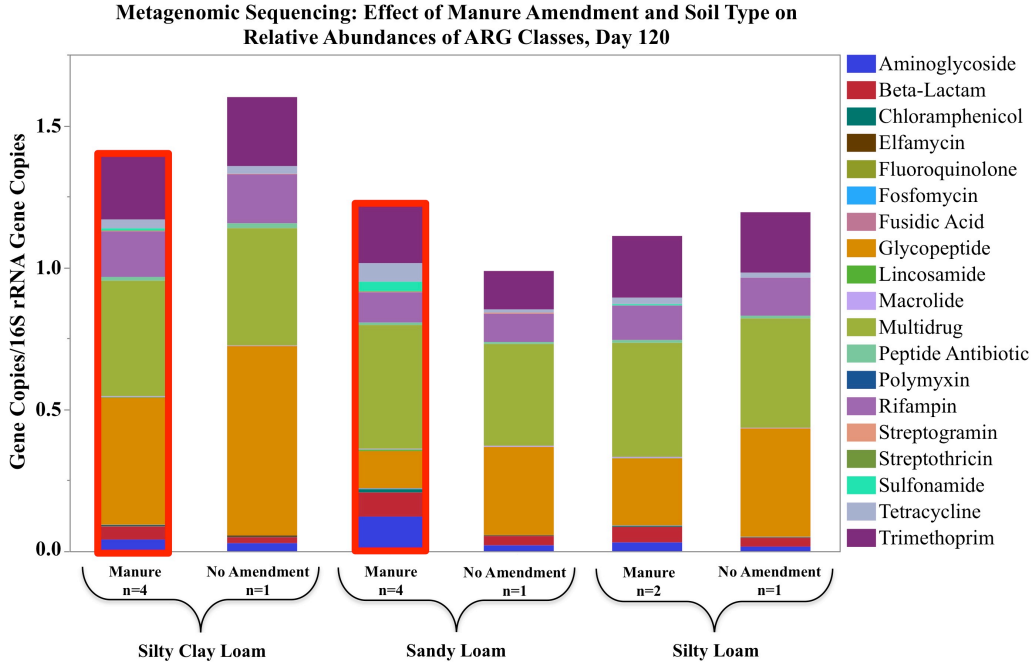
S1 Fig. This bar graph, with standard error bars, from the metagenomics data shows the mean relative abundance of CTX-M genes in samples (n) that had CTX-M present. The relative abundance of CTX-M was not significantly different among the treatments ($p>0.05$; Kruskal-Wallis), but the occurrence of CTX-M in the samples increased from day 1 to 120.



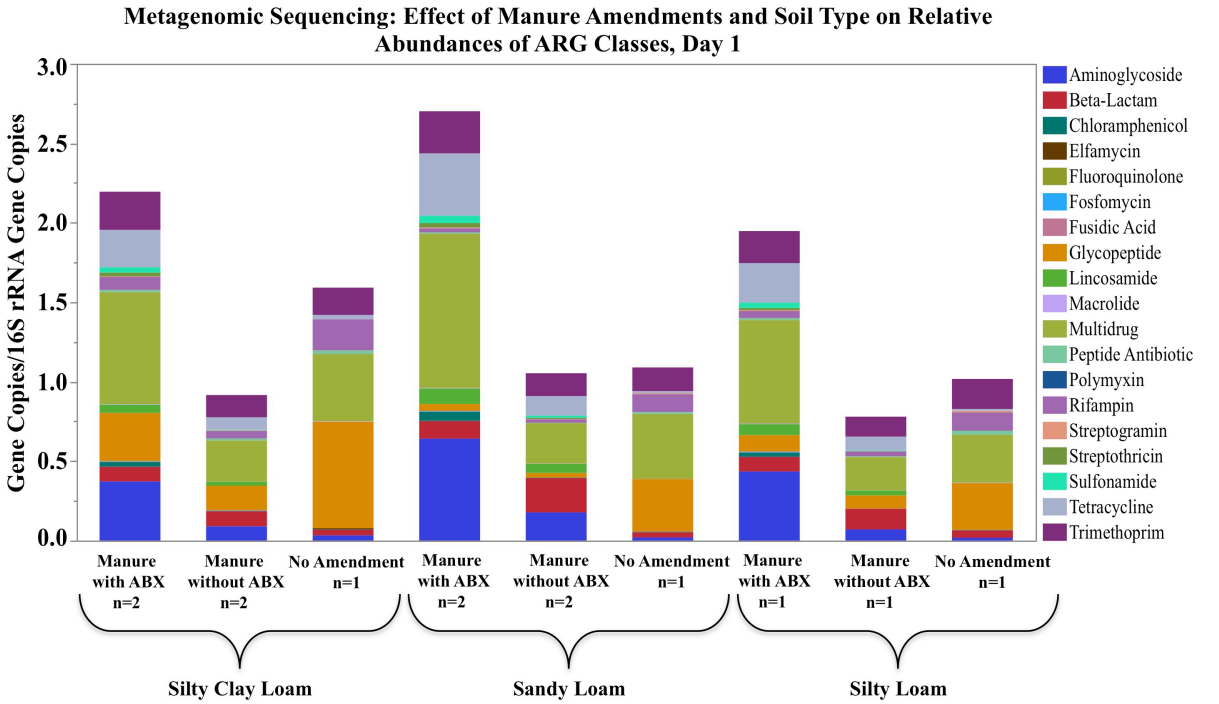
S2 Fig. Comparison of the relative abundance of total ARGs by compost treatment and soil type on day 1. The red box indicates significance between compost treatments on silty clay loam and sandy loam ($p=0.029$; ANOSIM).



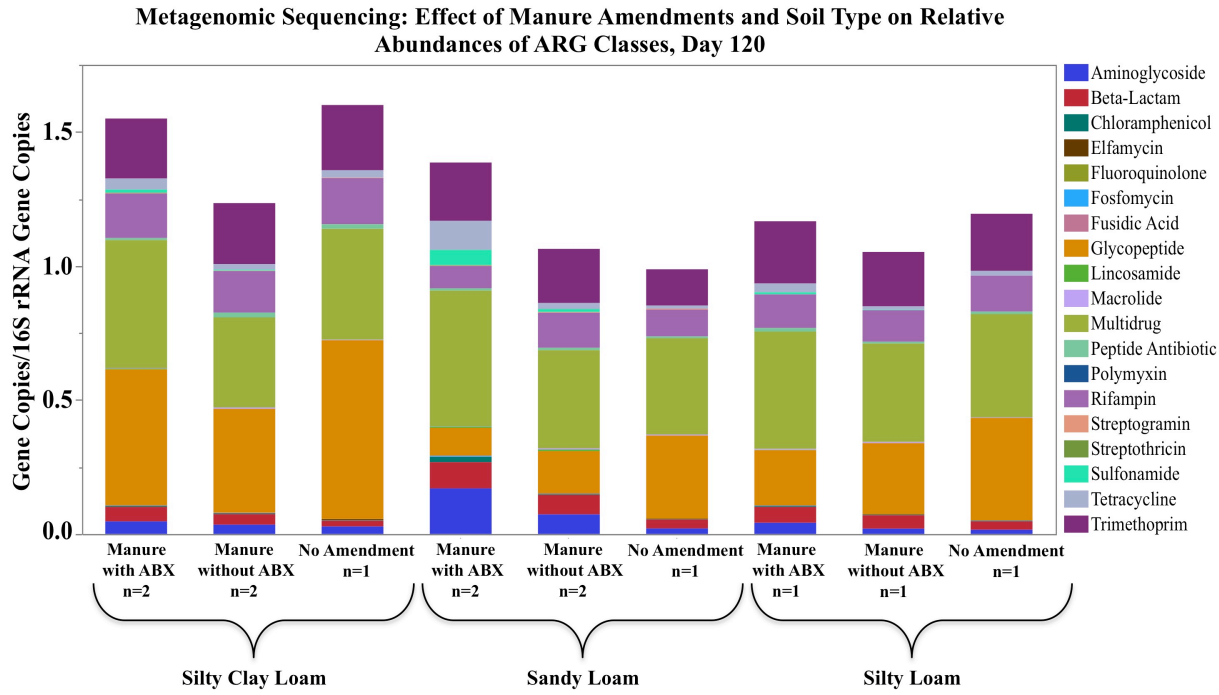
S3 Fig. Comparison of the relative abundance of total ARGs by compost treatment and soil type on day 120. The red box indicates significance between compost treatments on silty clay loam and sandy loam (p=0.029; ANOSIM).



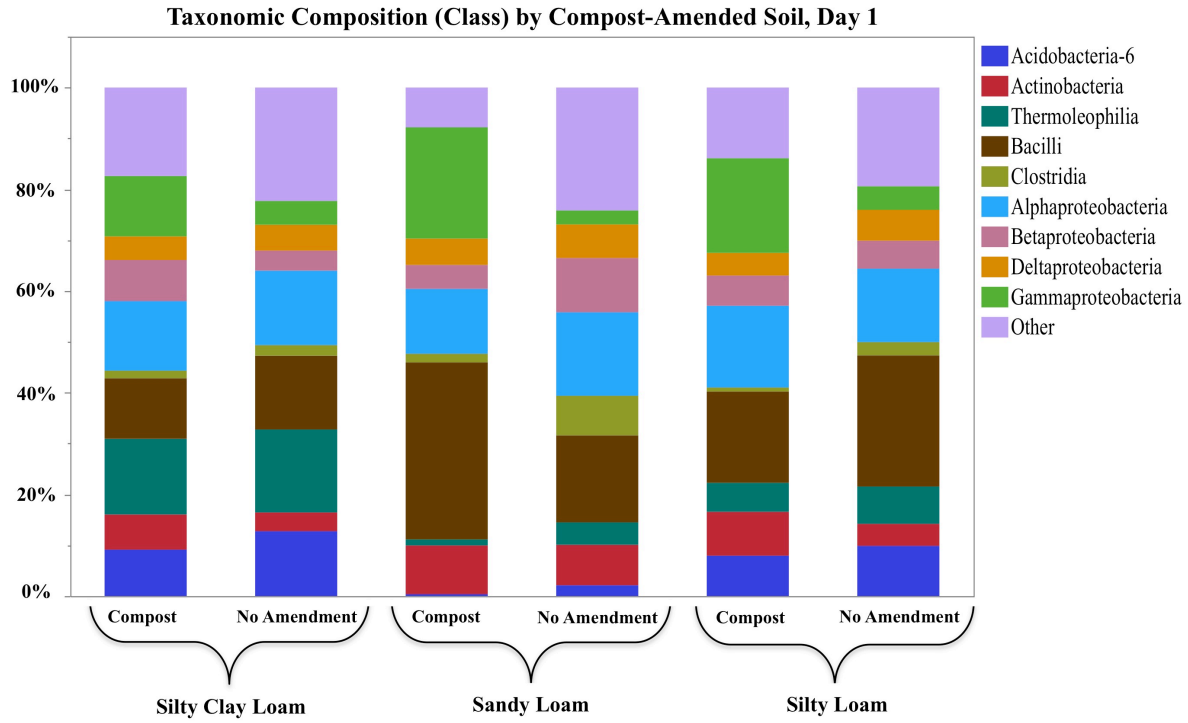
S4 Fig. Comparison of the relative abundance of total ARGs by manure treatment and soil type on day 120. The red box indicates significance between manure treatments on silty clay loam and sandy loam (p=0.029; ANOSIM).



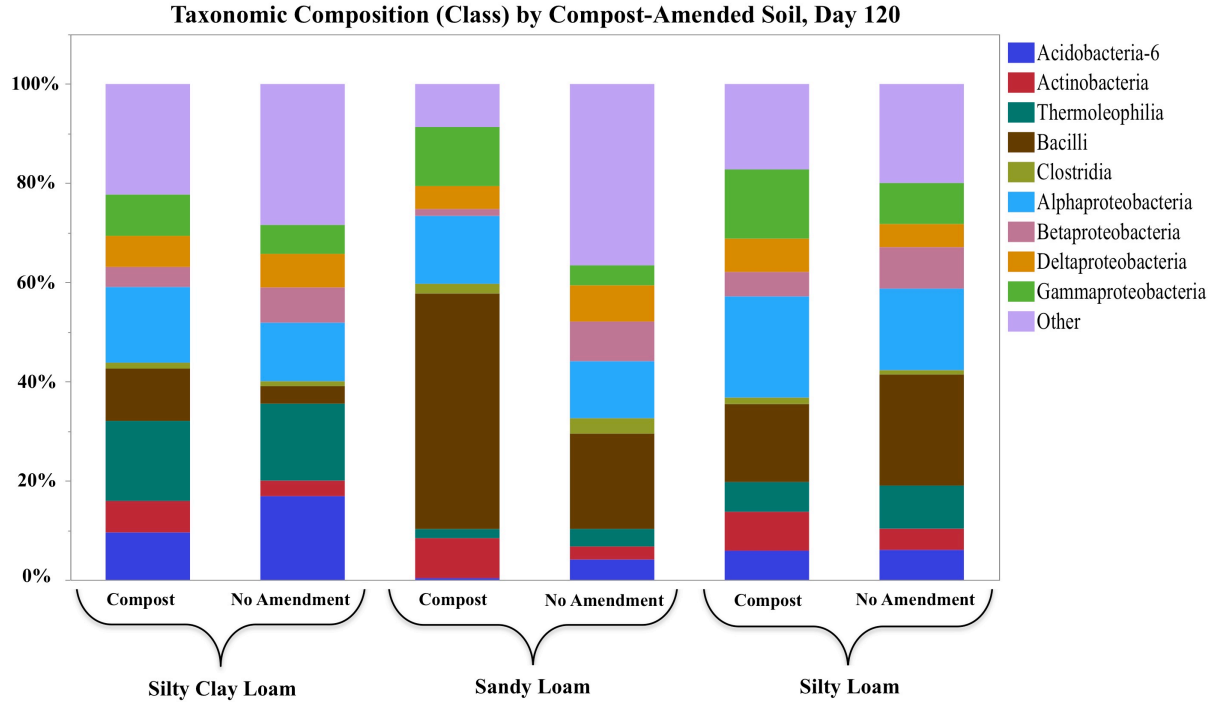
S5 Fig. Comparison of the relative abundance of total ARGs by manure treatments applied to each soil type at day 1. Qualitative comparison due to low replicates.



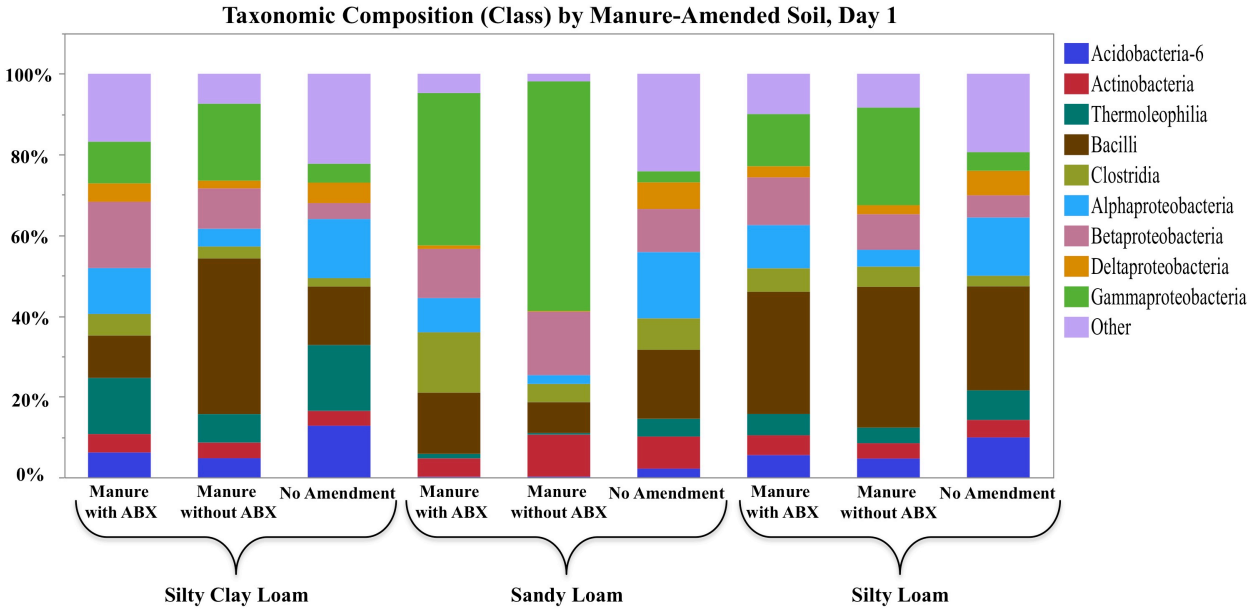
S6 Fig. Comparison of the relative abundance of total ARGs by manure treatments applied to each soil type at day 120. Qualitative comparison due to low replicates.



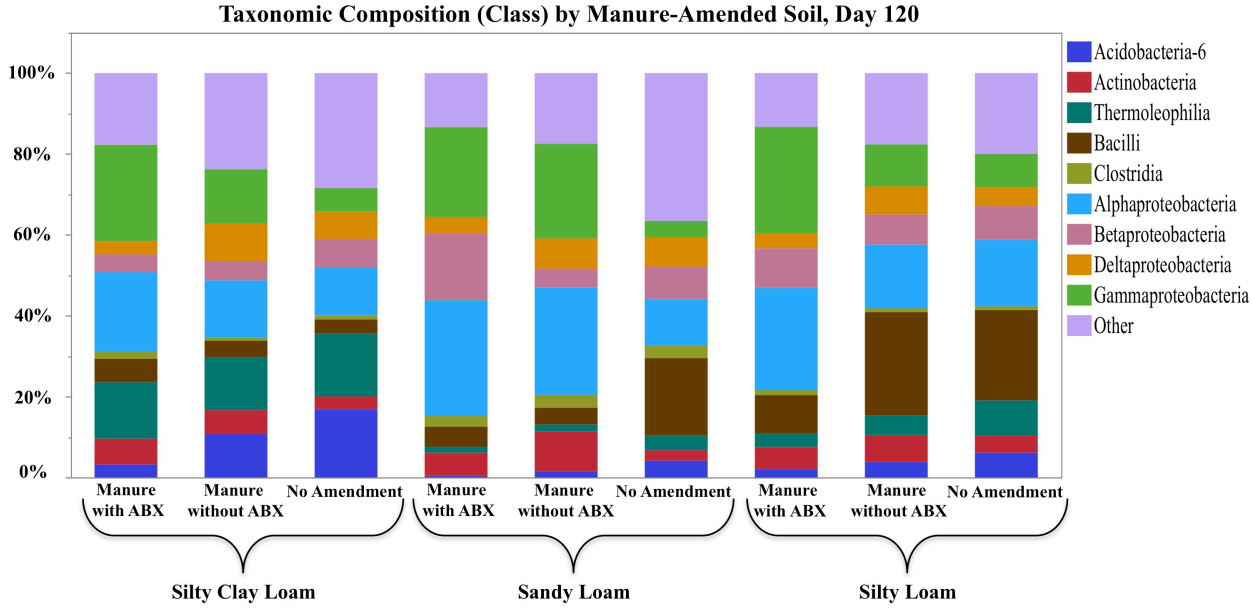
S7 Fig. Comparison of the class-level taxonomic compositions of the compost treatments applied to each soil type at day 1. All three soil types applied with compost were significantly different from each other (0.002; ANOSIM).



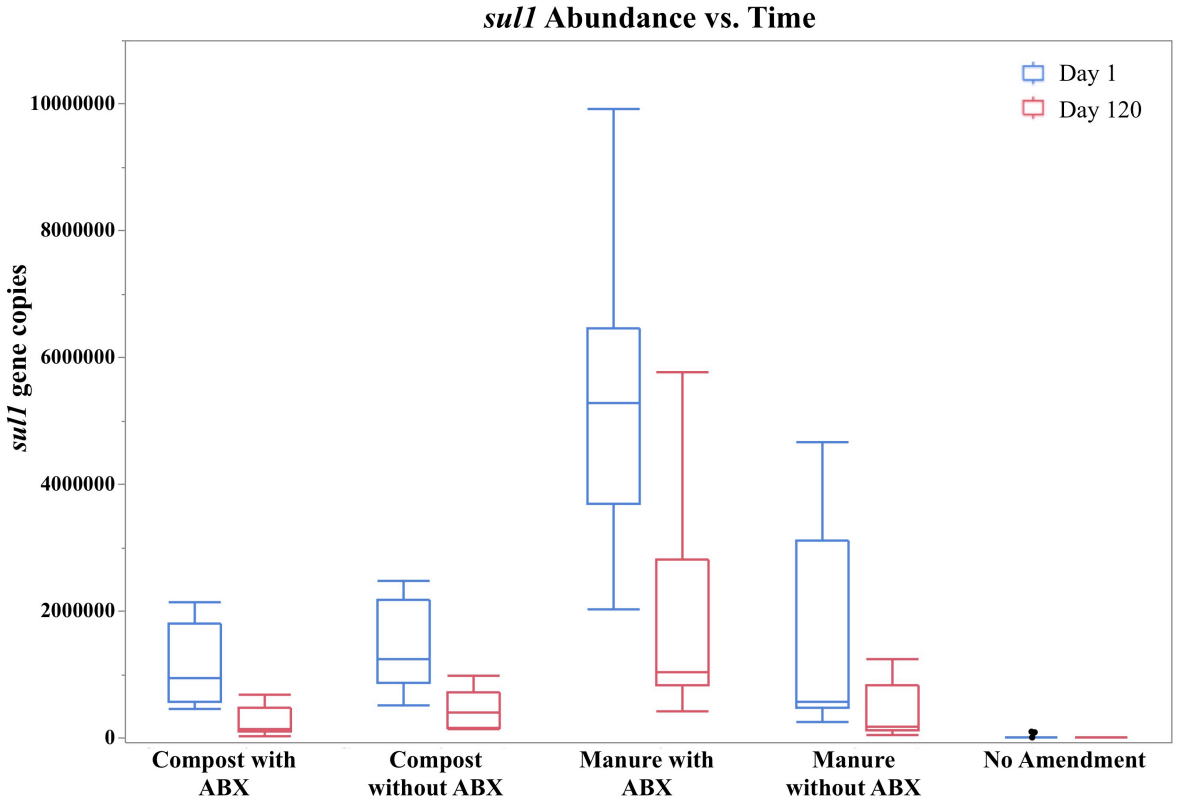
S8 Fig. Comparison of the class-level taxonomic compositions of the compost treatments applied to each soil type at day 120. All three soil types applied with compost were significantly different from each other (0.002; ANOSIM).



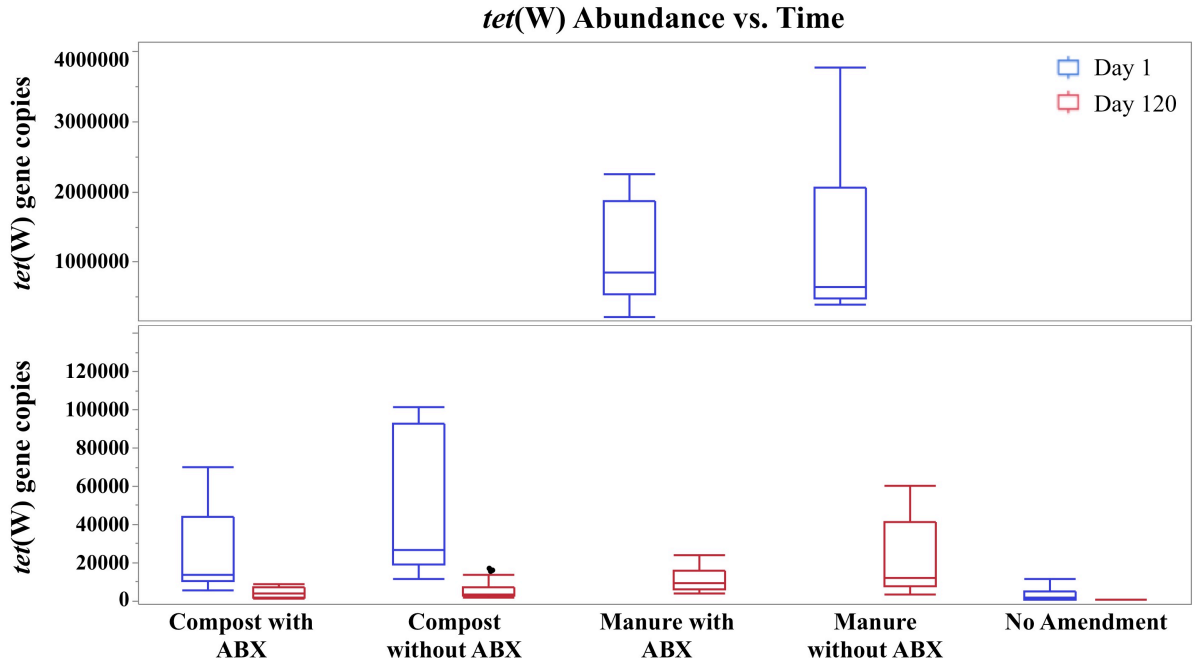
S9 Fig. Comparison of the class-level taxonomic compositions of the manure treatments applied to each soil type at day 1. Qualitative comparison due to low replicates



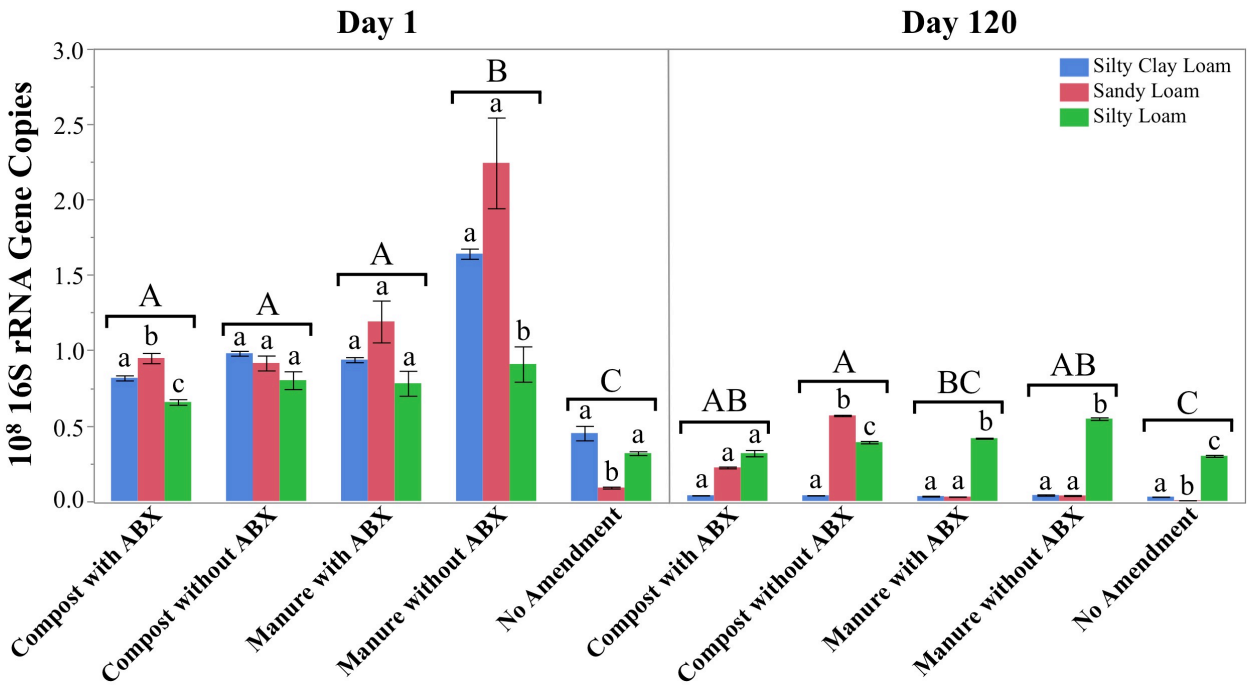
S10 Fig. Comparison of the class-level taxonomic compositions of the manure treatments applied to each soil type at day 120. Qualitative comparison due to low replicates



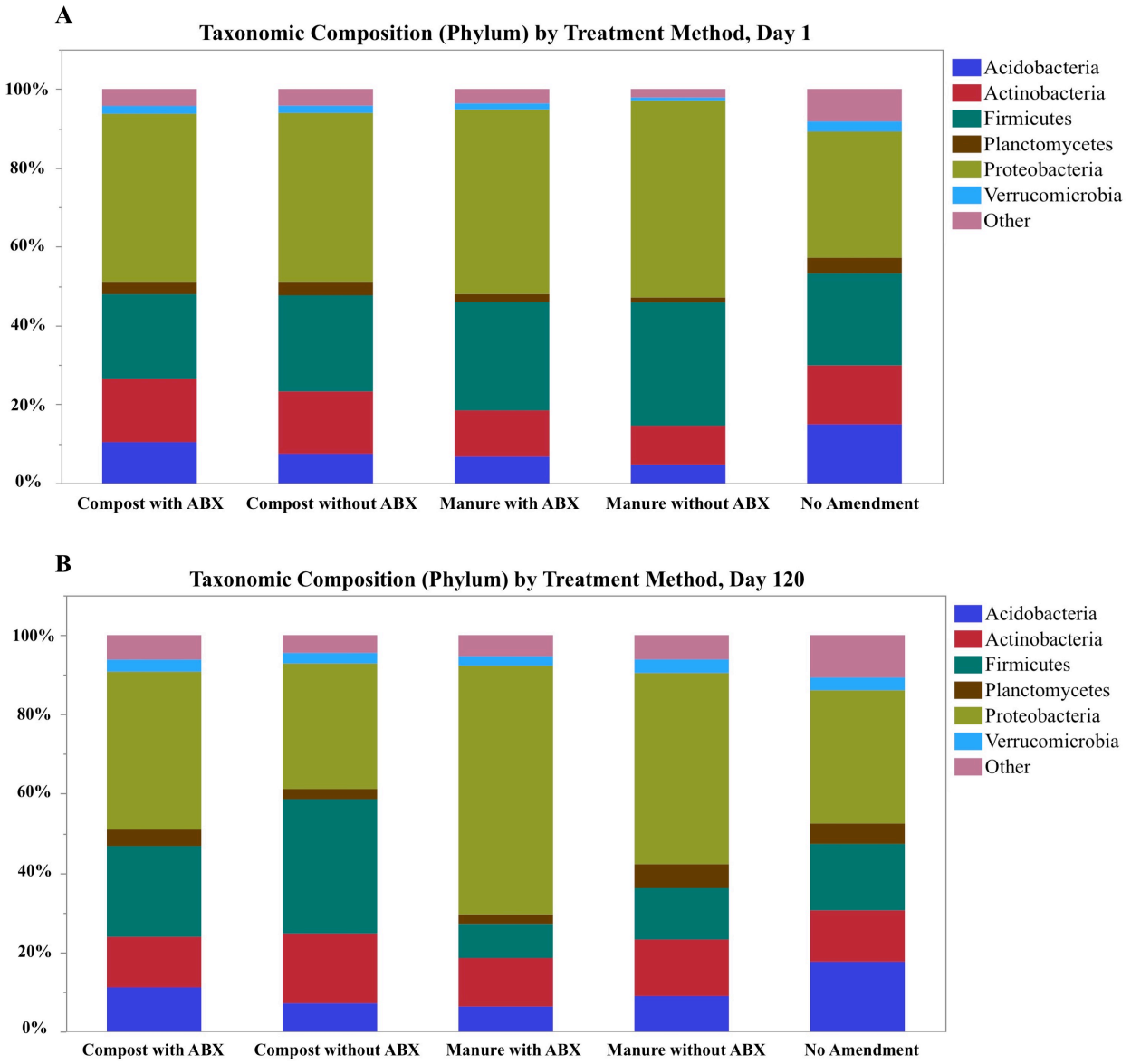
S11 Fig. Box plots of the *sul1* absolute abundances.



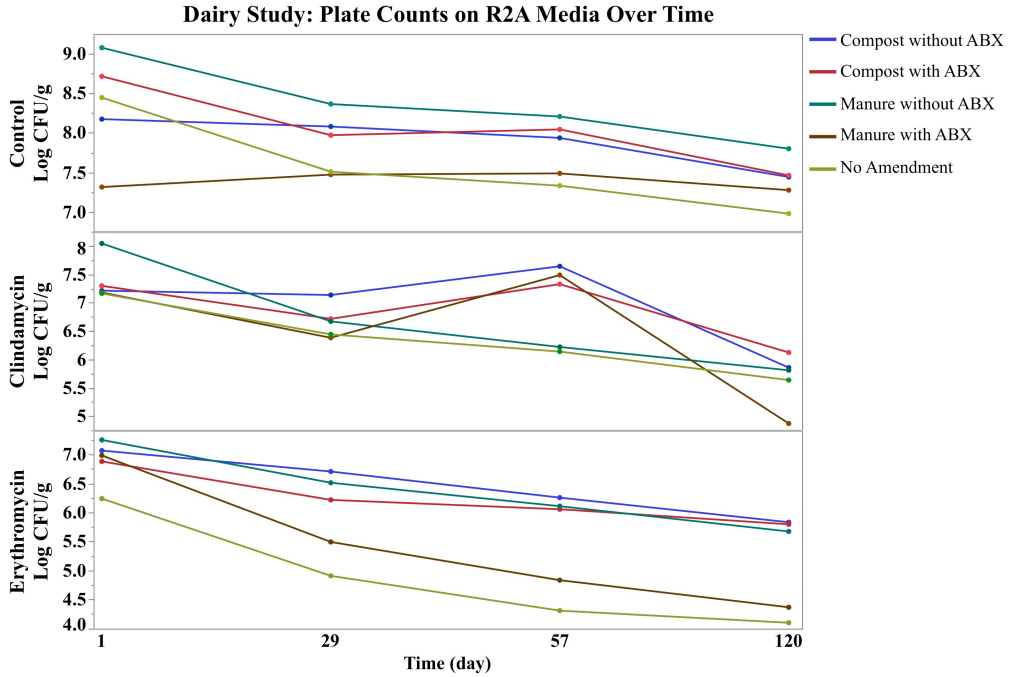
S12 Fig. Box plots of the *tet*(W) absolute abundances.



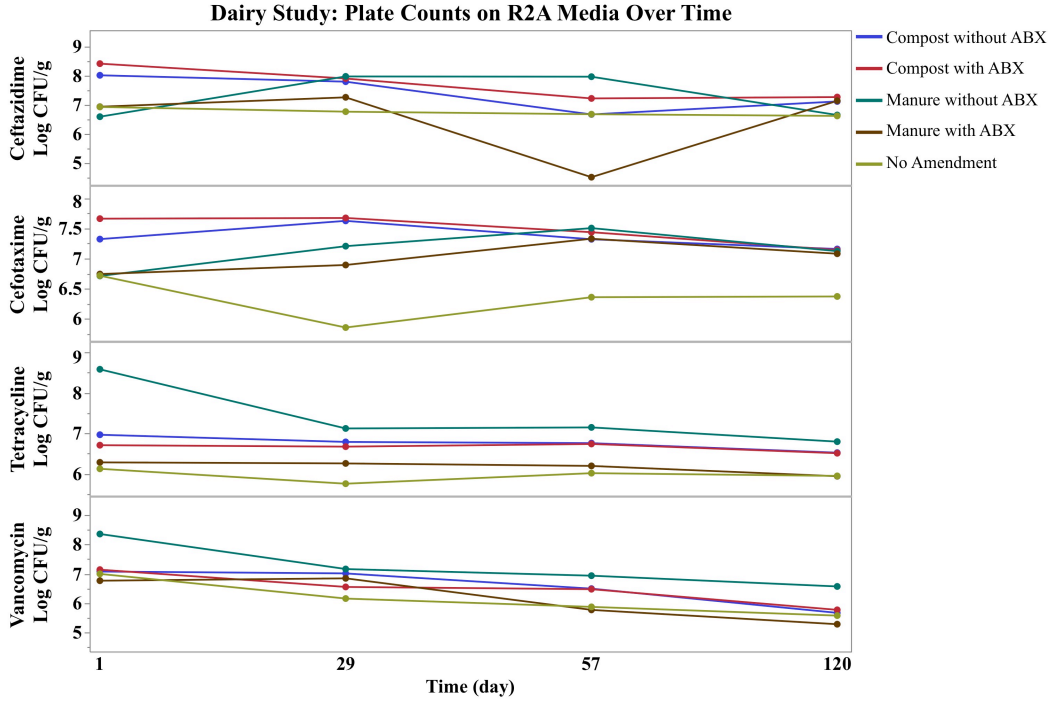
S13 Fig. Absolute abundances of 16S rRNA gene. Per timepoint, the capital letters indicate significance between soils applied with the different amendment conditions, and the lowercase letters indicate significance among soil types within each specific treatment ($p<0.05$; Steel-Dwass All Pairs).



S14 Fig. Comparison of the top five bacteria phyla compositions of the different treatments applied to soil at day 1 (A) and day 120 (B).



S15 Fig. R2A culturing results of the five different amended soil conditions on media infused with no antibiotics ("Control"), clindamycin, or erythromycin over four time points. The unamended soils ("No Amendment") frequently has the lowest Log CFU/g compared to the amended soils. Antibiotic concentrations within the media are listed in S3 Table.



S16 Fig. R2A culturing results of the five different amended soil conditions on media infused with ceftazidime, cefotaxime, tetracycline, or vancomycin over four time points. The unamended soils ("No Amendment") frequently has the lowest Log CFU/g compared to the amended soils. Antibiotic concentrations within the media are listed in S3 Table.

S1 Table. Properties of three soil types. The moisture content was tested when soil initially collected.

Soil Type	Label	Moisture (%)	pH	Field capacity (%)
Sandy loam	Sandy	N.A.	6.17	24.3±1.1
Silt loam	Silt	17.21	6.64	50.9±3.0
Silty clay loam	Clay	19.92	6.79	57.8±0.1

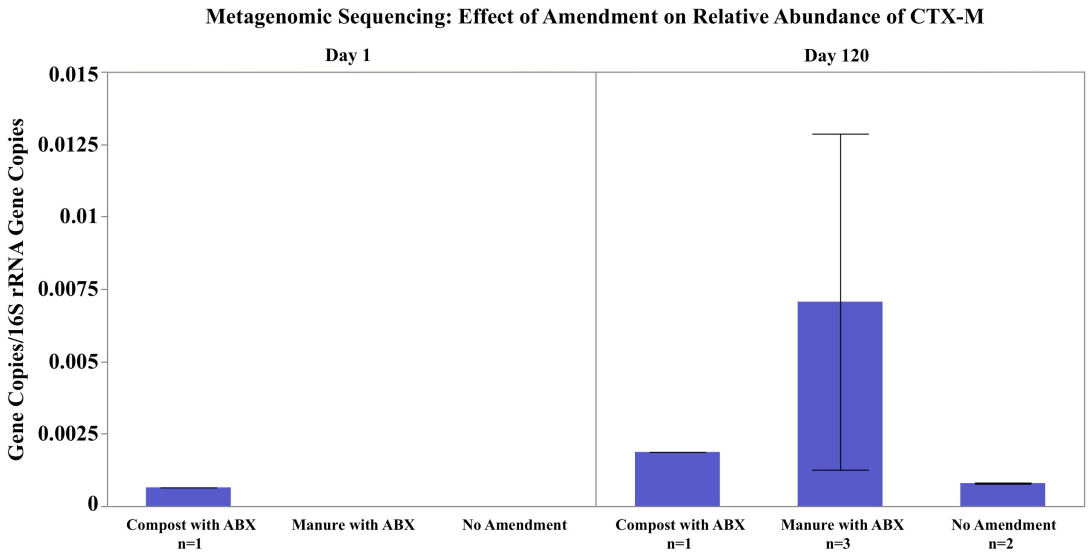
S2 Table. Characteristics of three soil types from USDA Web Soil Survey (Soil Survey Staff: Natural Resources Conservation Service. Web Soil Survey: United States Department of Agriculture; Accessed [03/02/2017]).

Soil Type	Coordinate	Classification	Clay (%)	Sandy (%)	Slit (%)	Organic matter content (%)	CEC (milliequivalents per 100 grams)
Sandy loam	37°12'40.6"N 80°26'37.1"W	Eunola loamy fine sand	7	83.5	9.5	1.25	4.6
Silt loam	36°39'45.5"N 76°44'02.3"W	Guernsey silt loam	20	26.5	53.5	2.00	9.0
Silty clay loam	37°12'54.0"N 80°26'32.2"W	Carbo and Chilhowie soils	35	16.9	48.1	1.75	16.0

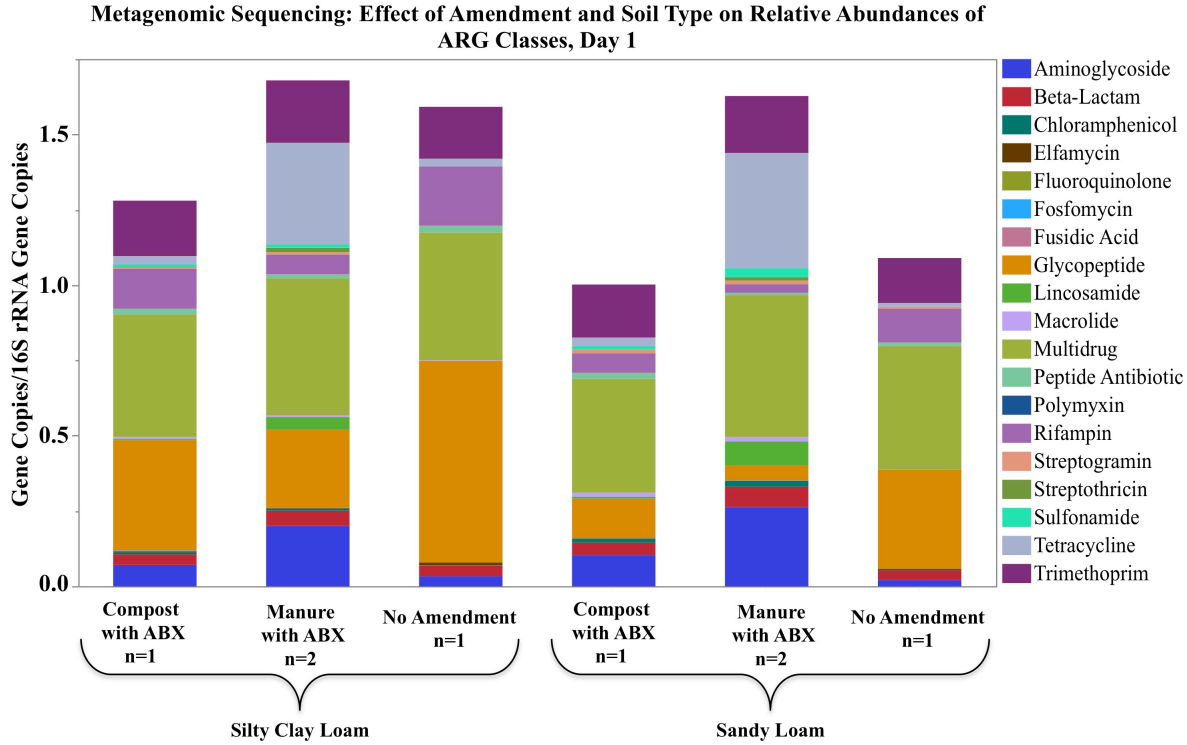
S3 Table. Concentrations of specific antibiotics within the R2A media. Since R2A media (heterotrophic bacteria) is not a selective or differential media, minimum inhibitory concentrations were derived empirically. Plates were incubated at 37°C for 24 hours.

Clindamycin (μg/mL)	Erythromycin (μg/mL)	Ceftazidime (μg/mL)	Cefotaxime (μg/mL)	Tetracycline (μg/mL)	Vancomycin (μg/mL)
25	25	10	10	3	11

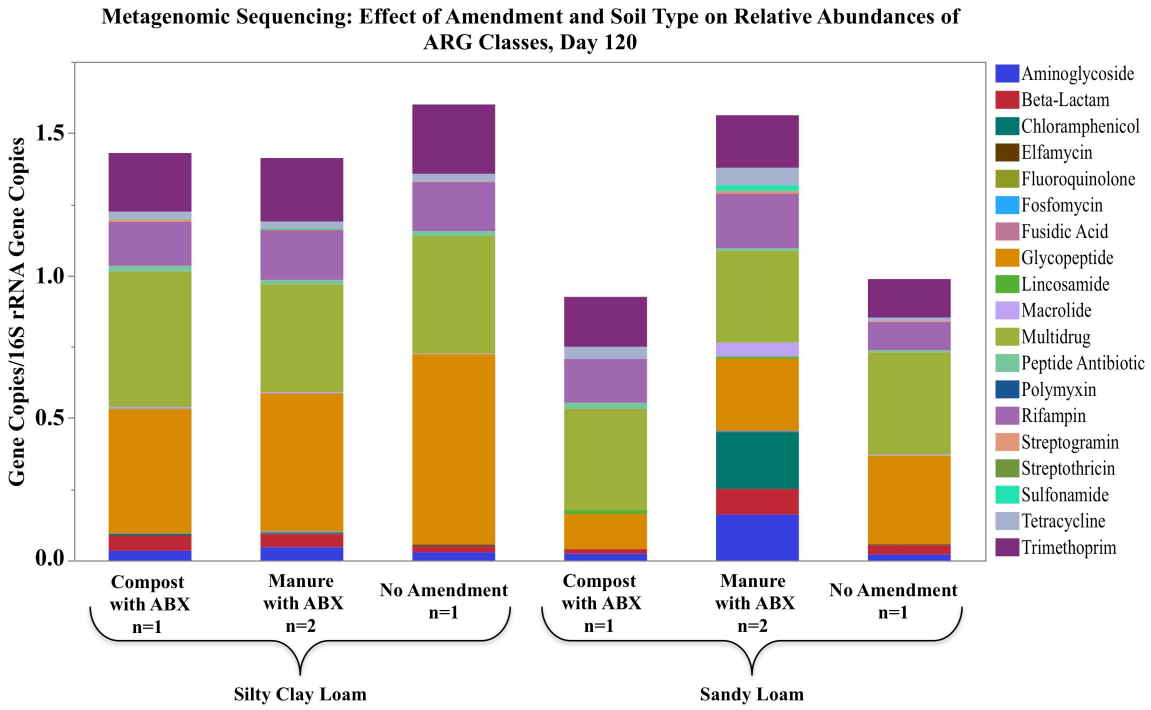
Chapter 3—Beef Supplementary Figures



S1 Fig. Bar graph, with standard error bars, showing average relative abundance of CTX-M genes in samples that were positive for the gene. The sample number (n) represents the number of samples within the treatment that had CTX-M. There was no significant difference between treatments ($p>0.05$; Kruskal-Wallis), but the number of samples with CTX-M increased from day 1 to 120.



S2 Fig. Comparison of the relative abundance of total ARGs among soils applied with raw manure or composted manure of beef cattle fed antibiotics and no amendment on day 1. Qualitative comparison due to low replicates.



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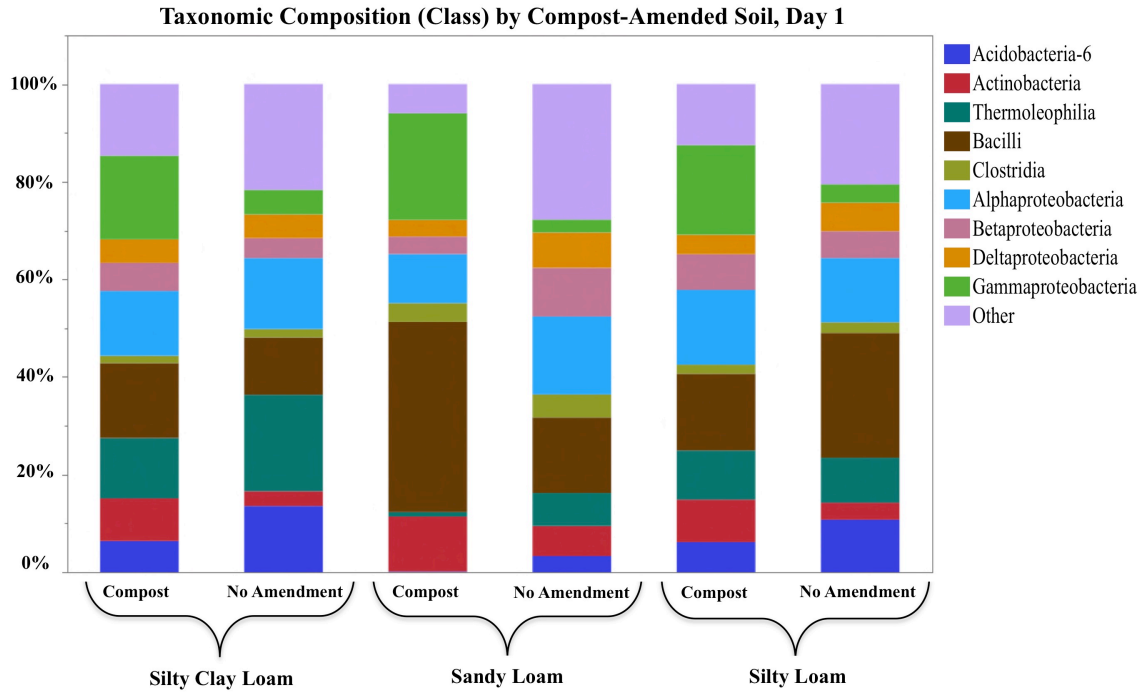
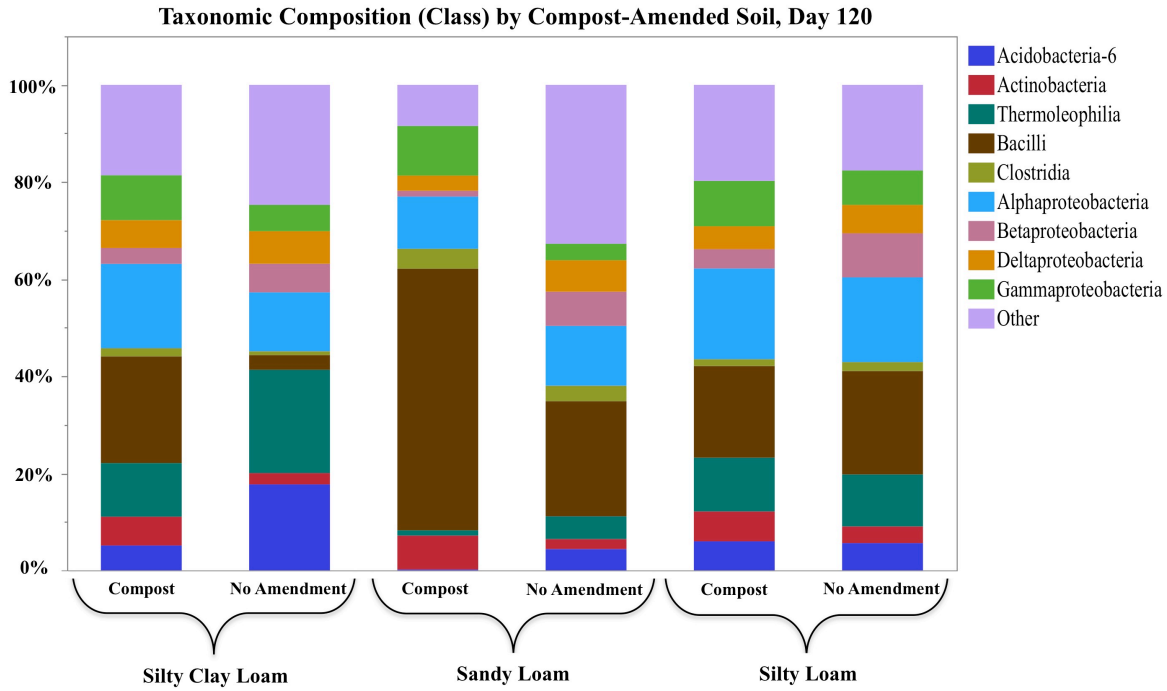
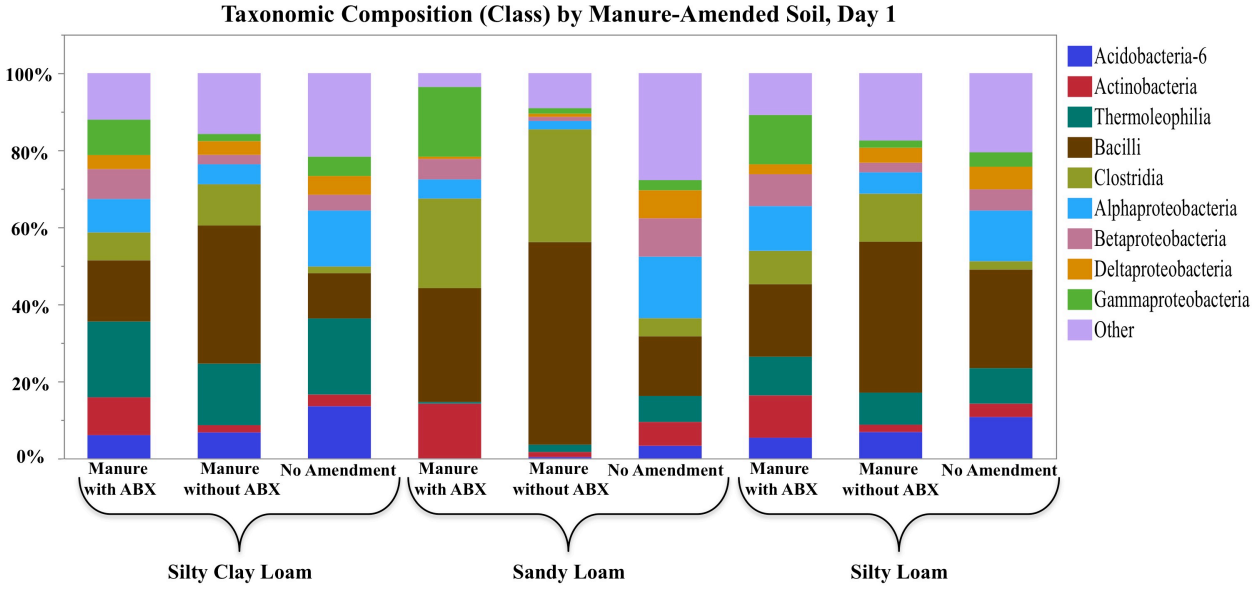


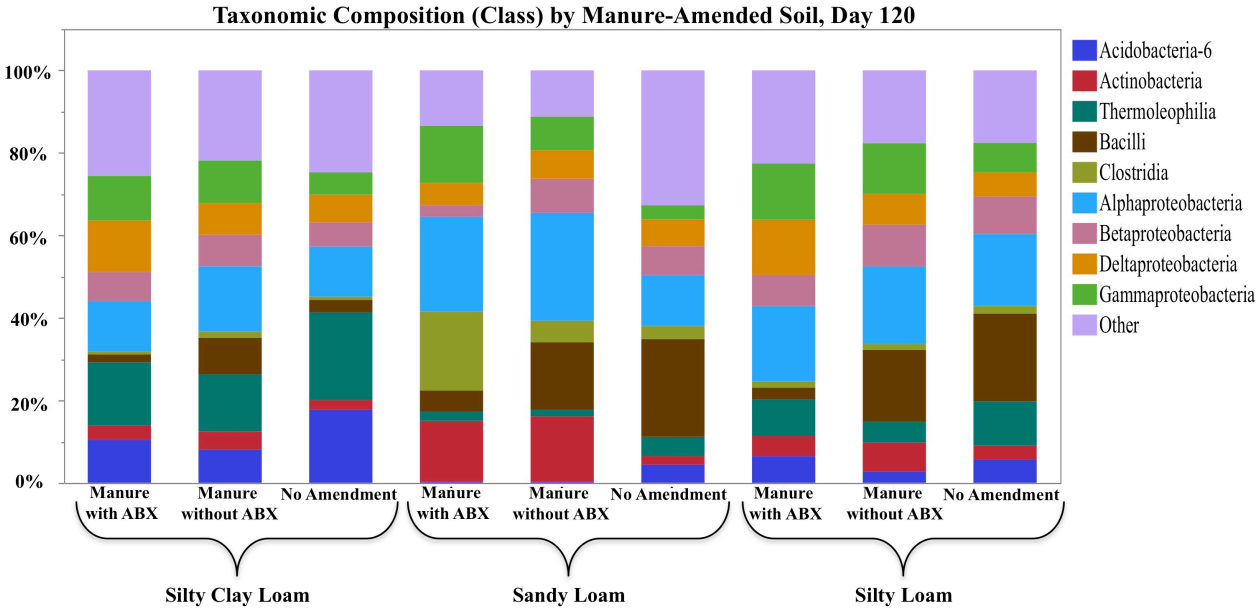
S4 Fig. Comparison of the three soil types' class-level taxonomic compositions when applied with compost on day 1. Sandy loam applied with either compost treatment was significantly different from the amended silty clay loam and silty loam (p=0.002; ANOSIM). Compost applied silty clay loam and silty loam were not significantly different from each other.



S5 Fig. Comparison of the three soil types' class-level taxonomic compositions when applied with compost on day 120. Sandy loam applied with either compost treatment was significantly different from the amended silty clay loam and silty loam (p=0.002; ANOSIM). Compost applied silty clay loam and silty loam were not significantly different from each other.



S6 Fig. Comparison of the three soil types' class-level taxonomic compositions when applied with either raw manure treatments on day 1. Qualitative comparison due to low replicates



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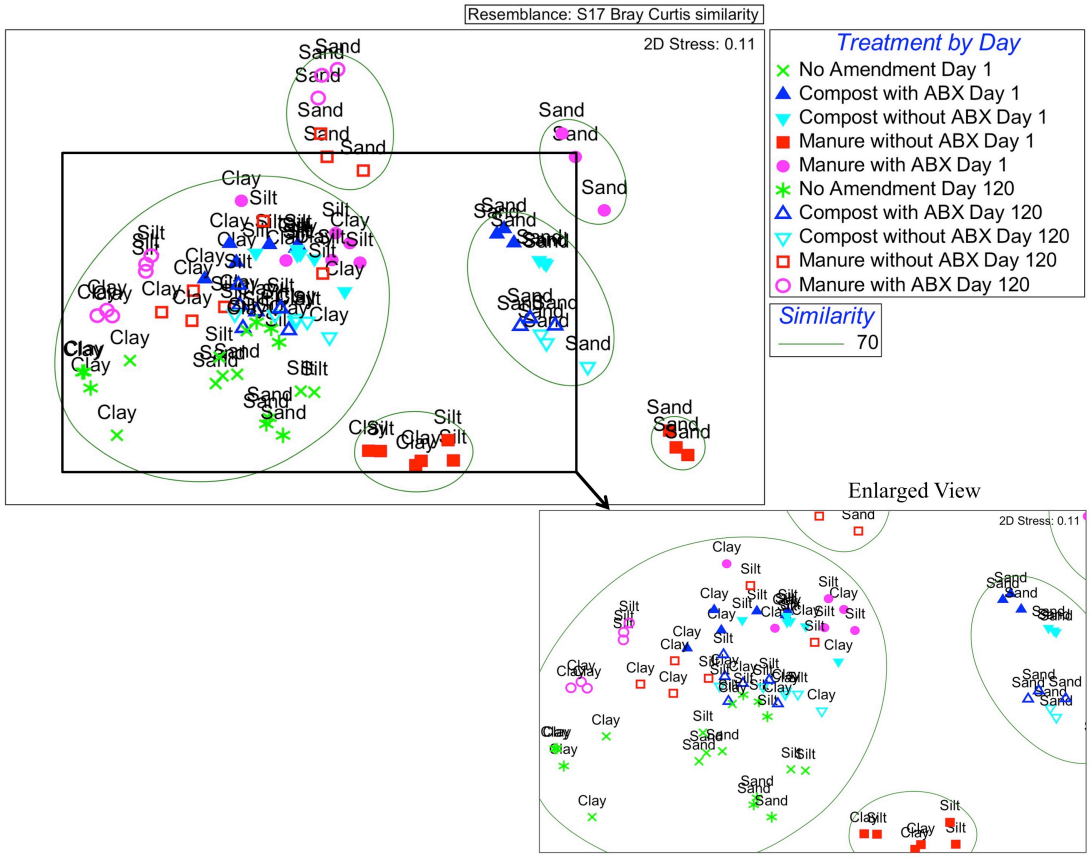
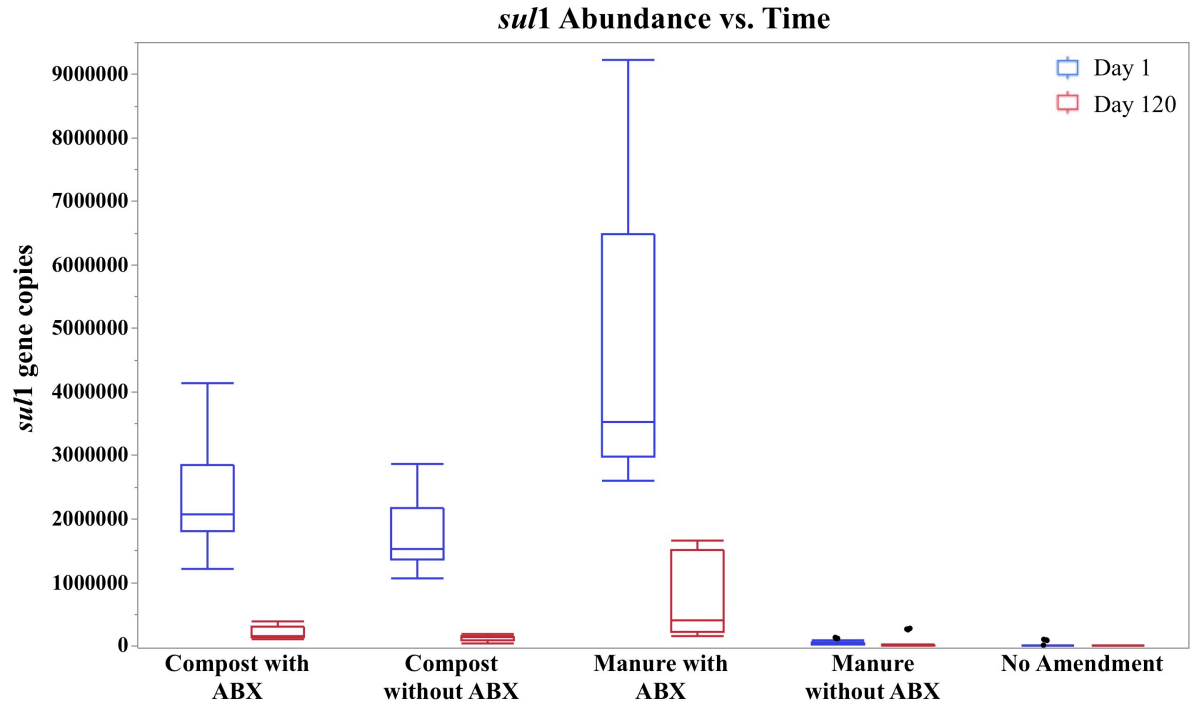
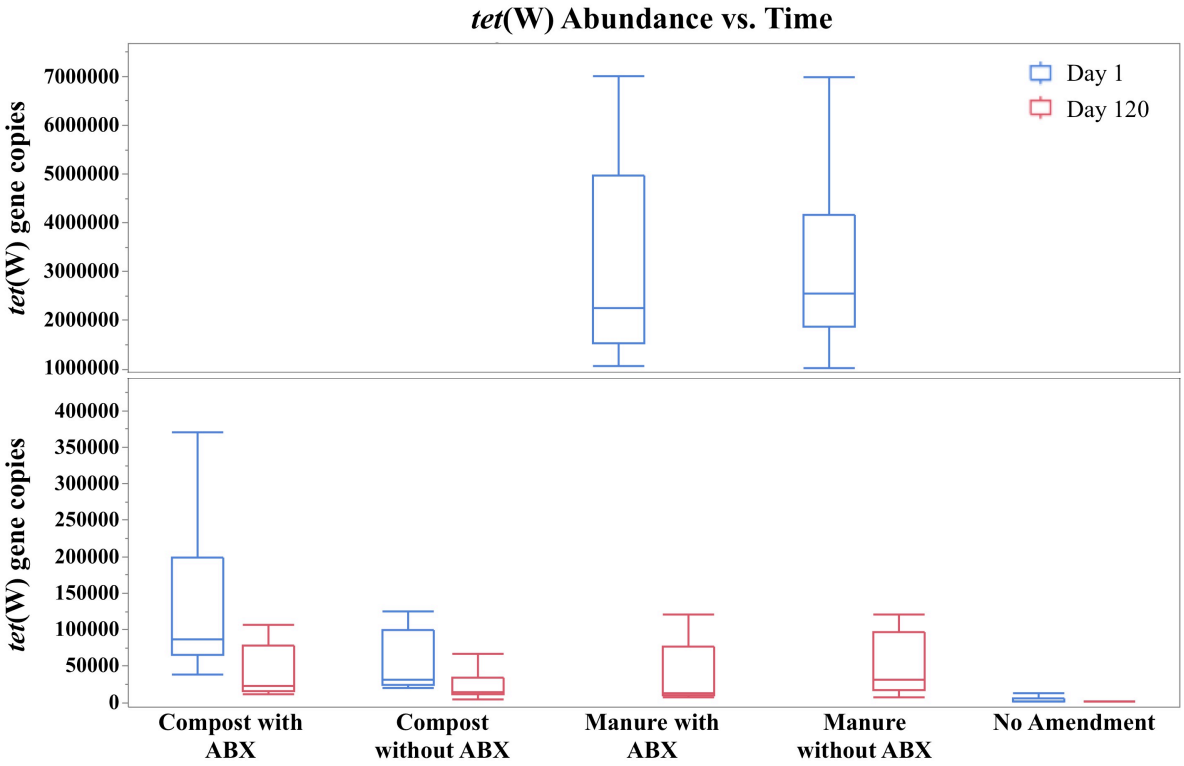


S8 Fig. Enlarged view of cluster in 16S rRNA amplicon sequencing MDS plot.



S9 Fig. Box plots of the *sul1* absolute abundances.



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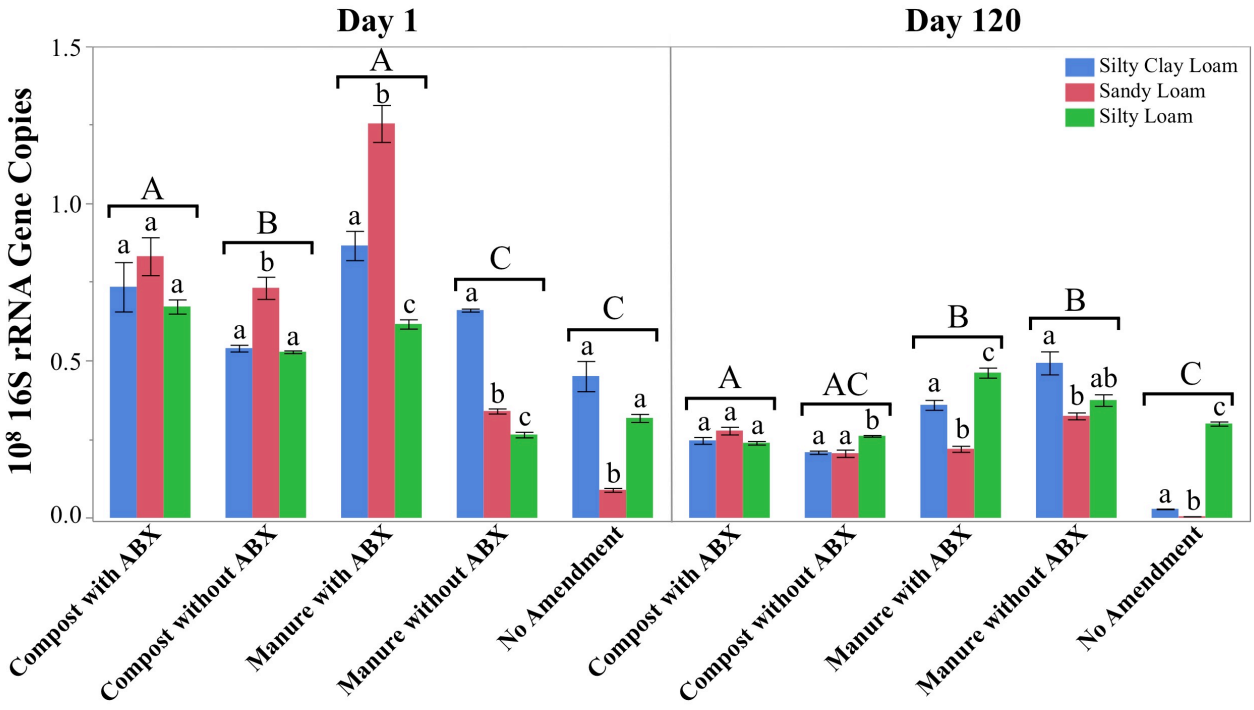
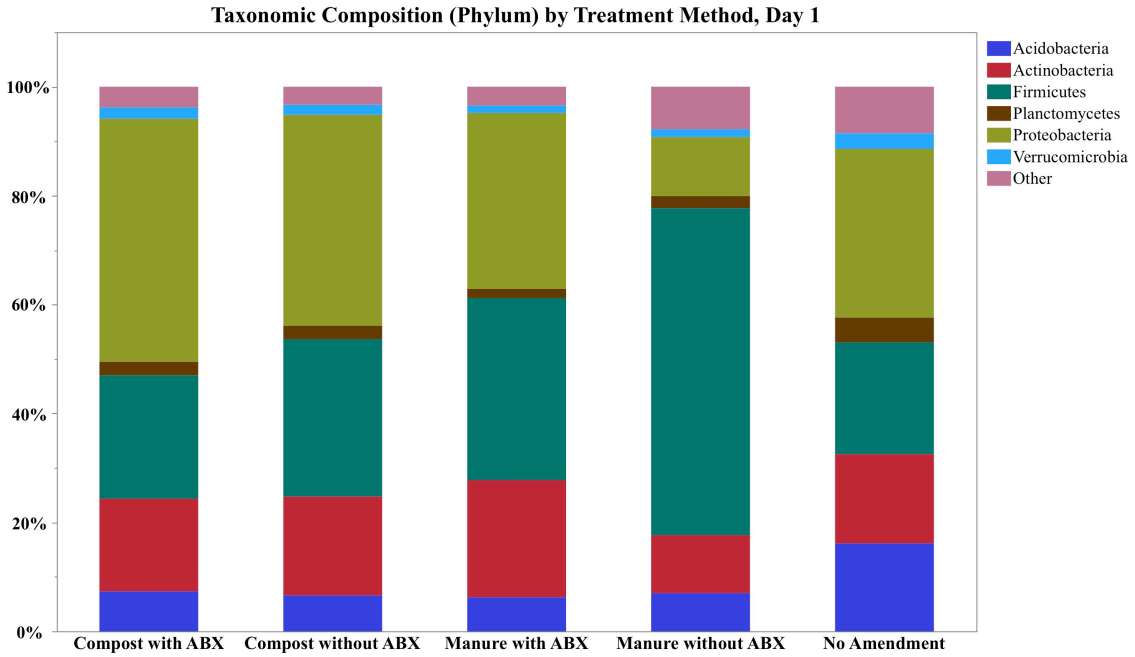
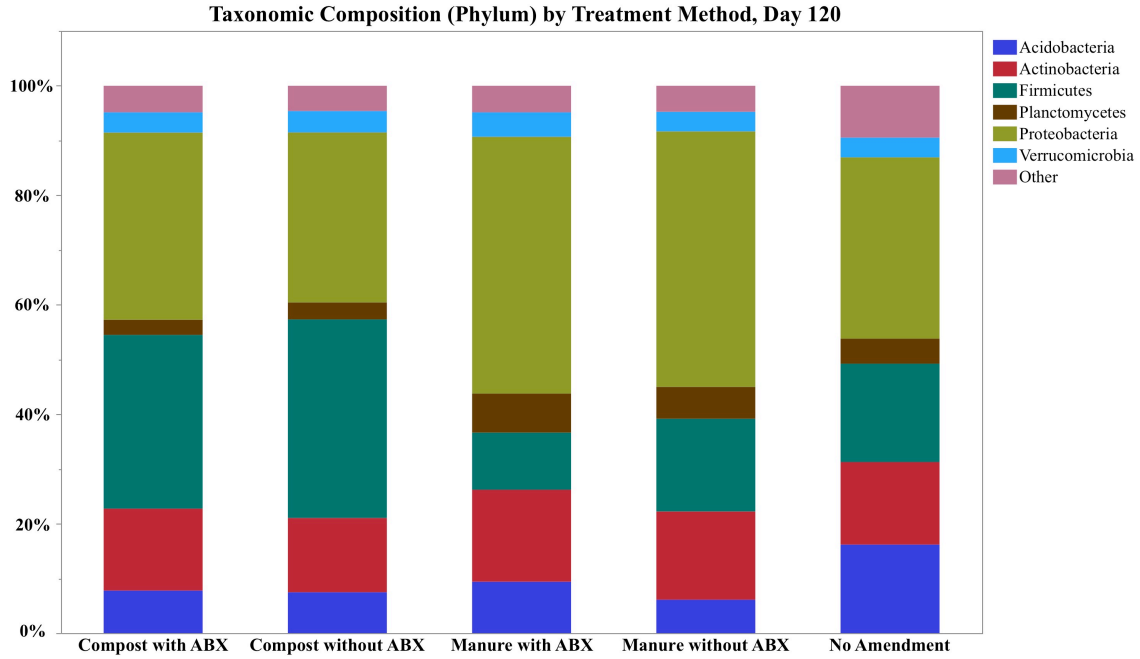


S11 Fig. Absolute abundances of 16S rRNA gene. Per timepoint, the capital letters indicate significance between soils applied with the different amendment conditions, and the lowercase letters indicate significance among soil types within each specific treatment ($p<0.05$; Steel-Dwass All Pairs).



S12 Fig. Compositional comparison of the top six most abundant phyla and remaining "other" phyla of the five different soil treatments on day 1.



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Chapter 4—Supplementary Information and Figures

Bacterial Culturing Methods

Media preparation

R2A (heterotrophs) and MacConkey media (enteric bacteria) dosed with different antibiotics were used. The R2A media contained the following antibiotics and concentrations: Clindamycin (25µg/mL), Erythromycin (25µg/mL), Ceftazidime (10µg/mL), Cefotaxime (10µg/mL), Tetracycline (3µg/mL), and Vancomycin (11µg/mL). MacConkey media contained the following antibiotics and concentrations: Clindamycin (35µg/mL), Erythromycin (40µg/mL), Ceftazidime (0.5µg/mL), Cefotaxime (0.25µg/mL), and Tetracycline (8µg/mL). For MacConkey media, since it is a selective and differential media for enteric bacteria (coliforms), the antibiotic concentrations (cefotaxime, ceftazidime, and tetracycline) were found using the minimum inhibitory concentration (MIC) listed by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for *E. coli*. The remaining antibiotic concentrations for MacConkey and all of R2A were based off of experimental tests and do not indicate true resistance.

After media was prepared, autoclaved, and cooled to 50°C, the antibiotics were added to the media from stock solutions. Before addition, the stock solutions were thawed at 4°C and filter sterilized through a syringe and 0.22µm nylon filter (CELLTREAT Scientific Products, Pepperell, MA).

Antibiotic Stock Solutions

Stock solutions of the antibiotics were made and stored at -20°C for no longer than 3 months. Before freezing, the stock solutions were aliquotted to screw cap test tubes to maintain

just one freeze thaw cycle and were wrapped in aluminum foil in order to reduce exposure to UV light.

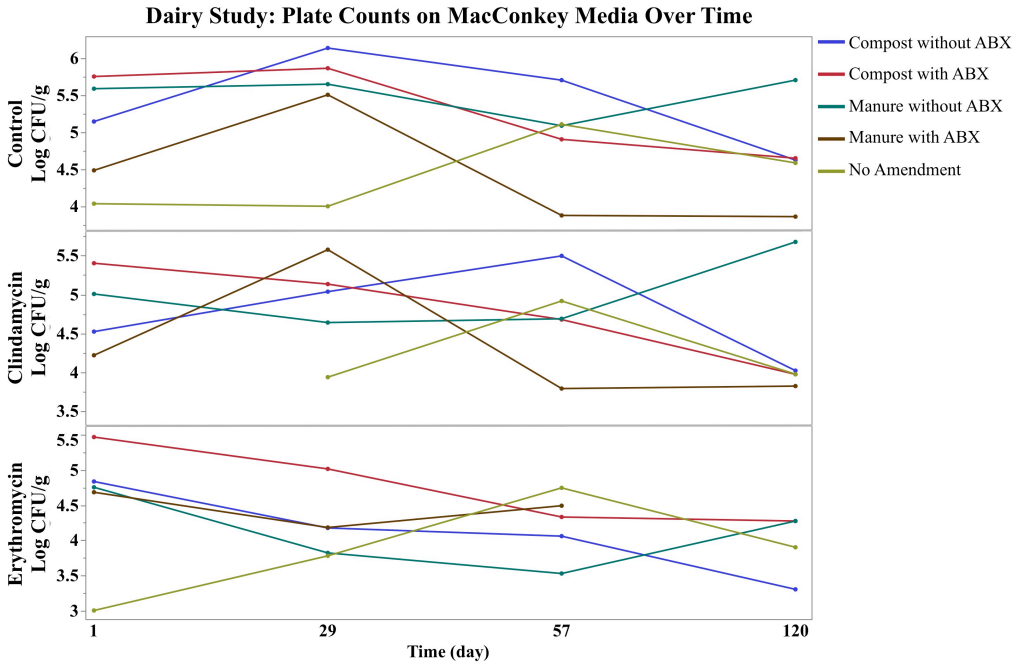
Sample Processing and Spread Plating

The 1-gram of homogenized sample from the corresponding microcosm was placed into a Stomacher® bag (VWR®, Radnor, PA). A 1:100 dilution was done by adding 99 mL of 0.1% peptone (Sigma-Aldrich, St. Louis, MO) to the Stomacher® bags and placed in a BagMixer® 400 W (interscience Laboratories Inc., Woburn, MA). The BagMixer® was run for 2 minutes at speed level 6. One milliliter of the stomached sample was pipetted out and serially diluted in test tubes containing 0.1% peptone. One hundred microliters of the corresponding dilution was pipetted onto the media, after 5-second vortex, and spread plated. The plates were inverted, placed in an opaque container and incubated at 37°C for 24 hours. After 24 hours the plates were removed for colony counting. Only pink or red colonies were counted on MacConkey plates as this indicated lactose fermentation, which differentiates the enteric bacteria.

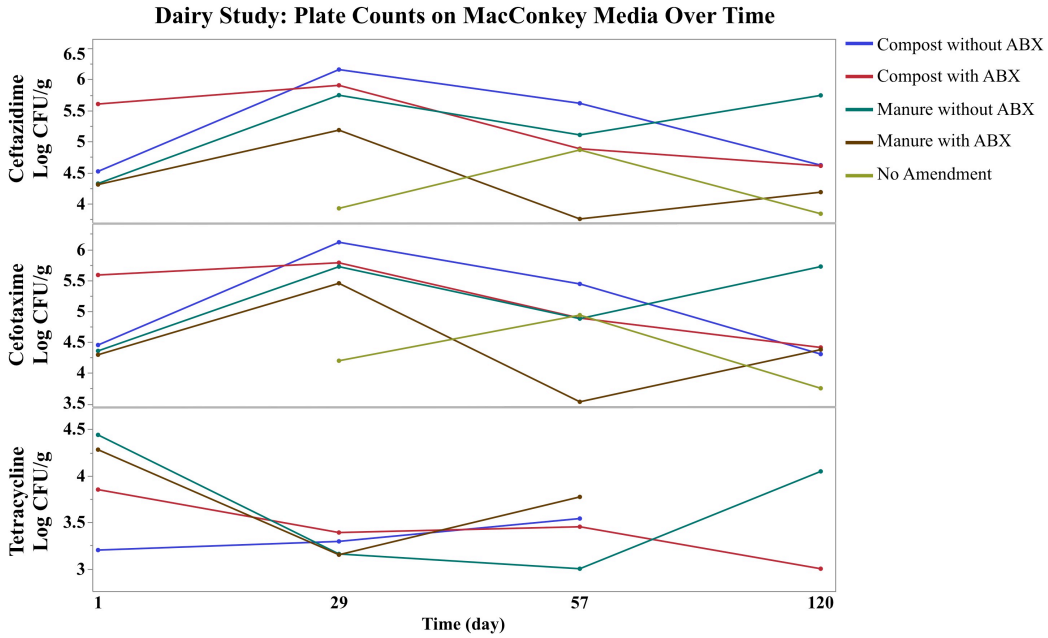
Statistical Analysis

For the R2A media, plates with colony counts between 25-300 were considered statistically valid. In order to include the plates that had too few colonies to count (TFTC) or too numerous to count (TNTC), interval censoring analysis was performed using the survival package (version 2.38-1) in R (version 2.1). The MacConkey plates, as a selective and differential media, did not have any plates that were TNTC. However, there were many plates that were below the detection limit (0 colonies). Thus, left censoring analysis was done in R with the same survival package. Data was log transformed and a Weibull distribution was used. The

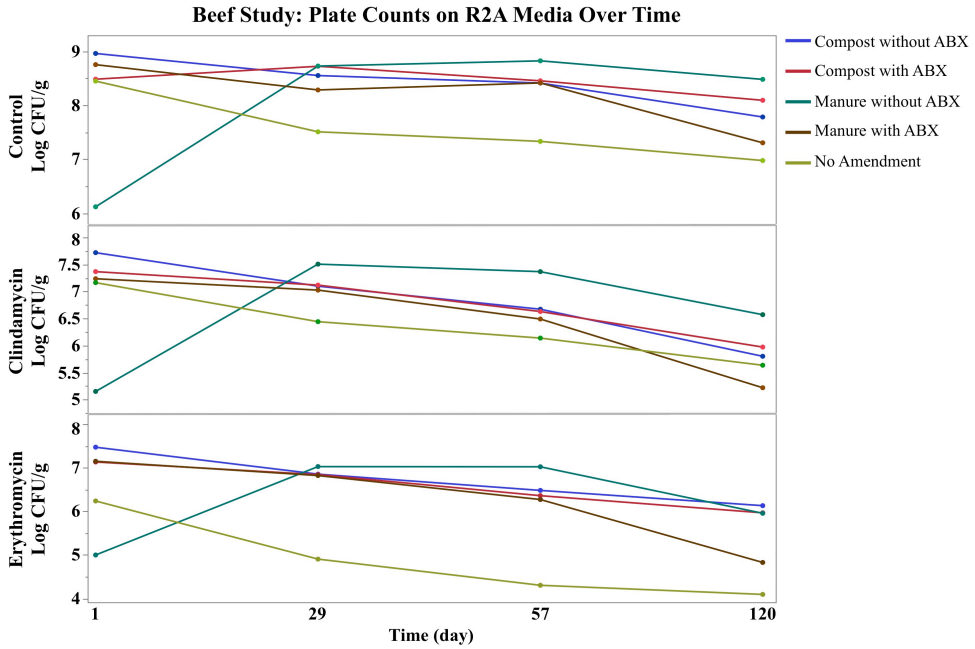
censored model was then run through ANOVA to evaluate the significance of the following factors: amendment type, soil type, and time.



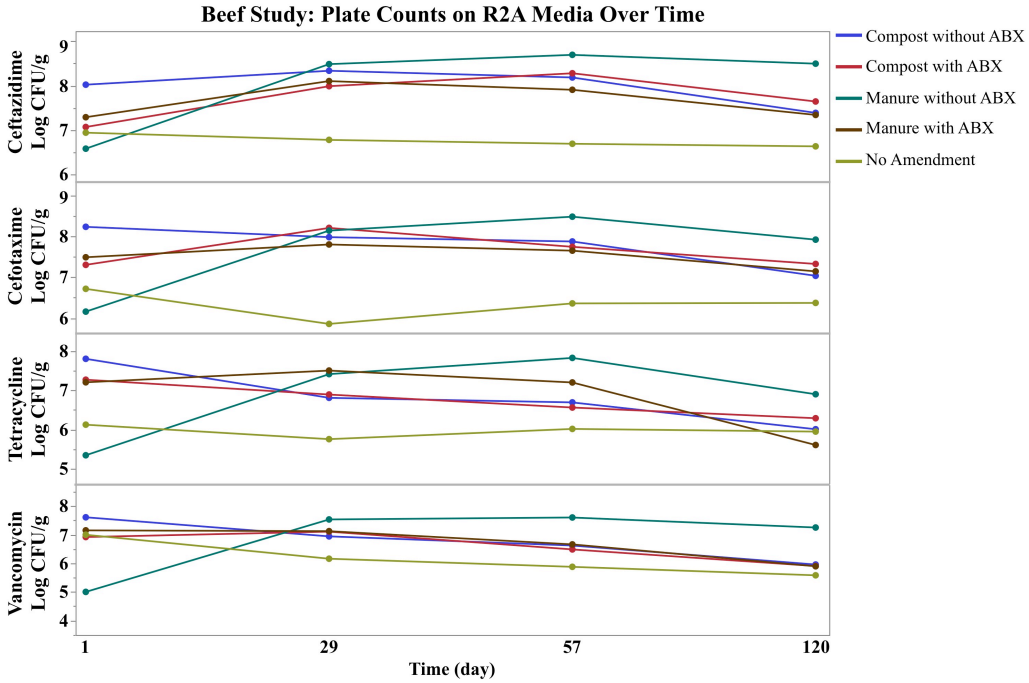
S1 Fig. Dairy study MacConkey media culturing results of the five different amended soil conditions on media infused with no antibiotics ("Control"), clindamycin (35µg/mL), or erythromycin (40µg/mL) over four time points.



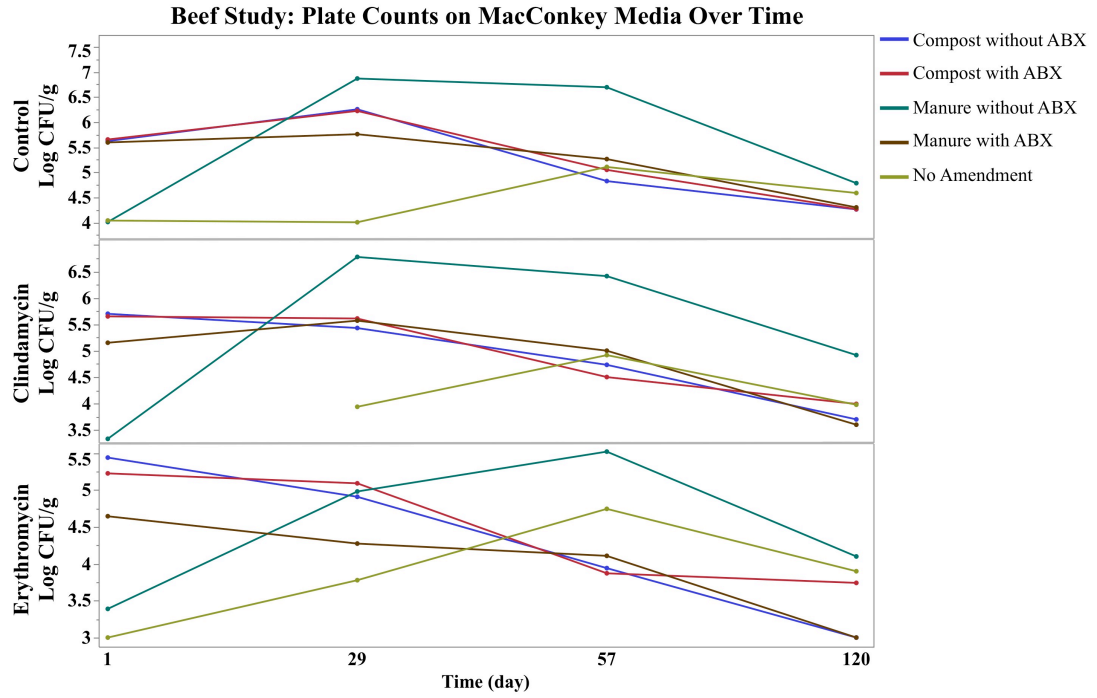
S2 Fig. Dairy study MacConkey media culturing results of the five different amended soil conditions on media infused with ceftazidime (0.5µg/mL), cefotaxime (0.25µg/mL), and tetracyline (8.0µg/mL) over four time points.



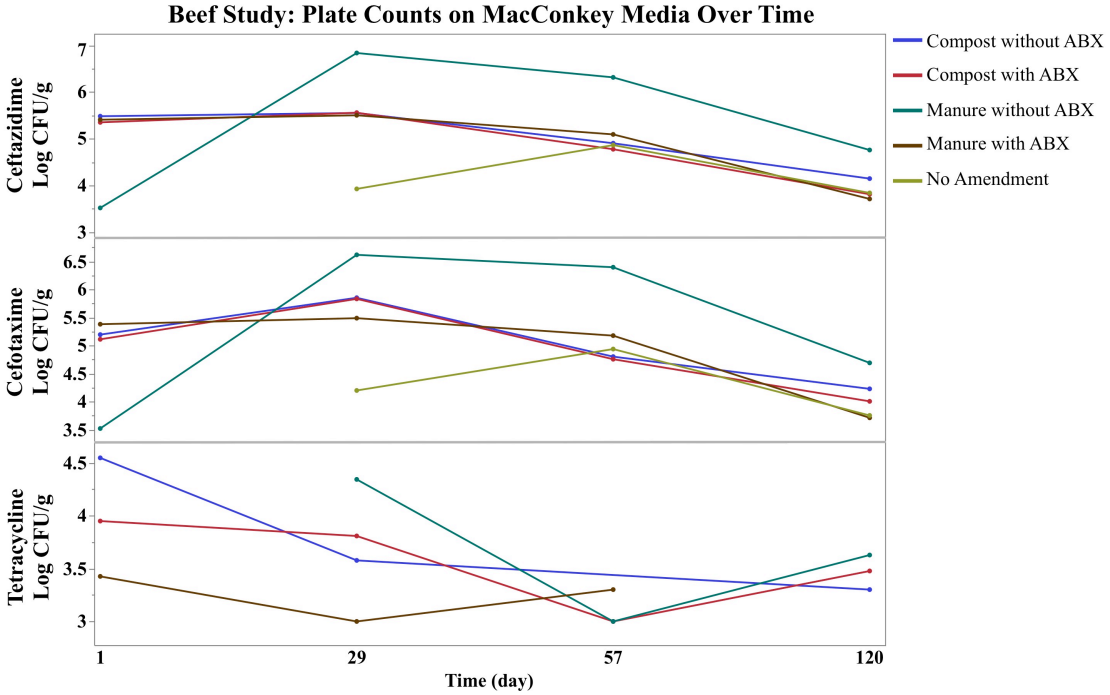
S3 Fig. Beef study R2A media culturing results of the five different amended soil conditions on media infused with no antibiotics ("Control"), clindamycin (25µg/mL), or erythromycin (25µg/mL) over four time points.



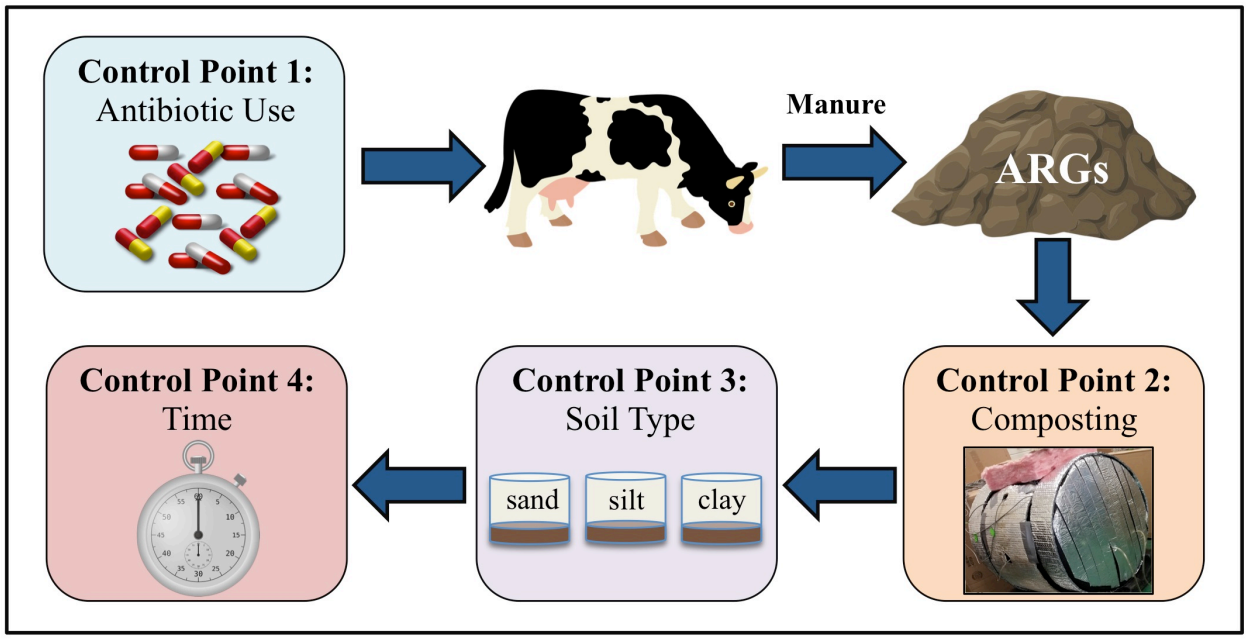
S4 Fig. Beef study R2A media culturing results of the five different amended soil conditions on media infused with ceftazidime (10µg/mL), cefotaxime (10µg/mL), tetracycline (3.0µg/mL), and vancomycin (11.0µg/mL)over four time points.



S5 Fig. Beef study MacConkey media culturing results of the five different amended soil conditions on media infused with no antibiotics ("Control"), clindamycin (35µg/mL), or erythromycin (40µg/mL) over four time points.



S6 Fig. Beef study MacConkey media culturing results of the five different amended soil conditions on media infused with ceftazidime (0.5µg/mL), cefotaxime (0.25µg/mL), and tetracyline (8.0µg/mL) over four time points.



S7 Fig. Graphical abstract.

S1 Table. Statistical comparison of dairy study's plate counts using the Survival package in R. The bolded antioibiotics were significant at the factor with $p \leq 0.01$ for the plate counts on that specific antibiotic infused media. Antibiotics that are not bolded were signifcant for the factor at $0.01 < p \leq 0.05$.

Factors	R2A Media	MacConkey Media
Soil Type * Treatment Type	**Control**, **Clindamycin**, **Erythromycin**, **Ceftazidime**, **Cefotaxime**, **Tetracycline**, **Vancomycin**	**Control**, **Clindamycin**, **Erythromycin**, **Ceftazidime**, **Cefotaxime**, **Tetracycline**
Time * Treatment Type	**Control**, **Clindamycin**, **Erythromycin**, **Ceftazidime**, **Cefotaxime**, **Tetracycline**, **Vancomycin**	**Clindamycin**, **Erythromycin**, **Ceftazidime, Cefotaxime**
Treatment Type	**Control**, **Clindamycin**, **Erythromycin**, **Ceftazidime**, **Cefotaxime**, **Tetracycline**, **Vancomycin**	**Control**, **Clindamycin**, **Erythromycin**, **Ceftazidime**, **Cefotaxime**, **Tetracycline**
Soil	--	**Erythromycin**, Tetracycline
Time	**Control**, **Clindamycin**, **Erythromycin**, **Ceftazidime**, Cefotaxime, **Tetracycline**, **Vancomycin**	Erythromycin, **Tetracycline**

S2 Table. Statistical comparison of beef study's plate counts using the Survival package in R. The bolded antioibiotics were significant at the factor with $p \leq 0.01$ for the plate counts on that specific antibiotic infused media. Antibiotics that are not bolded were signifcant for the factor at $0.01 < p \leq 0.05$

Factors	R2A Media	MacConkey Media
Soil Type * Treatment Type	**Control**, **Clindamycin**, **Erythromycin**, **Ceftazidime**, **Cefotaxime**, **Tetracycline**,	**Control**, **Clindamycin**, **Erythromycin**, **Ceftazidime**, **Cefotaxime**
Time * Treatment Type	**Control**, **Clindamycin**, **Erythromycin**, **Ceftazidime**, **Cefotaxime**, **Tetracycline**, **Vancomycin**	**Control**, **Clindamycin**, **Erythromycin**, **Ceftazidime,** **Cefotaxime**, **Tetracycline**
Treatment Type	**Control**, **Clindamycin**, **Erythromycin**, **Ceftazidime**, **Cefotaxime**, **Tetracycline**, **Vancomycin**	**Control**, **Clindamycin**, **Erythromycin**, **Ceftazidime**, **Cefotaxime**, **Tetracycline**
Soil	**Tetracycline**	Erythromycin
Time	**Control**, **Clindamycin**, **Erythromycin**, **Ceftazidime**, Cefotaxime, Tetracycline, **Vancomycin**	**Control**, Clindamycin, **Erythromycin**, Ceftazidime, **Cefotaxime**, Tetracycline