EFFECT OF COMPOSTING ON THE PREVALENCE OF ANTIBIOTIC RESISTANT BACTERIA AND RESISTANCE GENES IN CATTLE MANURE

By

Robert K. Williams

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Amy Pruden, Chair

Leigh-Anne Krometis

Gregory D. Boardman

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ABSTRACT

Antibiotic resistance is a growing human health threat, making infections more difficult to treat and increasing fatalities from and cost of treatment of associated diseases. The rise of multidrug resistant pathogens threatens a return to the pre-antibiotic era where even the most common infections may be impossible to treat. It is estimated that the majority of global antibiotic use, and use in the U.S., is dedicated towards livestock, where they are used to promote growth, treat, or prevent disease. Given that exposure to antibiotics selects for antibiotic resistant bacteria (ARBs) and can stimulate the horizontal transfer of their associated antibiotic resistance genes (ARGs), it is important to examine livestock operations as a reservoir of resistance. Correspondingly, there is growing interest in identifying how agricultural practices can limit the potential for spread of antibiotic resistance through the "farm to fork continuum," starting with antibiotic use practices, manure management and land application and ending with the spread of ARBs and ARGs present onto edible crops and serving as a route of exposure to consumers. This study focused specifically on the effect of composting on the prevalence of ARBs and ARGs in cattle manure. Three composting trials were performed: small-scale, heat-controlled, and large-scale. The small-scale composting trial compared dairy and beef manures, with or without antibiotic treatment (treated beef cattle received chlortetracycline, sulfamethazine, and tylosin while treated dairy cattle received cephapirin and pirlimycin), subject to either static or turned composting. The heat-controlled composting trial examined only dairy manure, with or without antibiotic treatment, subject to static composting, but using external heat tape applied to the composting tumblers to extend the duration of the thermophilic (>55°C) temperature range. The large-scale composting trial examined dairy manure, with or without antibiotic treatment, subject to static composting at a much larger scale that is more realistic to typical farm practices. Samples were analyzed to assess phenotypic resistance using the Kirby Bauer disk diffusion method and by diluting and plating onto antibiotic-supplemented agar. Genetic markers of resistance were also assessed using quantitative polymerase chain reaction (qPCR) to quantify *sul*1 and *tet*(W) ARGs;

metagenomic DNA sequencing and analysis were also performed to assess and compare total ARG abundance and types across all samples. Results indicate that composting can enrich indicators of phenotypic and genetic resistance traits to certain antibiotics, but that most ARGs are successfully attenuated during composting, as evidenced by the metagenomic sequencing. Maintaining thermophilic composting temperatures for adequate time is necessary for the effective elimination of enteric bacteria. This study suggests that indicator bacteria that survive composting tend to be more resistant than those in the original raw manure; however, extending the thermophilic stage of composting, as was done in the heat-controlled trial, can reduce target indicator bacteria below detection limits. Of the two ARGs specifically quantified via qPCR, prior administration of antibiotics to cattle only had a significant impact on *tet*(W). There was not an obvious difference in the final antibiotic resistance profiles in the finished beef versus dairy manure composts according to metagenomics analysis. Based on these results, composting is promising as a method of attenuating ARGs, but further research is necessary to examine in depth all of the complex interactions that occur during the composting process to maximize performance. If not applied appropriately, e.g., if time and temperature guidelines are not enforced, then there is potential that composting could exacerbate the spread of certain types of antibiotic resistance.

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GENERAL AUDIENCE ABSTRACT

Antibiotics are drugs that are used to treat bacterial infections by killing the bacteria that cause the infection. Bacterial infections now exist that are resistant to several antibiotics; which are extremely difficult and costly to treat. Many antibiotics are used in the agriculture industry where they are used to promote growth, treat, or prevent disease in livestock animals. The antibiotics may then cause an increase in antibiotic resistance in bacteria by encouraging changes to the DNA of the bacteria which allow them to survive in the presence of antibiotics that would normally kill them. These DNA segments are called antibiotic resistance genes. Once developed, bacteria can share resistance genes among themselves, allowing for single bacteria that can resist several types of antibiotics. For this reason, it is important to see if it is possible to prevent the spread of antibiotic resistance from animal agriculture to people. One way that people could be affected would be if produce were exposed to resistant bacteria when grown in soil that had been fertilized with manure or compost. This study looks at the impact of composting on the presence and amount of antibiotic resistance genes in composted cattle manure. Three composting trials were performed: small-scale, heat-controlled, and large-scale. The small-scale composting trial compared dairy and beef manures, with or without antibiotic treatment, with or without regular turning during composting. The heat-controlled composting trial examined only dairy manure, with or without antibiotic treatment, without regular turning during composting, but using external heat to maintain high temperatures. The large-scale composting trial examined dairy manure, with or without antibiotic treatment, without regular turning during composting, but at a larger scale that is more realistic to how composting is actually performed on farms. Antibiotic resistance of compost bacteria was tested by growing bacteria on nutrient-dense plates containing antibiotic disks and measuring how much each antibiotic prevented the growth of the bacteria, in terms of the diameter about each disk where bacteria did not grow. Individual target resistance genes were measured throughout the study by using a method called qPCR. Metagenomic analysis was performed to identify all of the genes, especially resistance genes, in each of the samples. Results

show that composting may increase antibiotic resistance in bacteria that survive the composting process, but that most resistance genes are themselves reduced. The key to successful composting is maintaining high temperatures for as long as possible; this is necessary to kill off infectious bacteria. Extending the high temperature (>55°C) phase of composting is a potential method for improving the effectiveness of composting in eliminating pathogens and destroying resistance genes. Results were not significantly affected by whether antibiotics were given to the cattle and were not different between dairy or beef cattle. Based on these results, composting is a promising method of reducing resistance genes in composted manure, but further research is necessary to maximize performance. If not performed correctly, composting could have the opposite effect and be detrimental.

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ANOSIM: Analysis of Similarities

ARBs: Antibiotic Resistant Bacteria

ARGs: Antibiotic Resistance Genes

CARD: Comprehensive Antibiotic Resistance Database

CFU: Colony Forming Unit

DNA: Deoxyribonucleic Acid

EUCAST: European Committee on Antimicrobial Susceptibility Testing

FSMA: Food Safety Modernization Act

HGT: Horizontal Gene Transfer

HPCs: Heterotrophic Plate Counts

LB: Luria Bertani (Broth)

MDS: Multidimensional Scaling

MG-RAST: Metagenome Project Rapid Annotation using Subsystems Technology

MIC: Minimum Inhibitory Concentration

MLS-B: Macrolide-Lincosamide-Streptogramin B

MPN: Most Probable Number

NCCLS: National Committee for Clinical Laboratory Standards

OTU: Operational Taxonomic Unit

PCR: Polymerase Chain Reaction

PVC: Polyvinyl Chloride

QIIME: Quantitative Insights into Microbial Ecology

qPCR: Quantitative Polymerase Chain Reaction

WHO: World Health Organization

1. ANTIBIOTIC RESISTANCE IN AGRICULTURAL COMPOST AND MANURE: BACKGROUND AND LITERATURE REVIEW

Antibiotic resistance presents a growing and serious human health risk by making treatment of serious infections more difficult, thus increasing the odds of complications and fatalities as a result of infections that do not respond to antibiotics (1). Even common infections, like *Staphylococcus aureus* and *Escherichia coli*, are becoming resistant to the most powerful antibiotics (2). Microorganisms can develop a number of mechanisms for counteracting the effects of antibiotics including the use of efflux pumps, synthesis of enzymes to break down the antibiotic, chemical alteration of the antibiotic, or through mutations to the antibiotic binding site that render it ineffective (3,4). All of these mechanisms, whether innate or acquired, are coded for in the DNA by genetic elements, or antibiotic resistance genes (ARGs). Microorganisms can spread ARGs to their daughter cells through typical cell division and reproduction (i.e. vertical gene transfer, or VGT), but of particular concern is the ability to share ARGs between bacteria through horizontal gene transfer (HGT) (5). Even ARGs lysed by dead bacteria may still pose a threat because they can be assimilated by living bacteria through the HGT process of transformation. For this reason ARGs themselves can be considered the primary contaminant of concern, rather than resistant bacteria (6).

Antibiotics are heavily relied upon in livestock agriculture. They are used at therapeutic levels to treat disease in animals, as well as at sub-therapeutic concentrations to prevent disease or to promote weight gain (7). It has been estimated that as much as 80% of the antibiotics sold in the United States, and more than 50% of antibiotics produced in the world, are administered to livestock (8,9). However, most of the administered antibiotic is later excreted by the animal in urine or feces (10). This residual antibiotic can persist in the environment and select for antibiotic resistance (11). When land-applied, livestock manure can lead to the spread of antibiotic resistance and ARGS directly into the soil, into the waterways via rainfall runoff, or onto vegetable crops (12,13). Livestock operations can therefore serve as reservoirs of antibiotic resistance (14). This has drawn attention to the need to understand the potential for antibiotic resistance to spread along the "farm to fork continuum," or from antibiotic use and manure management practices through crop production, post-harvest practice and consumer exposure.

Composting is the controlled process of aerobically biodegrading organic matter into a

humus-rich material that is ideal for use as a soil amendment. As composting already presents other benefits, including manure management and pathogen control, the potential for composting to also attenuate ARGs is of interest (15). During composting, bacteria and fungi grow rapidly, degrading the organic matter and producing heat, naturally elevating the temperature into the thermophilic range (55°C). While the thermophilic phase is known to be essential for killing off pathogens, little is known about the potential for composting to also reduce the concentration and bioavailability of antibiotics and the overall abundance of ARBs and ARGs (15,16). With regard to ARBs, one study looked at the effect of composting antibiotic-administered swine manure on the growth of erythromycin-resistant and tetracycline-resistant colonies on antibiotic supplemented tryptic soy agar and found that composting reduces ARB plate counts by 4-7 logs (17).

Prior studies of the effects of composting on ARG abundance have yielded mixed results. Selvam et al. (18) examined the effect of composting swine manure spiked with chlortetracycline, sulfadiazine, and ciprofloxacin on ARGs associated with tetracycline, sulfonamide, and fluoroquinolone resistance and found that after 28 to 42 days of composting all twelve genes, except for *par*C, were undetectable. Conversely, Su et al. studied the effect of composting municipal sewage sludge on antibiotics resistance using high-throughput quantitative polymerase chain reaction (qPCR array) of 156 ARGs and found an increase in ARG abundance and diversity following 50 days of composting (19). The present study seeks to explore the effect of manure composting on ARB and ARG abundance through the use of culture-based techniques, qPCR, and metagenomic sequencing to provide a comprehensive understanding of the effects of prior antibiotic use and composting on various measures of antibiotic resistance. In particular, metagenomics is a relatively new technology, derived from next-generation DNA sequencing, and can help to resolve prior discrepancies in the literature regarding the effects of composting on ARGs.

1.1. ANTIBIOTIC RESISTANCE

Antibiotics are powerful and life-saving drugs and their discovery has led to longer human life spans and quality of life by providing effective treatments for previously debilitating and fatal infectious diseases (20). However, resistance to medically important antibiotics was discovered

quickly thereafter when penicillin was found to be ineffective against previously susceptible bacteria (21). Since then, rates of antibiotic resistant infections have continued to increase while fewer new antibiotics are being discovered each year (22). Antibiotic resistance presents a growing and serious human health risk by making treatment of serious infections more difficult, thus increasing the odds of complications and fatality as a result of infections that do not respond to antibiotics (1). The rates of antibiotic resistance observed are increasing for both hospital- and community-acquired infections, (1). Even the most common infections, like *Staphylococcus aureus* and *Escherichia coli*, are becoming resistant to the most powerful antibiotics (2).

Many antibiotics are naturally occurring, produced by bacteria and fungi at relatively low concentrations as a means of attaining a competitive advantage in the environment (3). Antibiotic resistance is also a natural adaptation to allow bacteria to survive in the presence of these antibiotics, but the mass production and use of antimicrobial drugs exposes bacteria to levels of antibiotics orders of magnitude higher than what is experienced in nature. This imposes a strong selective pressure in which bacteria that adapt, mutate, or acquire ARGs have a competitive advantage and will increase in abundance (3,5).

In the United States, the overuse and misuse of antibiotics has resulted in increasing levels of antibiotic resistance in recent years (23), which has correspondingly led to increased cost for healthcare as infections become more difficult and costly to treat (24). Some infectious bacteria are developing multidrug resistance, which is of special concern because it even further limits the treatment options. Some Gram-negative rod-shaped bacteria have been found that are resistant to virtually all known antibiotics. This distressing observation could mean a future where antibiotics are no longer helpful and many infections cannot be treated (25).

1.1.1. ANTIBIOTIC RESISTANCE GENES

Microorganisms can develop a number of mechanisms to circumvent the effects of antibiotics. Some microorganisms possess an innate resistance to certain antibiotics, meaning that they are naturally immune to their effects either because they lack the target that the antibiotic works upon, or because they have an impermeable membrane that the antibiotic cannot penetrate (26). Other microorganisms can acquire resistance through the use of several mechanisms

including the use of efflux pumps, synthesis of enzymes to break down the antibiotic, chemical alteration of the antibiotic, or mutation of the antibiotic binding site, rendering the antibiotic ineffective (3,4). All of these mechanisms, whether innate or acquired, are coded for by genetic elements, or antibiotic resistance genes (ARGs). ARGs coding for innate resistance are located on the bacterial chromosome (4) while ARGs coding for acquired resistances may be located on either the bacterial chromosome or on mobile genetic elements, such as plasmids (3).

Plasmids are extra-chromosomal, circular elements of double-stranded DNA that often house ARGs; microorganisms can have multiple plasmids (4,26). Other mobile genetic elements that can house ARGs include transposons and integrons (26). Transposons are DNA segments that can change their position within a genome moving from chromosomes to plasmids or vice-versa (4). Integrons contain mobile gene cassettes that are often the site of ARGs.

Microorganisms can spread ARGs to their offspring through normal cell division processes, or can be shared between bacteria through horizontal gene transfer (HGT) (5). HGT is mediated through one of three processes: conjugation, transformation, or transduction. Conjugation is considered the most prevalent form of HGT, wherein bacterial DNA is shared between two bacteria through physical contact; DNA from either the chromosome or from transposons or plasmids can be shared this way (26). Transformation is the process wherein extracellular DNA from dead or lysed bacteria is incorporated into the genome of living bacteria (27). The ability for bacteria to uptake DNA through transformation means that even ARGs contained within dead bacteria still pose a threat and that ARGs themselves are the primary contaminant of concern, rather than resistant bacteria (6). Transduction is the process whereby DNA is exchanged from one bacterium to another by the actions of bacteriophages (14). HGT can be induced by selective pressure such as the presence of antibiotics. HGT allows ARGs to be spread between different species of bacteria in ways that may not have occurred through mutation and is therefore considered the most important mechanism in the spread of antibiotic resistance (22,28,29). This also means that even resistant but non-pathogenic bacteria can pose a health threat by potentially sharing their ARGs with pathogens (30).

Some ARGs may be located together on the same genetic element (31). The selection for a genetic element containing a certain ARG may therefore result in resistance to another, unrelated ARG as well; this can rapidly result in multidrug resistance (32).

1.2. ANTIBIOTIC RESISTANCE IN AGRICULTURE

Modern livestock agriculture is heavily reliant on antibiotics. They are used at therapeutic levels to treat disease in animals, as well as at sub-therapeutic concentrations to prevent disease or to promote weight gain (7). It has been estimated that as much as 80% of the antibiotics sold in the United States, and more than 50% of antibiotics produced in the world, are administered to livestock (8,9). However, most of the administered antibiotics, as well as metabolites, are later excreted by the animal in urine or feces (10). It has been shown that administration of antibiotic supplemented feed can significantly increase the prevalence of ARGs in the feces of feedlot cattle (33). One study demonstrated an increase in resistance to seven different antimicrobials in *E. coli* isolated from conventional farms where antibiotics were regularly used as compared to those isolated from organic farms without antibiotic use (34). This residual antibiotic can persist in the environment and select for antibiotic resistance (11). There have been several cases of antibiotic resistant pathogen transfer from animals to humans including the transfer of methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococci* infections (35,36).

When land applied, livestock manure can elevate ARGS in the soil where they can be transferred to crops or to adjacent waterways via runoff (12,13). It has been shown that land and waterways near farmland contain a higher prevalence of ARBs and ARGs than non-farm areas (13,37). Livestock operations can therefore serve as reservoirs of antibiotic resistance (14). Livestock is considered one of the most significant human actions contributing to the rapid spread of antibiotic resistance (23).

1.2.1. THE FARM TO FORK CONTINUUM

The farm to fork continuum is an important pathway to consider, through which there is the highly plausible likelihood that human exposure to livestock manure-derived ARGs could occur. Fecal contamination of produce is often the cause of outbreaks of foodborne illness (38). The sources for such outbreaks are typically contamination of irrigation water or soil amended with improperly composted or raw animal manures too near to harvest (39). It has been shown that enterohemorrhagic *E. coli* can survive for up to 159 days in untreated manures and that they can also survive and regrow in compost if not properly performed (40,41). If fecal-derived pathogens

can be transmitted to humans through fresh produce, then it stands to reason that humans could also be exposed to ARGs through the same route. Many animal antibiotics are similar classes to those used in human medicine, such as lincosamides and β-lactams that are used in the dairy industry (42). One study showed that *E. coli* isolated from cases of clinical mastitis displayed multidrug resistance and were resistant to both veterinary and human antibiotics (43). Within the farm to fork continuum, the area of manure management represents a critical control point with the potential for decrease the prevalence of ARGs and ARBs before they can spread to the consumer.

1.2.2. MANURE TREATMENT STRATEGIES

Manure management refers to the collection, storage, treatment, and subsequent use of animal manures with the goals of recycling nutrients, preventing the spread of pathogens, and protecting the environment. Manure treatment aims to reduce nutrient levels in collected animal manure in order to minimize nutrient runoff into waterways and reduce pathogen loads. Common options for treating livestock manures include lagoons, anaerobic digestion, and various composting methods (15,44).

Lagoons are a method for treating liquid or slurry waste by passing it through basins of slow moving water, allowing suspended solids to settle, and promoting the biological degradation of organic matter prior to application to cropland (44). One study has shown that simulated lagoon treatment resulted in a 1 to 2 log decrease in cultivated aerobic heterotrophic erythromycin-resistant bacteria and tetracycline-resistant bacteria over a 48 day period; however this same study showed no significant reduction in the absolute abundance of any of the *tet* and *erm* ARGs that were quantified (44).

Anaerobic digestion is a process most commonly used to treat municipal biosolids, but it is becoming more common as a livestock manure treatment (15). It involves blending biosolids into a liquid form and incubating in the absence of oxygen at either mesophilic temperatures of 35 to 37°C or at thermophilic temperatures of approximately 55°C. This process produces methane-rich biogas, which may be sold or burned to produce energy. Solids are separated from the remaining digestate and are used to produce fertilizer for agriculture (15). Adequate time is

required at a given temperature to eliminate pathogens, though less time is needed for higher temperatures. Past research has demonstrated reductions in ARBs of up to 90% following mesophilic digestion, and reductions below detection at higher temperatures (45). The majority of past research on the fate of ARGs during anaerobic digestion has used municipal biosolids and not livestock manure. Results of such treatments on ARGs have yielded mixed results, with *sul*, *tet*, and *erm* genes showing modest reduction in most studies, and an increase in others (15). One study on mesophilic swine manure digestion showed no significant change in the abundances of tested *erm* and *tet* genes (46).

Composting is the controlled process of aerobically biodegrading organic matter into a humus-rich material that is ideal for use as a soil amendment (15). It is the primary focus of the present study and is reviewed in depth in the following section.

1.2.3. MANURE COMPOSTING

Manure composting is an aerobic process that involves amending manure with materials such as mulch, sawdust, and hay, or other readily available organic materials, with the primary goal of adjusting the C:N ratio and increasing porosity to allow oxygen to penetrate the composting heap (16). Optimal composting will aim to achieve a carbon to nitrogen ratio (C:N) in the preferred range of 25:1 to 30:1 and moisture content in the range of 50-60% (16). During composting, bacteria and fungi will grow rapidly, degrading the organic matter and producing heat. The composting process takes weeks to months to complete; during this time the biologically generated heat will naturally raise the temperature into the thermophilic range (55°C). These high temperatures serve as the primary mechanism for pathogen removal, as well as the potential inactivation of ARBs, ARGs, and degradation of antibiotics (15,16). Once microbial growth and decomposition begins to slow, temperatures decline into the mesophilic stage, where they may remain for one to several weeks while they undergo the final curing process. During curing, recalcitrant compounds continue to degrade, the pH shifts toward neutral, the C:N continues to decrease, and the concentration of humus increases. Curing is considered complete when, while maintaining adequate moisture and oxygen, the compost returns to near ambient temperatures (16).

There are three primary types of thermophilic composting, which include: aerated static piles, turned piles or windrows, and in-vessel systems (15). Windrows are long, narrow piles of compost which are agitated or turned on a regular basis and are generally 3 feet high for manure-based composts (16). Currently in the Unites States, composting of animal manure is not regulated by any federal agency, though some state and local regulations do exist (47). The Food and Drug Administration (FDA) has proposed a rule for Biological Soil Amendments under Subpart F of its Food Safety Modernization Act (FSMA) (47). In this proposed rule, manure composting must either: 1) use a composting method that can be proven scientifically valid by demonstrating that the finished compost meets certain microbial pathogen standards, i.e. detection of less than 3 MPN per 4 grams of dry weight solids *Salmonella* species and less than 1,000 MPN fecal coliforms per gram dry weight solids (47); or 2) follow one of two composting methods outlined in the rule that are considered scientifically valid and are designed to meet the aforementioned microbial standards. These methods include either static composting that maintains aerobic conditions at a minimum of 55°C for three days, followed by adequate curing, or turned composting that maintains aerobic conditions at a minimum of 55°C for 15 days, with a minimum of 5 turnings, followed by adequate curing (47).

Studies examining the effect of composting on the degradation of antibiotics have consistently shown a reduction, sometimes below detection levels, of sulfonamide, tetracycline, macrolide, and quinolone antibiotics (48–50).

A limited number studies have demonstrated ARB reductions following composting (15). Wang et al. found that composting reduced levels of cultivated aerobic heterotrophic tetracycline-resistant and erythromycin-resistant bacteria isolated on selective tryptic soy agar in swine manure from pigs administered tetracyclines and tylosin by 4-7 logs over 48 days (17). Sharma et al. explored the effect of composting on resistant *E. coli* levels in cattle manure compost from cattle administered either tylosin, chlortetracycline and sulfamethazine, or no antibiotics, and found that composting reduced levels of ampicillin-resistant and tetracycline-resistant *E. coli* (51). Guan et al. found that composting at temperatures of 50°C or above prevented the transfer of plasmids containing multiple antibiotic resistances and reduced their *E. coli* hosts to non-detect levels (52).

Evidence of the effects of composting on ARG abundance are mixed. Some studies show that composting may have a beneficial effect on ARG attenuation. Holman et al. studied the effects

of co-composting cattle manure with construction waste on antimicrobial resistance and found that composting reduced the abundance of 10 ARGs including *erm, sul, and tet* genes (53). Selvam et al. (18) examined the effect of composting swine manure spiked with chlortetracycline, sulfadiazine, and ciprofloxacin on ARGs associated with tetracycline, sulfonamide, and fluoroquinolone resistance and found that after 28 to 42 days of composting all twelve genes, except for *par*C, were undetectable. Yu et al. and Chen et al. found a reduction in tetracycline resistance genes and macrolide-lincosamide-streptogramin B resistance genes, respectively, after composting of swine manure (46,54). Storteboom et al. (55) studied the effect of high and low intensity manure management techniques on the abundance of ARGs in horse manure spiked with chlortetracycline, tylosin, and monensin and found a decrease in *tet*(W) and *tet*(O) resistance genes after 6 months of treatment.

Yet other studies show that composting increases ARG abundance. Qian et al. explored the effect of oxytetracycline concentration on the composting process and its ability to attenuate ARGs and found that the relative abundances of the ARGs *tet*(C)*, tet*(X)*, sul*1*, sul*2*,* and *intI*1 increased while the relative abundances of *tet*(Q)*, tet*(M)*,* and *tet*(W) decreased (56). Sharma et al. found an increase in the genes *tet*(A)*, tet*(C), and *erm*(A), but a decrease in the remaining seven genes that were tested; however, the inability to maintain thermophilic temperatures a full fifteen days may have limited the ability of composting to degrade all ARGs in this case (51). Su et al. studied the effect of municipal sewage sludge composting on antibiotics resistance using high-throughput quantitative polymerase chain reaction (qPCR) of 156 unique ARGs and found an increase in ARG abundance and diversity following 50 days of composting (19).

While these studies do shed some light on the potential effectiveness of composting in the attenuation of ARBs and ARGs, the mixed results for ARB attenuation suggest a need for further research. Specifically, the composting process must be optimized to ensure thermophilic temperatures are maintained long enough to degrade ARGs. Several of these studies involved spiking antibiotics into manure while others administered the antibiotics to the livestock; administration to livestock mirrors real world situations more closely and is the preferred approach where possible. The aforementioned studies on ARGs in compost primarily quantified ARGs using targeted qPCR of specific genes, with the exception of Su et al.; the use of metagenomic sequencing and analysis to get a better overall picture of the antibiotic resistome throughout the

composting process would provide enhanced understanding of the complex microbial interactions that take place during composting (19). It is also helpful to assess resistance using both culture-based and genetic techniques simultaneously: culture-based techniques demonstrate phenotypic expression of resistance, while genetic techniques provide an insight into the majority of the microbial community that is unculturable. Together, these will give a more complete picture of the resistome.

1.3. SELECT CLASSES OF ANTIBIOTICS AND BACTERIAL MODES OF RESISTANCE

In general, antibiotics can be classified into one of four main categories based upon their method of action and their site of activity within the bacterial cell, i.e. antibiotics may: inhibit cell wall synthesis, inhibit protein synthesis, inhibit nucleic acid synthesis, or weaken the integrity of the cell membrane (57). Of the following antibiotics, the World Health Organization has named 3rd and 4th generation cephalosporins, glycopeptides, macrolides, and fluoroquinolones the highest priority critically important antimicrobials; this means that a large number of people are affected by diseases for which these antibiotics are the sole or one of few antibiotics available for treatment, that they have a high frequency of use, and that there is a greater degree of confidence that non-human sources, like the livestock industry, play a role in increasing the transfer of associated ARGs and ARBs (58).

1.3.1. β-LACTAMS

The β-lactam class of antibiotics includes penicillins, cephalosporins, monobactams, and carbapenems; β-lactam antibiotics are structurally similar in that they all contain a β-lactam ring (57). This is the most commonly used class of antibiotics with cephalosporins alone accounting for more than 28% of antibiotic sales (59). β-lactam antibiotics work by inhibiting the formation of peptidoglycan in the bacterial cell wall; they do this by binding to enzymes that produce peptidoglycan, called penicillin binding proteins (57). Resistance to β-lactams occurs through one of four primary mechanisms: through a mutation in penicillin binding proteins which prevents the antibiotic from being able to bind to it, through the production of β-lactamase enzymes that

degrade the antibiotic, through the use of efflux pumps, or by altering cell wall channels called porins to prevent the antibiotic from permeating deep enough into the cell wall to reach the target site (57).

Of these mechanisms, the production of β-lactamase enzymes is the most important factor contributing to β-lactam resistance, especially in gram negative organisms (60). Extended spectrum β-lactamases (ESBL) are of special concern because they have the ability to degrade and cause resistance to a wide array of β-lactam antibiotics including the relatively newer 3rd and 4th generation cephalosporins (60). Most ESBLs can be broken down into three distinct types: CTX-M, TEM, and SHV types. The most common producer of CTX-M type ESBLs is *E. coli* (60). Carbapenems are generally considered the last line of defense when treating serious gram-negative infections because they are unaffected by most β-lactamases; however, recently the New Delhi metallo-β-lactamase (NDM) gene has appeared which confers resistance to carbapenems (1). Of particular concern is the spread of a multidrug resistant NDM strain of *E. coli* across continents as it is resistant to virtually all antimicrobials (58). In the United States, the use of penicillins and all four generations of cephalosporins are approved for use in cattle, swine, and poultry (61). Due to their extensive use in the dairy industry to treat bacterial mastitis, the potential exists for β-lactam resistance to be enriched in animal manure (42).

1.3.2. GLYCOPEPTIDES

Glycopeptides are composed of glycosylated cyclic or polycyclic non-ribosomal peptides; they are generally too large to infiltrate the gram-negative outer membrane and therefore their action is limited to gram-positive microorganisms (57). Glycopeptides function by binding to the end of peptidoglycan penta-peptide side chains and preventing reactions necessary to form more peptidoglycan, thereby weakening the cell wall (57). Bacterial resistance depends upon the ability to produce a D-Ala-D-Lac dipeptide terminus, rather than the typical D-Ala-D-Ala terminus, thus depriving the glycopeptides of a suitable target site (57). The most well-known glycopeptides antibiotic is vancomycin; there are 6 known vancomycin resistance types, with *van*(A) being the most common. In humans, glycopeptides are among the only available therapies for serious enterococcal infections; they are also the first line treatment for methicillin resistant *Staphylococcus aureus* (1,35,58). It has been shown that transmission of glycopeptide-resistant

Enterococcal infections from animals to people can occur, such as the transmission of vancomycin resistant Enterococcal infections from chickens that had received avoparcin treatment (35).

1.3.3. FLUOROQUINOLONES

Fluoroquinolones, and other quinolones, are a class of synthetic, broad-spectrum antibiotics that share a similar structure to nalidixic acid, the first antibiotic of their class (62). Fluoroquinolones have a rapid bactericidal effect and function by binding to DNA topoisomerase enzymes and inhibiting their activity, thereby inhibiting nucleic acid metabolism (57,62). Resistance to fluoroquinolones can develop through the use of three mechanisms: developing a mutation in the target topoisomerase enzymes, decreasing bacterial cell wall permeability, and through the use of efflux pumps (57). Each of these mechanisms of resistance can occur simultaneously within the same cell, leading to high level resistance (57). In humans, use of fluoroquinolones is generally reserved for cases of hospital acquired infections or infections where resistance to older antibiotics is suspected (1). In the United States, quinolones are approved only for use in cattle to treat respiratory diseases, and off-label use is banned (61,63).

1.3.4. MACROLIDES AND LINCOSAMIDES

Macrolides are a class of antibiotics that are distinguished structurally by having a large macrocyclic lactone ring; lincosamides are structurally different from macrolides and instead have a sulfur containing octose ring (57,64). Macrolides and lincosamides share a similar mechanism of action: they bind to the 50S subunit on bacterial transfer RNA (tRNA) which causes the tRNA to dissociate from ribosomes and therefore inhibit protein synthesis (57). Bacterial mechanisms of resistance include mutations of the 23S RNA subunit or the use of efflux pumps (57). In human medicine macrolides and lincosamides are used to treat gram positive infections such as respiratory tract and soft tissue infections (1). In the United States, both macrolides and lincosamides are approved for use in cattle, swine, and poultry (61).

1.3.5. SULFONAMIDES

Sulfonamide antibiotics are synthetic antibiotics that contain a sulfonyl group connected to an amine group; they are structurally similar to p-aminobenzoic acid (PABA) (57). Due to this similarity, sulfonamides compete with PABA for the dihydropteroate synthase (DHPS) enzyme site, blocking the formation of nucleotide precursors (57). Bacteria can become resistant to sulfonamides by producing a new variant of the DHPS enzyme that is incompatible with sulfonamides (57). Two common resistant DHPS enzymes are encoded by the *sul*1 and sul2 genes, which have been found in gram negative bacteria and can exist on conjugative plasmids (5). Sulfonamide resistance is widespread, so they are rarely used in the treatment of human infections anymore (1,65). In livestock, they are still regularly used in the cattle, swine, and poultry industries (61).

1.3.6. TETRACYCLINES

Tetracyclines are a class of broad spectrum antibiotics that structurally consist of four hydrocarbon rings; they are bacteriostatic and block protein synthesis by blocking aminoacyl tRNA binding at the 30S RNA subunit (57). Tetracyclines are effective against a wide range of gram negative and gram positive bacteria as well as protozoa (57). Bacterial mechanisms of resistance to tetracyclines include efflux pumps or the production of ribosomal protection proteins that prevent tetracycline from binding at the target site (5,57). In human medicine, tetracyclines are not considered a first line of treatment, but are used when resistance to other antimicrobials is indicated; they are often administered in combination with other drugs because tetracyclines do not distribute evenly throughout the body (1). In livestock, tetracyclines have been regularly used both therapeutically and sub-therapeutically in feed to enhance growth of animals (57,66). In the United States they are approved for use in the cattle, swine, and poultry industries (61).

1.4. METHODS FOR THE DETECTION AND QUANTIFICATION OF ANTIBIOTIC RESISTANCE

Given the diversity of antibiotics in use, as well as the inability to culture the majority of bacteria in a lab setting, there is no standard method for the assessment of overall resistance potential; therefore, the following summary reviews both culture-based techniques, which target the phenotypic display of resistance by living and culturable bacteria, as well as genetic markers of resistance potential like the presence of ARGs.

1.4.1. CULTURE BASED TECHNIQUES

Culture-based techniques for assessing antibiotic resistance depend on the phenotypic expression of resistance and involve exposing live bacterial isolates to known concentrations of antibiotics and determining the level of growth inhibition that results (67,68). From this information a minimum inhibitory concentration (MIC) can be determined; the MIC is the lowest concentration of a specific antibiotic required to visibly inhibit bacterial growth (69). The National Committee for Clinical Laboratory Standards (NCCLS) has established standards for the preparation of media that must be followed in order to obtain an accurate MIC (67). There are two primary culture techniques for assessing resistance: disk diffusion methods, and dilution methods (70). Disk diffusion methods, such as the Kirby Bauer disk diffusion method, allow a single bacterial isolate to be tested for resistance to many antibiotics at once (70). These methods utilize paper disks containing a known concentration of antibiotic; the antibiotic diffuses into the agar creating a concentration gradient (69). When a bacterial isolate is grown onto an agar plate containing antibiotic disks, zones of inhibition will appear around each paper disk; the diameter of each zone of inhibition is measured used to determine resistance; smaller zones of inhibition indicate increased resistance while larger zones indicate susceptibility (69). The zone of inhibition may be used to mathematically estimate the MIC for that isolate to each antibiotic tested (70). Dilution methods involve inoculating a series of solid or liquid media containing serial dilutions of antibiotic concentrations with a known quantity of the bacterial isolate of interest; the MIC can then be directly determined when the concentration of antibiotic is sufficiently high to prevent the isolate from growing (69,70). While culture based methods are important for assessing phenotypic resistance, they are limited by the necessity for the target bacteria to be culturable; it is worth

noting that the vast majority of bacteria are therefore not quantifiable by these strategies, as it is estimated that fewer than 1% can be cultured using current methods (68).

1.4.2. GENETIC TECHNIQUES

Genetic techniques for detecting and quantifying resistance focus on identifying ARGs within a sample. Polymerase Chain Reaction (PCR) is a technique used to identify the presence of a target gene within a sample (68). During PCR, extracted DNA is heated to cause the unwinding of double-stranded DNA into single stranded DNA. The DNA is then cooled to allow gene-specific primers to anneal to a target site within the DNA sequence. DNA polymerase adds complementary nucleotides to complete the sequence, thereby amplifying the gene of interest. This cycle is repeated many times, resulting in an exponential increase in the number of target gene copies (71). When the PCR cycles are complete, it is necessary to verify that the correct gene sequence was amplified by performing gel electrophoresis to ensure that the resulting amplicon is the correct size (67). Real-time PCR, also known as quantitative PCR (qPCR), follows the same general process but allows for quantification of the gene of interest rather than mere detection; it does this through the use of fluorescent hybridization probes (72). Each time that the gene of interest is replicated, fluorescent light is emitted and measured; this information is plotted onto an amplification curve based upon target gene standards of a known concentration, and from this curve the number of target gene copies present in the DNA sample can be mathematically determined (72). With either PCR or qPCR specially designed primers must be used to target a specific, known gene; therefore, these technologies are therefore not useful for discovering novel ARGs (68).

Metagenomics is the analysis of all of the genes within a sample through direct shot-gun sequencing of DNA extracts, without prior PCR amplification, performed by using next-generation sequencing technologies, like 454-Pyrosequencing or Illumina. A main advantage of the metagenomics approach is that it amplifies all genes within a sample without the requirement of specific, targeted primers, as is the case in PCR-based techniques (73). When sequencing is complete, sequenced reads are compared against online databases to determine the identity and function of each gene. The Meta-Genome Rapid Annotation using Subsystems Technology (MG-RAST) database is a well-known open-source database that can be used for a variety of

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metagenomic analyses including phylogenetic comparisons, functional annotations, and metabolic reconstructions (74). There are also databases dedicated specifically to the genetics and genomics of antibiotic resistance, such as the Comprehensive Antibiotic Resistance Database (CARD), which compiles data about antibiotics, their targets, associated resistance genes, and associated proteins (75). The primary drawback to metagenomic sequencing and analysis currently is its high cost, which is expected to decrease with time. Another issue is that databases do not contain information on every possible gene sequence, but this also will improve with time as more studies are done and databases are populated with more information (74).

1.5. GOALS AND OBJECTIVES

The overall goal of this project was to investigate the effect of various composting practices on the prevalence of ARBs (operationally defined as capable of growth on antibiotic-amended media or characterized using the Kirby-Bauer disk diffusion method) and ARGs. To achieve this goal, the following objectives were specified:

1) Compare the occurrence of ARBs and ARGs in cattle manure and corresponding composts generated using various methods: small-scale static, small-scale turned, temperature-controlled, large-scale static.

2) Determine the effect of prior antibiotic administration on the prevalence of ARBs and ARGs during composting of corresponding dairy and beef manures.

3) Compare effectiveness of composting for reducing levels of ARBs and ARGs in dairy versus beef manures.

4) Compare the effect of different DNA extraction methods on the characterization of the taxonomic composition of dairy and beef manures and corresponding composts.

1.6. HYPOTHESES

The following hypotheses were tested:

1) Composting reduces the prevalence of ARBs and ARGs relative to non-composted, raw cattle manures.

2) Static versus turned composting approaches influence the occurrence of ARBs and ARGs in resulting compost.

3) Prior administration of antibiotics increases the prevalence of ARBs and ARGs in resulting manure and corresponding composts.

4) DNA extraction significantly influences characterization of the taxonomic composition of microbial communities in manure and compost.

5) There is a difference in the prevalence of ARBs and ARGs in beef manure, and derived products, when compared to dairy manure, and derived products.

2. METHODS AND MATERIALS

Three trials of composting experiments were performed to assess the impact of cattle manure composting on various measures of antibiotic resistance. The first trial was a small scale composting experiment performed in compost tumblers designed to: 1) test the impact of two distinct composting methods recommended in the Food Safety Modernization Act (FSMA) guidelines for biological soil amendments – an aerobic, static composting method where compost is maintained at or above 55°C for 3 days, followed by adequate curing, and an aerobic, turned composting method where compost is maintained at or above 55°C for 15 days and is turned at least five times during this phase, followed by adequate curing; 2) assess whether the use of manure from dairy cattle or beef cattle impacts the degree of antibiotic resistance in the finished compost and finally, 3) assess whether manure from antibiotic-treated cattle would result in finished compost with significantly different levels of antibiotic resistance than finished compost created from untreated cattle manure. This trial was allowed to heat naturally.

The second trial was a heat-controlled trial, also performed at bench-scale, but with FSMA-required thermophilic temperatures imposed using an external heat source. This trial focused only on dairy manure treated via the static composting method, but still compared composting of manure from antibiotic-treated versus untreated cattle manure. This trial was performed in response to the unexpected survival of *E. coli* through the end of the first small-scale trial. Given difficulties in maintaining recommended temperatures in the first trial, external heat in this effort served to extend the time at thermophilic temperatures in attempt to eliminate *E. coli*.

The third trial was conducted at full-scale and focused only on static composting of dairy manure from cattle with and without prior administration of antibiotics. A full-scale non-composted stockpile of antibiotic-treated cattle manure was also set up to serve as a positive control.

The cattle used in this study had no prior history of antibiotic treatment. To generate beef manure, nine healthy, yearling Hereford steers were fed a basal diet of corn silage and medicated or non-medicated grain mix. Three steers were fed 350 mg each of chlortetracycline and sulfamethazine per day; three steers were fed 11mg Tylosin per kg feed, and three steers were fed the basal diet containing non-medicated grain mix. Urine and feces were collected from day 3 to

day 7 post treatment when peak excretion of antibiotics and ARGs was expected. To generate dairy manure, six healthy, peak lactation dairy cows and three cows at the end of their current lactation cycle were used. Three cows received two intramammary therapeutic doses of 50 mg each of pirlimycin at peak lactation; three cows received a single intramammary therapeutic dose of 300 mg cephapirin at end of lactation; and three peak lactation cows received no antibiotic treatment. Both urine and feces were collected and composited to achieve homogeneous manures for each cattle and treatment type. Select properties of the four manures are outlined in Table 2.1.

Table 2.1. Select properties of raw manures.

Manure	pH	Moisture (%)	Carbon (%)	Nitrogen (%)
Beef Antibiotic	7.26	54.6	48.1	2.01
Beef Control	7.22	54.6	47.8	2.1
Dairy Antibiotic	6.79	56.4	47.8	2.12
Dairy Control	6.99	58.1	47.9	2.08

2.1. SMALL-SCALE COMPOSTING SETUP

The small-scale composting trial was set up to compare the effects of a static versus turned composting method. Four different manures (beef with or without chlortetracycline, sulfamethazine, and tylosin or dairy with or without pirlimycin and cephapirin antibiotic treatment) were mixed with alfalfa hay, pine bark mulch, and sawdust to achieve a C:N ratio of 23-26 and a moisture content of 55-65% and were composted in triplicate using both static and turned composting methods for a total of 24 independent composters (wet mass=20-22 kg). Static composters were aerated using an air pump and perforated polyvinyl chloride (PVC) pipes. Turned composters were mixed well by hand daily. Temperature sensors were placed at four locations within each composter to record and monitor temperature over time. Compost samples were taken on days 0, 4, 7, 14, 21, 28, 35 and 42. On day 0 samples were also taken of each non-composted, raw manure for comparison with the finished composts. Samples were used immediately for analysis by culture techniques and additional sample was stored at -20°C for subsequent molecular analysis.

2.2. HEAT CONTROLLED COMPOSTING SETUP

The heat-controlled trial tested dairy manures (with and without pirlimycin and cephapirin) and only used the static composting method; each combination was composted in triplicate for a total of 6 composters. Manures were mixed with grass hay and sawdust at ratios that were set to achieve a C:N ratio of 25-30 and a moisture content of 55-65%. Each composter was aerated using an air pump and perforated PVC pipes. Temperature sensors were placed at two locations within each composter to record and monitor temperature over time. Heat tape was applied externally to control the compost temperature; it was not turned on for the first 72 hours to allow self-heating of compost, but were then turned on to maintain the thermophilic stage (>55°C) for 15 days, after which a mesophilic temperature range (35-45°C) was maintained for three weeks before allowing compost to cool to room temperature. Samples were collected on days 0, 1, 3, 7, 14, 21, 28, 35, and 42 and raw manure samples were collected on day 0 for comparison with finished composts. Samples were used immediately for analysis by culture techniques and additional sample was stored at -20°C for subsequent molecular analysis.

2.3. LARGE-SCALE COMPOSTING SETUP

The large-scale composting took place outdoors at Kentland Farm in southwest Virginia. The compost piles were formed in early January. Dairy manure (with and without pirlimycin and cephapirin antibiotic treatment) was mixed with alfalfa hay and sawdust at a ratio of 4:1:4.3 to achieve a moisture content of 55-65% and a C:N ratio of 25-30. Compost piles were formed in large metal containers (20 × 8 × 4 ft.) on top of a system of perforated PCV pipes which supplied forced aeration using an attached air pump. A stockpile of antibiotic manure without any amendments or aeration was also formed in a metal container to serve as a non-composted positive control. Compost and stockpile samples were taken on days 0, 4, 7, 14, 28, 42, 56, and 63. Samples were used to enumerate *E. coli* using the Colilert defined substrate method on days 0 and 63 (www.idexx.com, Wetsbrook, MN). The remaining samples were stored at -20°C for subsequent molecular analysis.

2.4. BACTERIAL CULTURING AND ENUMERATION

Compost samples from the small-scale composting trial were plated onto both R2A and MacConkey agars to enumerate total heterotrophs and fecal coliforms, respectively. The fecal coliform count was intended to serve as a rough proxy for *E. coli*, though MacConkey is not specific to only *E. coli*. Heat controlled composting samples were plated onto MacConkey agar and MacConkey agar that had been supplemented with one of several antibiotics: 40 µg/ml erythromycin, 35 µg/ml clindamycin, 8 µg/ml tetracycline, 0.25 µg/ml cefotaxime, or 0.5 µg/ml ceftazidime to enumerate resistance of fecal coliforms. These concentrations were determined from EUCAST MICs for *E. coli* (tetracycline, cefotaxime, ceftazidime) and concentrations for those antibiotics without known *E. coli* MICs (erythromycin and clindamycin) were determined experimentally and therefore are not MICs indicating true resistance.

For both small-scale and heat-controlled compost samples, 10g of compost or manure were added to a sterile blender bag with 90 mL of 0.1% peptone solution to make a 1:10 dilution. The bag was mixed in a bag mixer for 2 minutes. A serial dilution was then performed in test tubes containing 0.1% peptone solution. The dilutions were then plated onto the appropriate media. Plates were incubated for 24 hours at 37°C. Only plates containing between 30 and 300 colonies were counted. All colonies were counted on R2A plates and only colonies appearing pink or red (indicating lactose fermentation) were counted on MacConkey plates.

2.5. IDEXX ENUMERATION OF *E. COLI*

Due to the non-specificity of MacConkey Agar to *E. coli*, the Colilert defined substrate method with Quanti-Tray 2000s was used to verify that the fecal coliform count from the MacConkey plates did in fact indicate survival of *E. coli* following the thermophilic stage of composting. A 1:10 dilution was made using 0.1% peptone solution via the bag mixer as for the plating method. This was further diluted to an appropriate concentration and combined with the Colilert powder in a 100ml vial and mixed thoroughly by hand until the powder dissolved completely. The resulting solution was poured into a Quanti-Tray 2000 tray and heat sealed. Trays were incubated for 24 hours at 37°C. The number of large and small wells that fluoresced under

ultraviolet light were counted and *E. coli* counts were determined using the IDEXX MPN Generator Software.

2.6. KIRBY BAUER DISK DIFFUSION TEST

The Kirby Bauer disk diffusion test was used to assess antibiotic resistance of fecal coliform bacteria collected from small-scale composting MacConkey plates on day 42, representing conditions at the end of composting. Ten colonies were selected randomly from plates from each compost or manure treatment and were streaked to isolation onto a fresh MacConkey plate and incubated for 24 hours at 37°C. Isolated colonies were added to tubes of sterile saline (0.9% NaCl) and compared to a 0.5 McFarland standard to ensure a standard cell density. Isolated colonies were also grown up in Luria-Bertani (LB) broth and preserved in glycerol stock at -20°C for future analysis. Large (15 cm diameter) plates of Mueller Hinton agar were made with a standard agar thickness of 4 mm. A Mueller Hinton plate was inoculated from the saline solution by using a cotton swab to streak the full area of the plate. Antibiotic disks were placed onto the inoculated plate using a disk dispenser to ensure even spacing. The antibiotics disks used were: 10 µg ampicillin, 5 µg cefotaxime, 30 µg chloramphenicol, 5 µg ciprofloxacin, 10 µg gentamicin, and 10 µg imipenem. Plates were incubated for 24 hours at 37°C. Results were read as the diameter of the resulting zone of inhibition formed around each disk in millimeters, with a smaller zone of inhibition indicating greater resistance. Results were compared to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoint values for *E. coli* for each antibiotic to determine the proportion of samples from each treatment that are considered resistant. The breakpoint diameters used were: ampicillin: 14 mm, cefotaxime: 23 mm, chloramphenicol: 17 mm, ciprofloxacin: 25 mm, gentamicin: 16 mm, and imipenem: 24mm.

2.7. DNA EXTRACTION

DNA from compost and manure samples was extracted using two extraction kits: the FastDNA Spin Kit for Soil, Catalog #116560200 (MP Biomedicals, Solon, OH), which served as the primary extraction kit and was used for all samples, and the ZR Fecal DNA MiniPrep Kit, Catalog #D6010 (Zymo Research Corporation, Irvine, CA) which served as a secondary kit for

comparison purposes and was only used to extract 10% of samples. Compost samples were first blended thoroughly to break down large pieces of mulch and hay into a more uniform and homogeneous consistency that could be easily extracted. Following blending, 500 mg of compost or manure was aseptically transferred into an appropriate test tube for extraction. Extraction followed manufacturer's instructions, except that a 2-hour incubation period was added to the protocol immediately following the bead-beating step to ensure unbiased lysing of both gram-positive and gram-negative microbial cells, and the final centrifugation step was extended to 3 minutes to ensure complete capture of DNA. The OneStep PCR Inhibitor Removal Kit, Catalog #D6030 (Zymo Research Corporation, Irvine, CA) was used as an additional cleanup step.

DNA was also extracted from the Kirby Bauer isolates using the FastDNA Spin Kit for Soil by streaking the stored isolates from glycerol stock onto a fresh MacConkey agar plate and incubating overnight. The plate was then rinsed with 500 ml of deionized (DI) water and mixed using a sterile plate spreader. The resulting suspension fluid was then pipetted into the extraction kit's Lysing Matrix E tube and DNA was then extracted following the manufacturer's protocol and the Zymo OneStep PCR Inhibitor Removal Kit was used as an additional cleanup step.

2.8. WHOLE GENOME SEQUENCING OF KIRBY BAUER ISOLATES

Illumina whole genome sequencing of the Kirby Bauer isolates will be carried out in the future to explore the genotypes of putative *E. coli* colonies. This can help validate the use of the selected antibiotic MICs and also provide evidence of whether gene transfer was at play in the composting process. Eighty isolates have been submitted to MicrobesNG at the University of Birmingham, England for whole-genome sequencing.

2.9. METAGENOMIC SEQUENCING AND ANALYSIS

A set of 60 samples was selected from among all of the small-scale and heat-controlled compost and manure samples to provide a cross-section of the overall composting process with time. Samples were selected to be equally representative of all manure types and composting methods at each time point. Samples were then submitted to the Virginia Bioinformatics Institute,

Blacksburg, VA, for Illumina HiSeq High Output 2x100 paired end cycle clustering and sequencing per lane and run as 5 lanes of 12 samples each. Sequencing results were stitched together and analyzed by uploading to MetaStorm, an online metagenomics analysis tool which compares gene sequence reads against several databases for both taxonomic and ARG analysis and facilitates visualization of the results (76). ARG sequences were identified through comparison with the comprehensive antibiotics resistance database (CARD).

2.10. 16S rRNA GENE AMPLICON SEQUENCING

All extracted DNA samples from each composting trial were submitted to the Virginia Bioinformatics Institute for 16S rRNA gen amplicon sequencing. Samples were submitted in 2 lanes of 150 samples each sequenced using Illumina MiSeq V3 2x300 paired end cycle clustering and sequencing per lane. Sequencing results were stitched together and taxonomic analysis was performed using QIIME(77). All singleton reads and chimeric sequences were removed and OTU tables were generated allowing for taxonomic composition analysis. Jackknifed beta diversity analysis was performed to calculate unweighted UniFrac distance matrices for the comparison of sample taxonomic similarity.

2.11. QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR)

A qPCR dilution test was performed on extracted DNA to minimize inhibition by running a plate with a range of dilutions and determining which dilution resulted in the highest initial concentrations of 16s rRNA. Samples were then diluted 1:100 based upon this result. QPCR was then performed in triplicate on all diluted samples using the CFX96 Touch Real-Time PCR Detection System (BioRad Laboratories, Hercules, CA) to quantify initial concentrations of 16S rRNA genes, *tet*(W), and *sul*1. *Tet*(W) and *sul*1 were chosen because the treated beef cattle were administered chlortetracycline and sulfamethazine, so an effect on tetracycline and sulfonamide resistance was expected; these two genes are also well represented in the current research. *Tet*(W) codes for ribosomal protection proteins, while *sul*1 codes for sulfonamide-resistant dihydropteroate synthase. Primer sequences used were those outlined by Ma et al. (78). The

SsoFast Evagreen Supermix, Catalog #1725204 (BioRad Laboratories, Hercules, CA) was used according to manufacturer instruction to create the qPCR mastermix.

2.12. STATISTICAL ANALYSES

Statistical analyses were performed using the JMP statistics software (SAS Institute Inc., Cary, NC). Statistical differences in the number of colonies enumerated on R2A and MacConkey agars were calculated using a Kruskal-Wallis non-parametric rank test. Zero-Inflated Poisson analysis was used to test for differences in the antibiotic supplemented plates due to the presence of many "below detection limit" measurements. A significance value of $\alpha = 0.05$ (i.e. $p < 0.05$) was considered significant. Statistical differences between qPCR ARG concentrations were also calculated using the Kruskal-Wallis nonparametric rank test. The test was applied to the log starting quantity as well as to the starting quantity normalized to the number of 16S rRNA gene copies. Statistical differences in the proportion of Kirby Bauer isolates resistant to each of the tested antibiotics were calculated using a Pearson's Chi-Squared analysis. Statistical comparisons of the similarity of ARG profiles of metagenomic data and of similarity of taxonomic class data and UniFrac distances were determined using an analysis of similarities (ANOSIM) test performed using the software Primer 6 (79). The ANOSIM test is a nonparametric test comparing samples based upon similarity using a similarity matrix. The ANOSIM test is more sensitive to presence or absence of a parameter (bacterial species or ARG) than to differences in the abundance of the most abundant parameters.

3. RESULTS: EFFECT OF COMPOSTING CATTLE MANURE ON THE PREVALENCE OF ARBs AND ARGs

3.1. CULTURE-BASED ENUMERATION AND RESISTANCE TESTING

3.1.1. SMALL-SCALE COMPOSTING

For the small-scale composting trial, total heterotrophic plate counts (HPCs) were enumerated using R2A agar, with results shown in Figure 3.1 and Figure 3.2. It was found that the number of HPCs increased rapidly at the onset of composting and ultimately stabilized 2 logs higher than initial day 0 values (Figure 3.1). There was significant variation in the number of HPCs in each of the four manure types subject to composting at day 0 ($p<0.0001$; Kruskal-Wallis), but by day 42 there was no significant difference among composts derived from the different manure types (Figure 3.2.).

The small-scale composting trial achieved the FSMA required 3 days at thermophilic temperatures for the statically composted treatments, but the turned treatments failed to maintain thermophilic temperatures for the required 15 days. Fecal coliforms enumerated using MacConkey agar (Figures 3.3 and 3.4) behaved in a similar manner as observed for HPCs. Despite a decrease in fecal coliforms on day 4 of static composting, counts rebounded and finished 2 logs higher than initial day 0 values finishing at 9 log CFU/g. This change was significant ($p<0.0001$; Kruskal-Wallis). The fecal coliform count, therefore, did not only fail to meet the FSMA required 3 log MPN/g, but the composting process appeared to increase the number of indicator bacteria. There was no significant difference in MacConkey counts between the two composting methods, i.e. the maintenance of target temperatures did not improve inactivation of indicator bacteria. The high remaining abundance of fecal coliforms prompted the use of IDEXX with Colilert to specifically quantify *E. coli* (Figure 3.5 and 3.6), which confirmed that *E. coli* were present in the 3-4 log MPN/g range in the finished day 42 compost.

The Kirby Bauer disk diffusion test was applied to a randomly selected subset of 10 MacConkey isolates corresponding to each treatment at day 42 for compost samples and day 0 for non-composted manure samples to characterize resistance patterns pre- and post-composting, as outlined in Table 3.1. The number of isolates classified as resistant to each of the six antibiotics is shown in Figures 3.7 and 3.8, organized by composting method and manure type, respectively.

Isolates from day 42 composts indicated significant increases in resistance to ampicillin (p<0.0001; Chi-Squared), chloramphenicol (p<0.0001; Chi-Squared), and cefotaxime (p<0.0001; Chi-Squared) as compared to raw manure (Figure 3.7); composting was also associated with increased rates of multi-antibiotic resistance (p<0.0001; Kruskal-Wallis) (Figure 3.8). There was no observable effect of manure type.
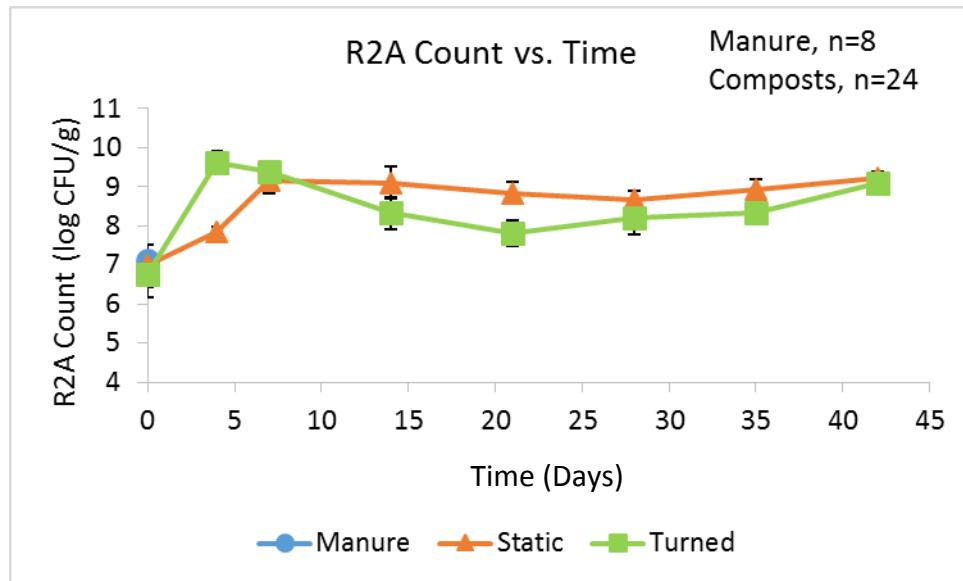

Figure 3.1. Total heterotrophic plate counts (HPCs) over time for raw manure (day 0 only) and both static and turned composting methods. The increase in total HPCs over time was significant (p<0.0001; Kruskal-Wallis), and composting resulted in higher plate counts on day 42 compared with raw manure on day 0 (p<0.0001; Kruskal-Wallis), but there was no significant difference between static versus turned composting HPCs on day 42 (p=0.1465; Kruskal-Wallis).
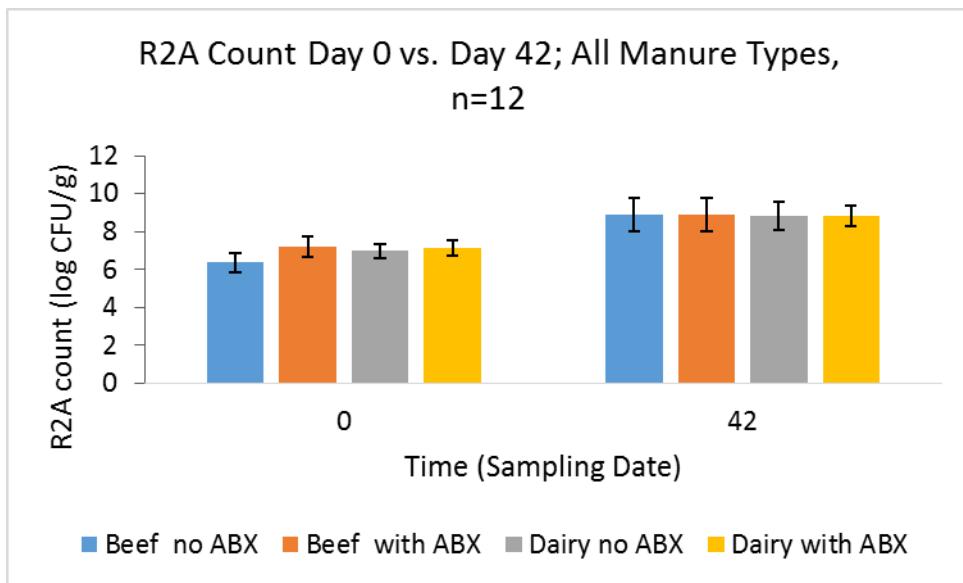
Figure 3.2. Comparison of total heterotrophic plate counts (HPCs) on day 0 with those on day 42 by manure type. On day 0, composts using beef manure without antibiotics had significantly fewer HPCs than other manure types subject to composting (p=0.0003; Kruskal-Wallis). No significant differences were observed among manure types on day 42 (p=0.1376; Kruskal-Wallis). All manure types showed significantly increased HPCs abundance after 42 days of composting (p=0.0001; Kruskal-Wallis)**.**
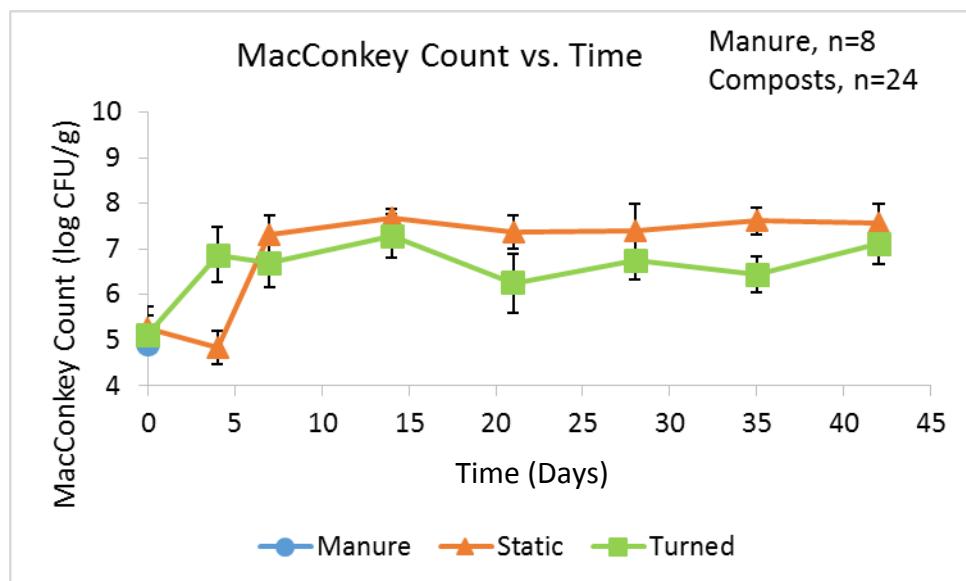

Figure 3.3. Fecal coliforms, as determined by MacConkey plate counts, for raw manure (day 0 only) as well as static and turned composting methods over time. Fecal coliform abundance

increased significantly with time (p<0.0001; Kruskal-Wallis). Both composting methods resulted in a significant increase in fecal coliforms by day 42 as compared to raw manure (p<0.0001; Kruskal-Wallis). Fecal coliform abundance was not significantly different between static and turned composting methods on day 42 (p=0.8855; Kruskal-Wallis), nor for all time points overall (p=0.0658; Kruskal-Wallis).
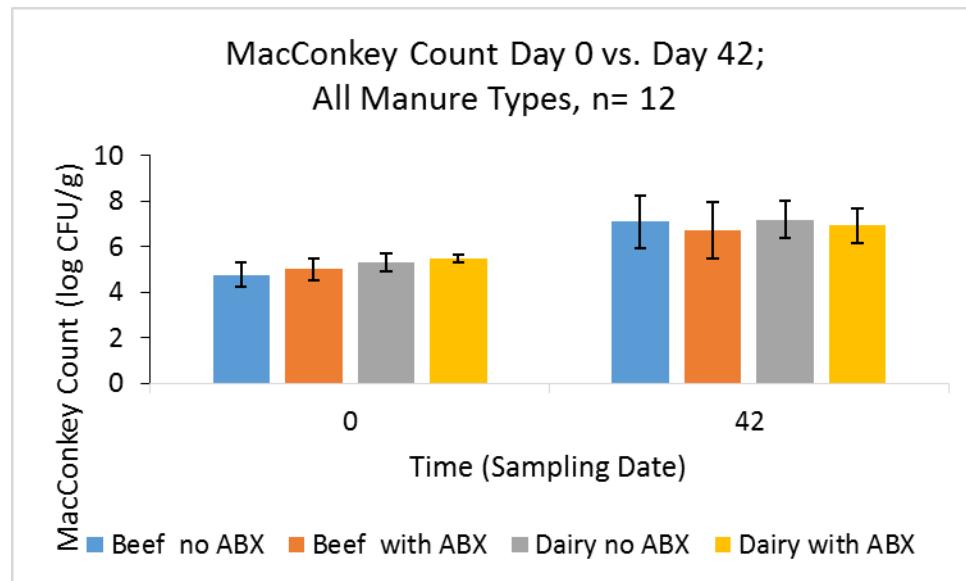

Figure 3.4. Comparison of fecal coliforms, as determined by MacConkey plate counts, on day 0 with those on day 42 by manure type. On day 0, there was a significant difference in fecal coliform abundance among the four manure types (p=0.0003; Kruskal-Wallis). No significant differences were observed among the composts generated from the four manure types on day 42 (p=0.3036; Kruskal-Wallis). All manure types showed significantly increased fecal coliform abundance after 42 days of composting (p=0.0001; Kruskal-Wallis)**.**
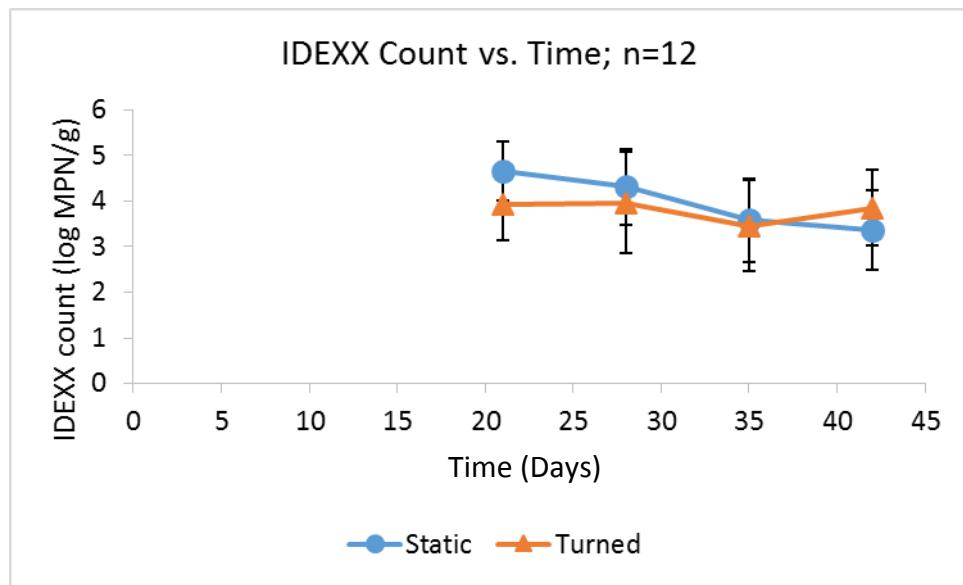
Figure 3.5. Abundance of *E. coli* measured using IDEXX with Colilert. Time was a significant factor impacting change in abundance (p=0.0087; Kruskal-Wallis). No significant difference was observed between static and turned composting methods (p=0.3664; Kruskal-Wallis).
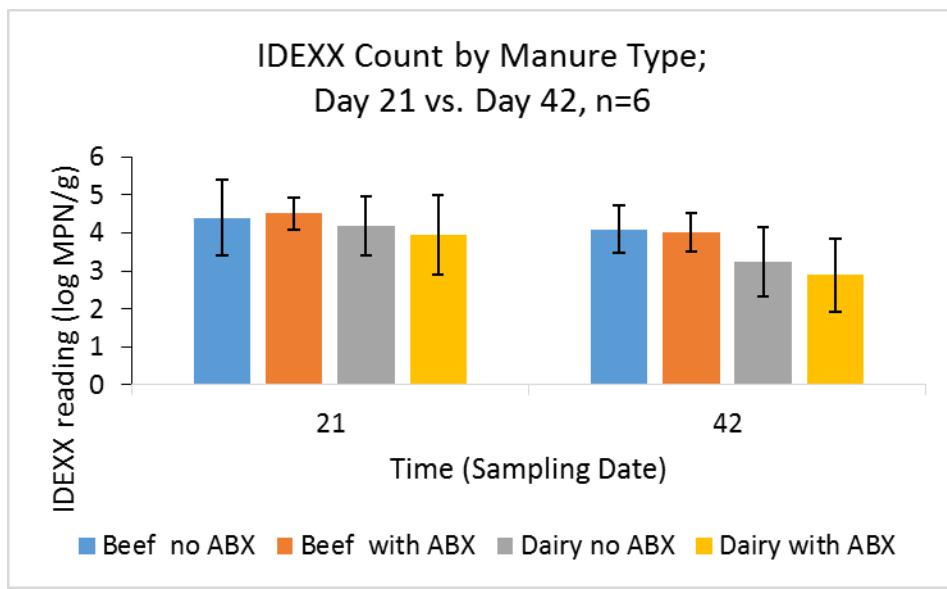

Figure 3.6. Comparison of *E. coli* counts between day 21 and day 42 of composting, organized by manure type. No significant difference was observed among *E. coli* counts of the various manure types subject to composting on day 21 (p=0.7623; Kruskal-Wallis) or day 42 (0.0747; Kruskal-Wallis). There was no significant difference in abundance on day 42 compared to day 21 with respect to any of the manure types subject to composting.

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Table 3.1. Summary of Kirby Bauer disk diffusion results for each of antibiotic tested. The proportion of isolates that tested as resistant is reported. Ten total isolates were tested for each reported sample type.

| Sample Type	Ampicillin	Ciprofloxacin	Chloramphenicol	Gentamicin	Imipenem	Cefotaxime
Beef Antibiotic Manure	0.0	0.0	0.0	0.0	0.0	0.0
Beef Control Manure	0.0	0.1	0.0	0.0	0.0	0.0
Dairy Antibiotic Manure	0.0	0.0	0.0	0.0	0.0	0.0
Dairy Control Manure	0.1	0.1	0.0	0.0	0.0	0.1
Static Beef Antibiotic Compost	1.0	0.1	0.7	0.0	0.0	0.6
Static Beef Control Compost	1.0	0.1	0.7	0.0	0.1	0.7
Static Dairy Antibiotic Compost	1.0	0.0	0.9	0.0	0.0	0.9
Static Dairy Control Compost	1.0	0.0	0.8	0.0	0.0	0.3
Turned Beef Antibiotic Compost	1.0	0.0	0.5	0.0	0.0	0.5
Turned Beef Control Compost	1.0	0.3	0.9	0.0	0.0	0.8
Turned Dairy Antibiotic Compost	0.6	0.2	0.3	0.0	0.1	0.2
Turned Dairy Control Compost	1.0	0.0	0.7	0.0	0.0	0.7
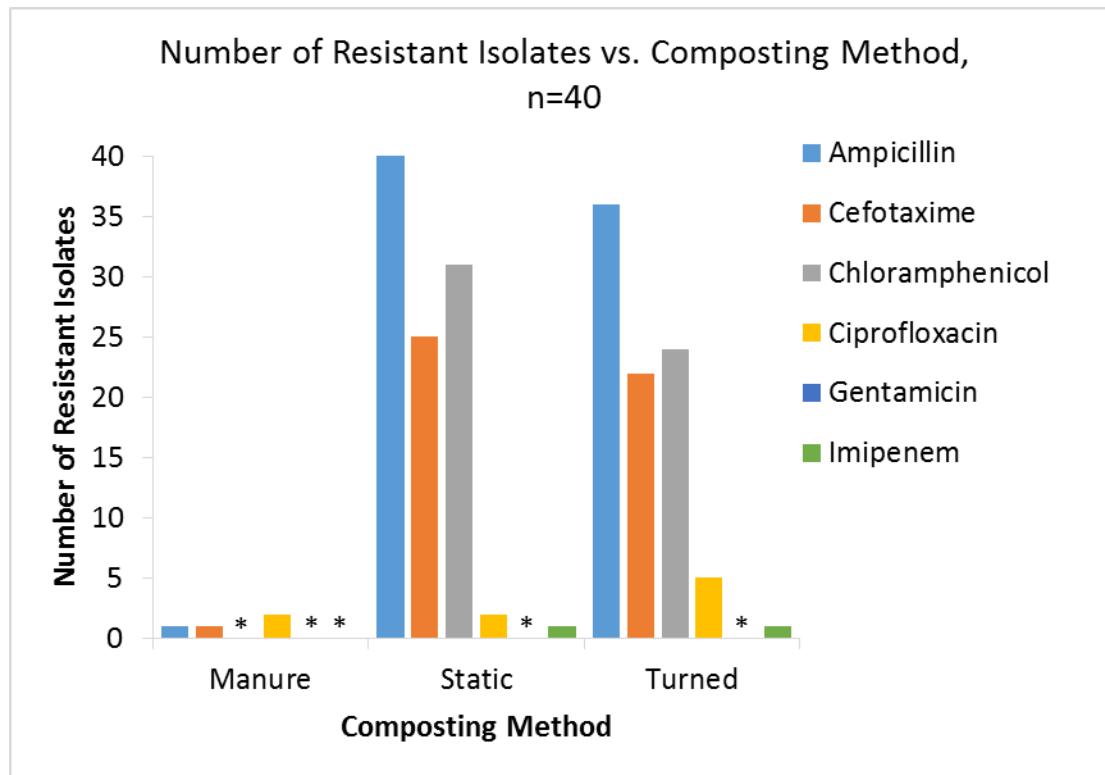
Figure 3.7. Number of MacConkey isolates classified as resistant to each of six antibiotics according to the Kirby-Bauer disk diffusion assay, organized by composting method. Composting, relative to raw manure, was associated with a significant increase in the number of isolates resistant to ampicillin (p<0.0001; Chi-squared), chloramphenicol (p<0.0001; Chi-squared), and cefotaxime

(p<0.0001; Chi-squared), but not ciprofloxacin (0.3392; Chi-squared) or imipenem (0.0806; Chi-squared) and gentamicin resistance was not detected in any of the isolates.
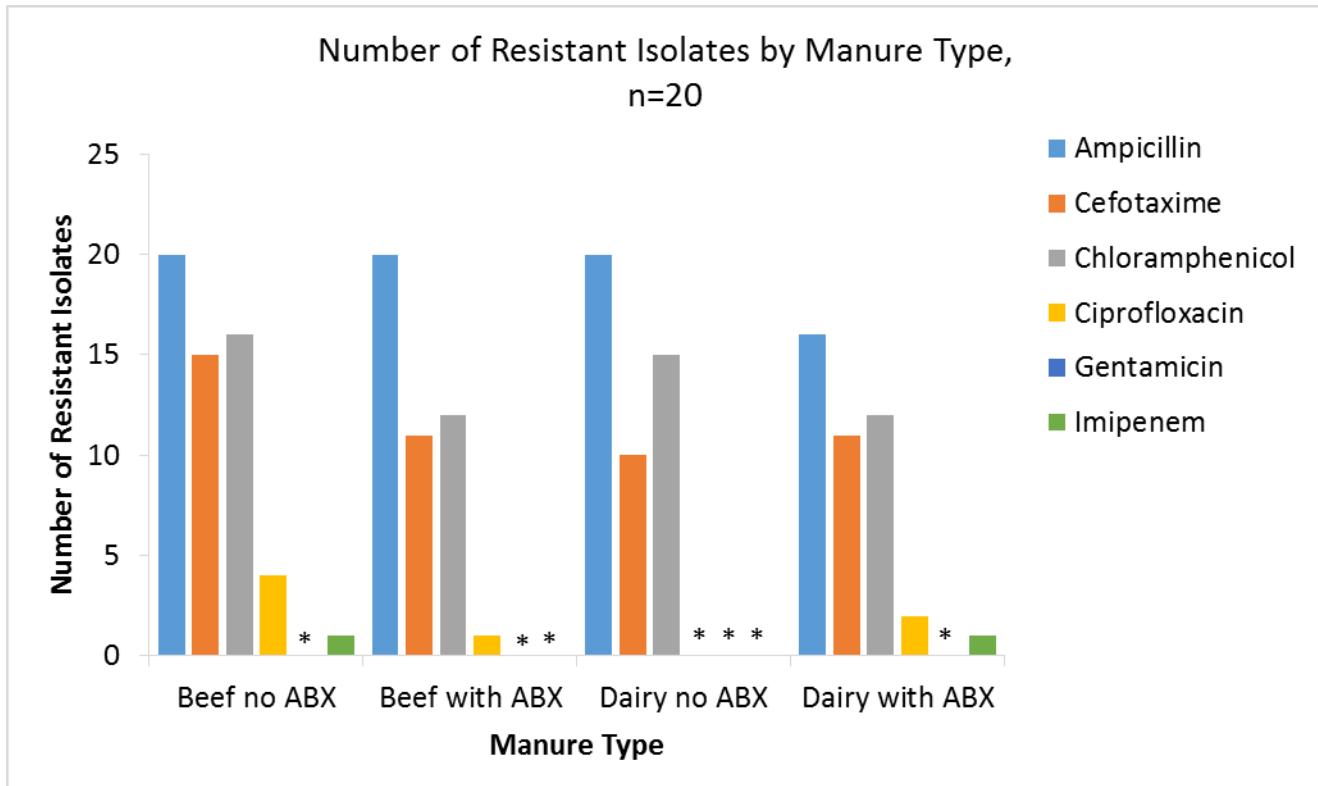

Figure 3.8. Number of isolates classified as resistant to each of six antibiotics according to the Kirby-Bauer disk diffusion assay, in finished day 42 compost derived from the four manure types. There was no apparent association of manure type on resistance of MacConkey isolates in finished compost to any of the tested antibiotics: ampicillin (p=0.5442; Chi-squared), ciprofloxacin (p=0.5159; Chi-squared), chloramphenicol (p=0.6343; Chi-squared), imipenem (p=0.5654; Chi-squared), cefotaxime (p=0.6444; Chi-squared). Gentamicin resistance was not detected in any of the isolates.
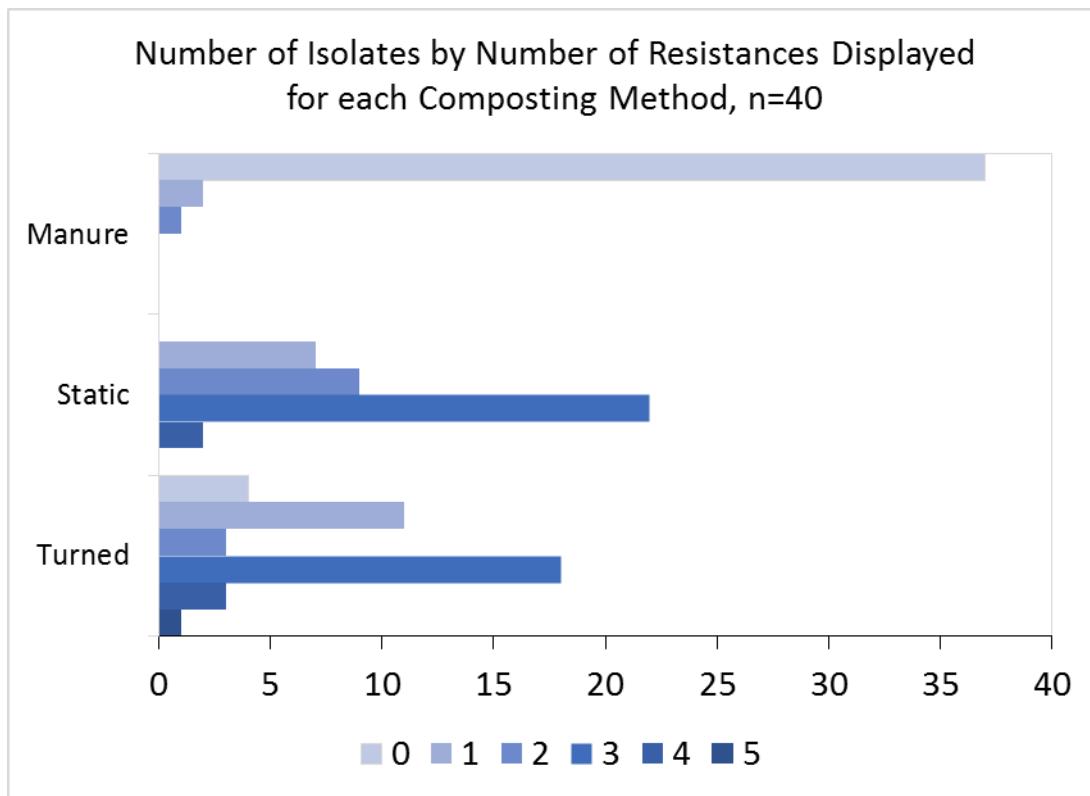
Figure 3.9. Comparison of multi-antibiotic resistance occurrence among the MacConkey isolates, as determined by the Kirby-Bauer disk diffusion assay, organized by composting method. Isolates derived from compost tested resistant to a significantly greater mean number of antibiotics than those taken from raw manure (p<0.0001; Kruskal-Wallis).
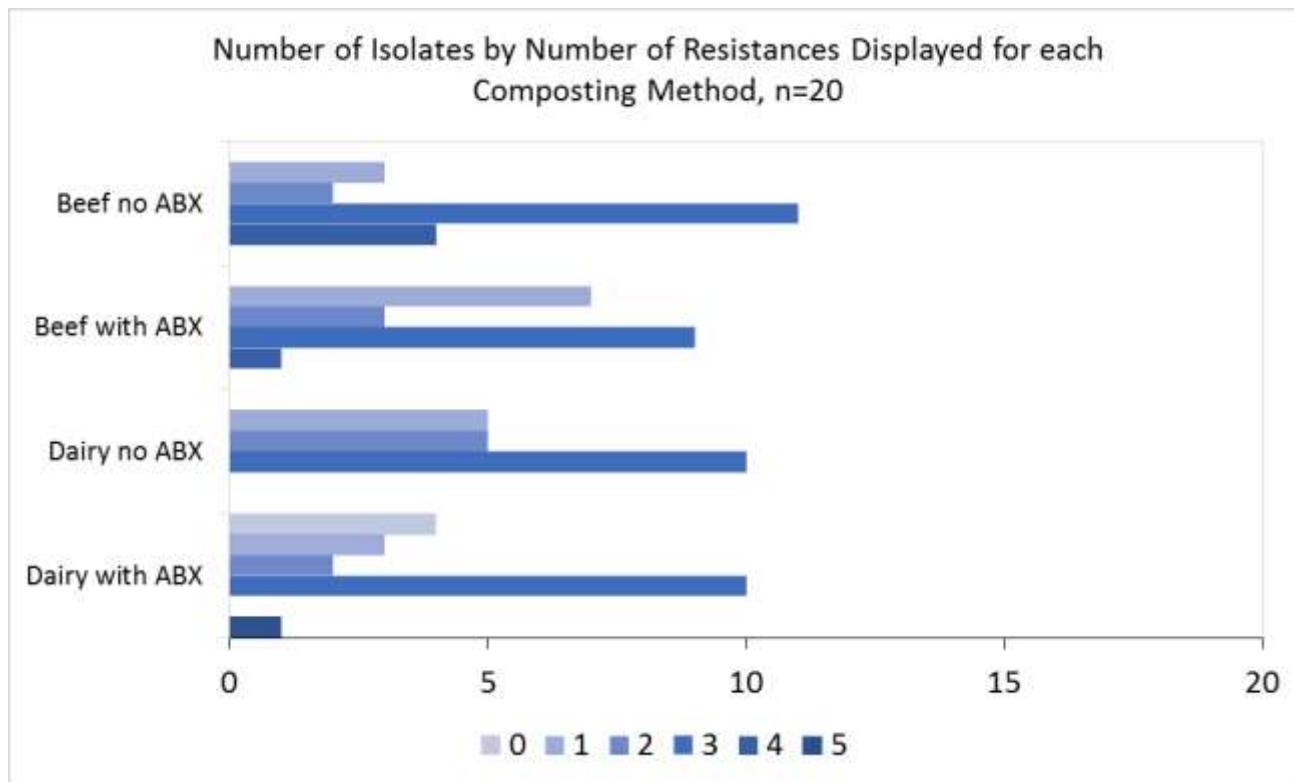
Figure 3.10. Comparison of multi-antibiotic resistance occurrence among the MacConkey isolates, as determined by the Kirby-Bauer disk diffusion assay, organized by the type of manure subject to composting. Manure type is not associated with significant differences in the number of resistances observed (p=0.5719; Kruskal-Wallis).

3.1.2. HEAT-CONTROLLED COMPOSTING

During heat controlled composting, resistant fecal coliforms were enumerated using antibiotic-supplemented MacConkey agar containing one of five different antibiotics as well as a control plate without antibiotics, and the ratio of the number of resistant colonies to control colonies for each of the antibiotics was determined (Figures 3.11 – 3.15). The temperature was maintained above thermophilic temperatures for the 3 days required by FSMA for static composting. For the 3rd generation cephalosporins, ceftazidime and cefotaxime (Figure 3.11 and 3.12, respectively), composting significantly increased the proportion of resistant colonies by day 3 (p<0.0001; Zero-Inflated Poisson), before counts fell below detection limits on day 7, while there was no effect of antibiotic treatment. Tetracycline resistance behaved similarly with a sharp increase on day 3 of composting (p<0.0001; Zero-Inflated Poisson) before falling below detection

on day 7 (Figure 3.13), but this time antibiotic administration did result in a significant increase in the proportion of tetracycline resistant isolates observed (p=0.0003; Zero-Inflated Poisson). The proportion of resistant isolates to erythromycin, a macrolide, and clindamycin, a lincosamide, behaved differently from the others (Figures 3.14 and 3.15, respectively). In each, there was no effect of composting, while antibiotic administration resulted in higher proportions of resistant isolates (p=0.0287 and p=0.0297 for erythromycin and clindamycin, respectively; Zero-Inflated Poisson), before falling below detection levels.

Once MacConkey agar counts fell below detection levels, IDEXX was then used to enumerate *E. coli* for the remainder of the composting trial (Figure 3.16). This time, *E. coli* abundance was reduced to non-detect levels by day 42 of composting, as expected, and there was again no significant impact of prior antibiotic administration. The reduction of *E. coli* below detection indicates a likely decrease in fecal coliforms but it cannot be definitively said whether fecal coliforms fell below the FSMA target of 1000 MPN/g since they were not measured directly.
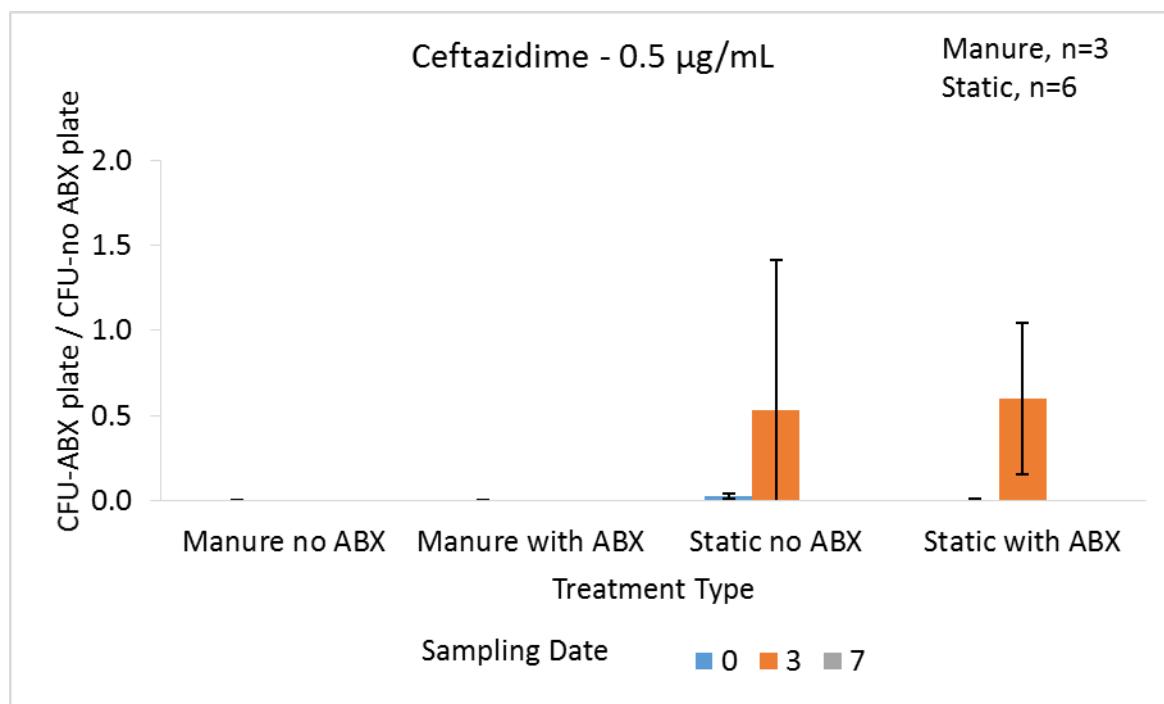

Figure 3.11. Proportion of ceftazidime resistant fecal coliforms relative to total fecal coliforms, as determined by MacConkey plate counts. Error bars represent standard deviation; error propagated in both numerator and denominator. Time and composting method significantly impacted the

proportion of fecal coliforms capable of growth on plates containing ceftazidime (p<0.0001; Zero-inflated Poisson). Prior antibiotic administration did not have a significant effect (p=0.6724; Zero-inflated Poisson).
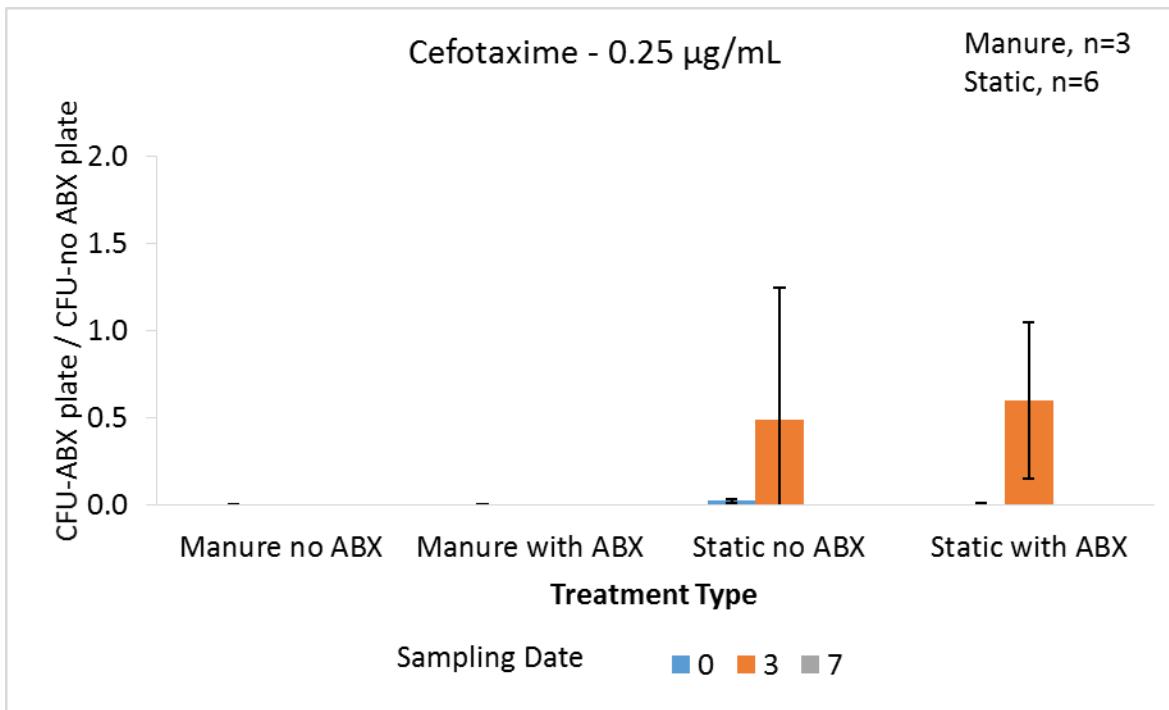

Figure 3.12. Proportion of cefotaxime resistant fecal coliforms relative to total fecal coliforms, as determined by MacConkey plate counts. Error bars represent standard deviation; error propagated in both numerator and denominator. Time and composting method significantly impact relative resistance of fecal coliforms to cefotaxime (p<0.0001; Zero-inflated Poisson), antibiotic treatment has no significant impact (p=0.5905; Zero-inflated Poisson).
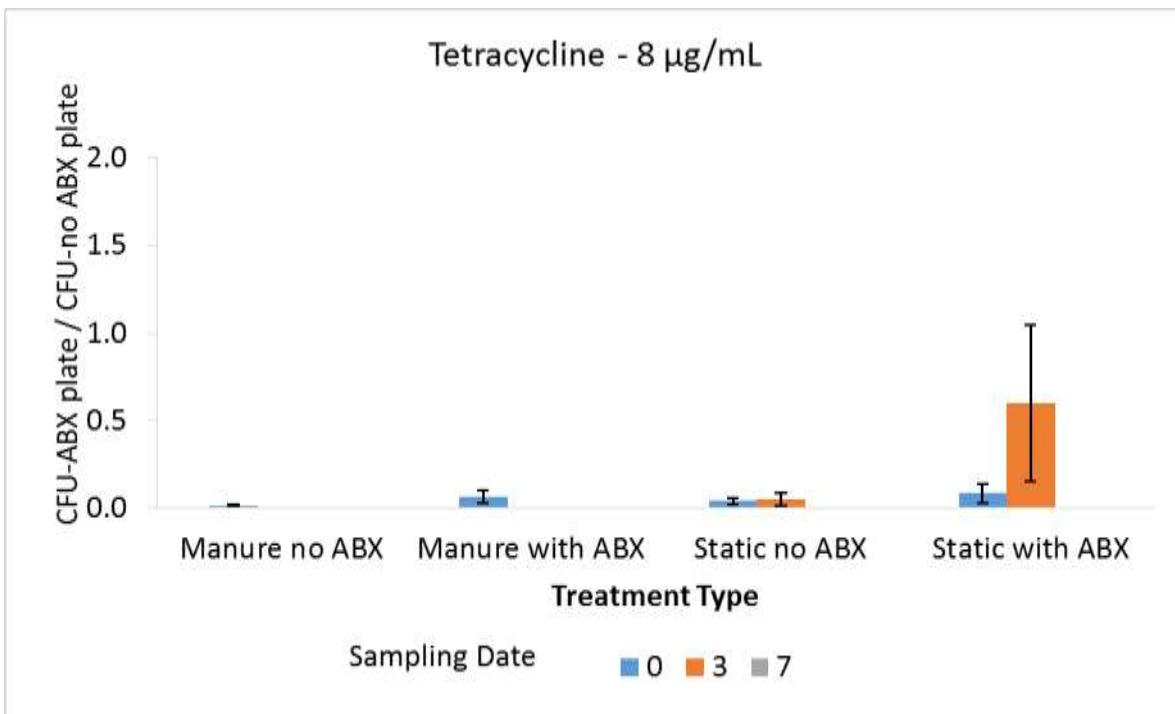
Figure 3.13. Proportion of tetracycline resistant fecal coliforms relative to total fecal coliforms, as determined by MacConkey plate counts. Error bars represent standard deviation; error propagated in both numerator and denominator. Composting method and antibiotic treatment significantly impact relative resistance of fecal coliforms to tetracycline (p=0.0274 and p=0.0003, respectively; Zero-inflated Poisson), time has no significant impact (p=0.0726; Zero-inflated Poisson).
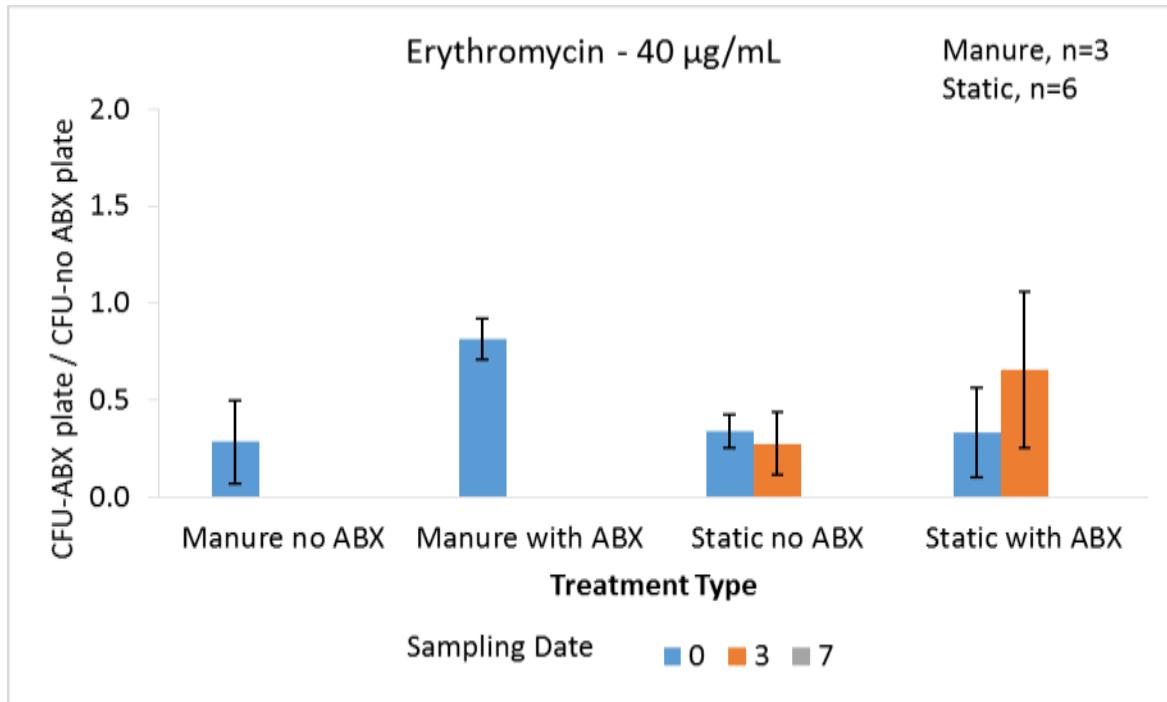
Figure 3.14. Proportion of erythromycin resistant fecal coliforms relative to total fecal coliforms, as determined by MacConkey plate counts. Error bars represent standard deviation; error propagated in both numerator and denominator. Antibiotic treatment significantly increases relative resistance of fecal coliforms to erythromycin ($p < 0.0287$; Zero-inflated Poisson), whereas time and composting have no significant impacts ($p = 0.8083$ and $p = 0.3272$, respectively; Zero-inflated Poisson).
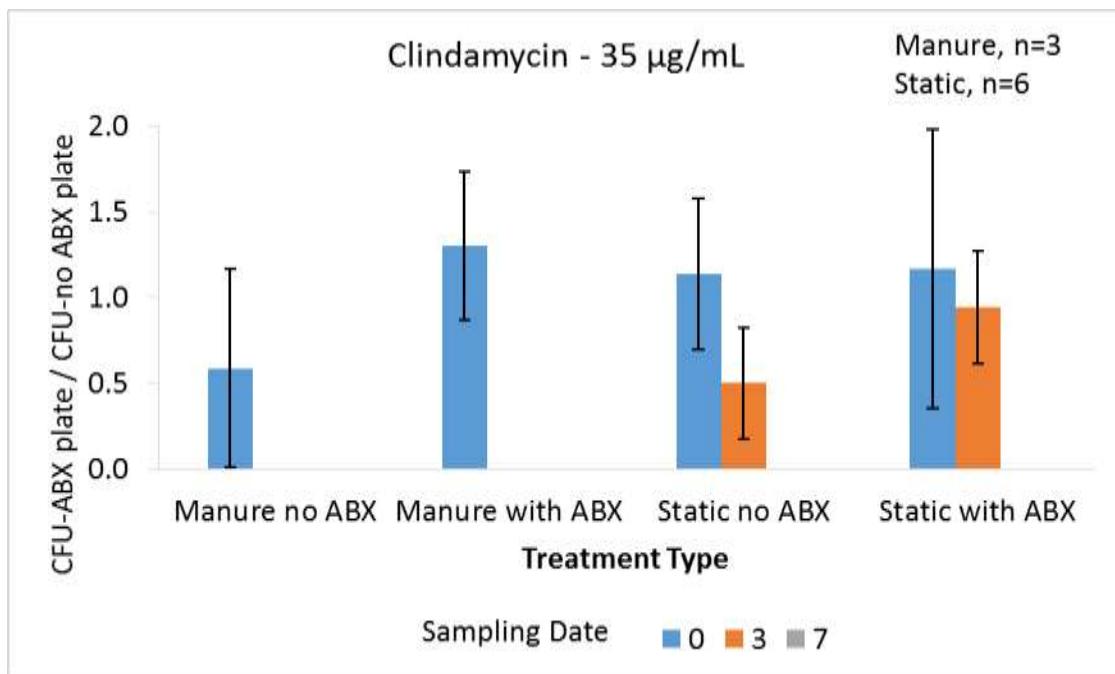
Figure 3.15. Proportion of clindamycin resistant fecal coliforms relative to total fecal coliforms, as determined by MacConkey plate counts. Error bars represent standard deviation; error propagated in both numerator and denominator. Antibiotic treatment significantly impacts the relative resistance of fecal coliforms to clindamycin ($p<0.0297$; Zero-inflated Poisson), whereas time and composting have no significant impact ($p=0.2378$ and $p=0.8272$, respectively; Zero-inflated Poisson).
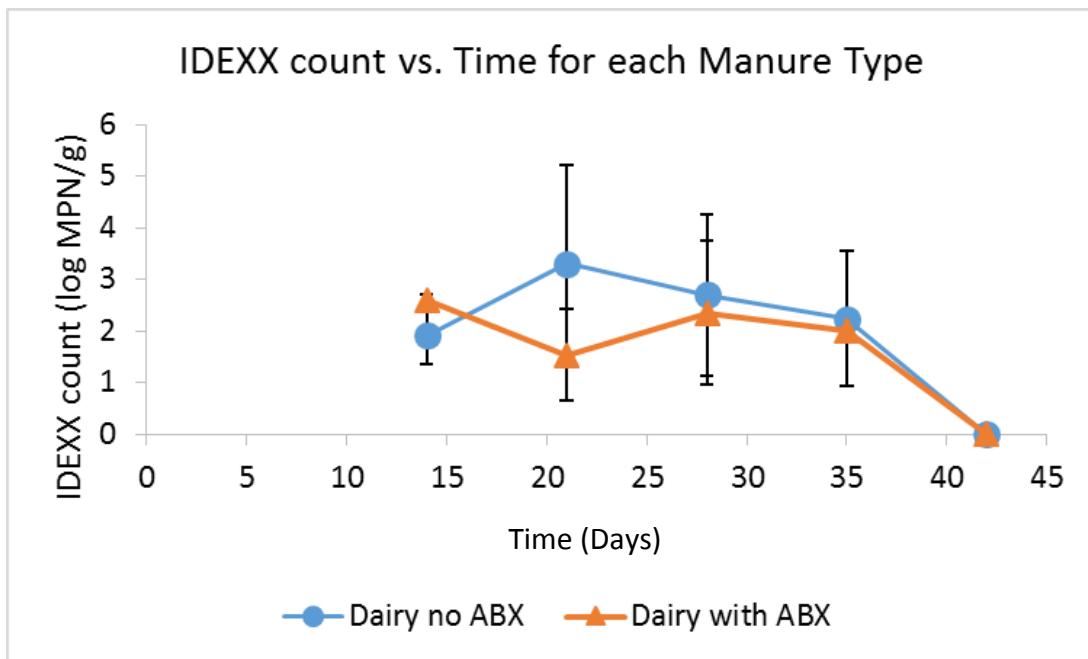
Figure 3.16. Abundance of *E. coli* measured using IDEXX with Colilert. Time was a significant factor impacting change in abundance (p=0.0222; Kruskal-Wallis). No significant impact of prior antibiotic administration was observed (p=0.8906; Kruskal-Wallis).

3.1.3. LARGE-SCALE COMPOSTING

IDEXX was used to enumerate *E. coli* on day 0 and day 63 of large scale composting (Figure 3.17). Composted treatments were maintained at or above thermophilic temperatures for the 3 days required by FSMA. Both composting (static composting vs. stockpiling manure) and prior antibiotic administration had a significant impact on day 63 *E. coli* counts (p<0.0001; Kruskal-Wallis), with counts falling below detection limits for the static compost condition with antibiotics by day 63. The reduction of *E. coli* below detection indicates a likely decrease in fecal coliforms but it cannot be definitively said whether fecal coliforms fell below the FSMA target of 1000 MPN/g for either the antibiotic treated or untreated composts since fecal coliforms were not measured directly.
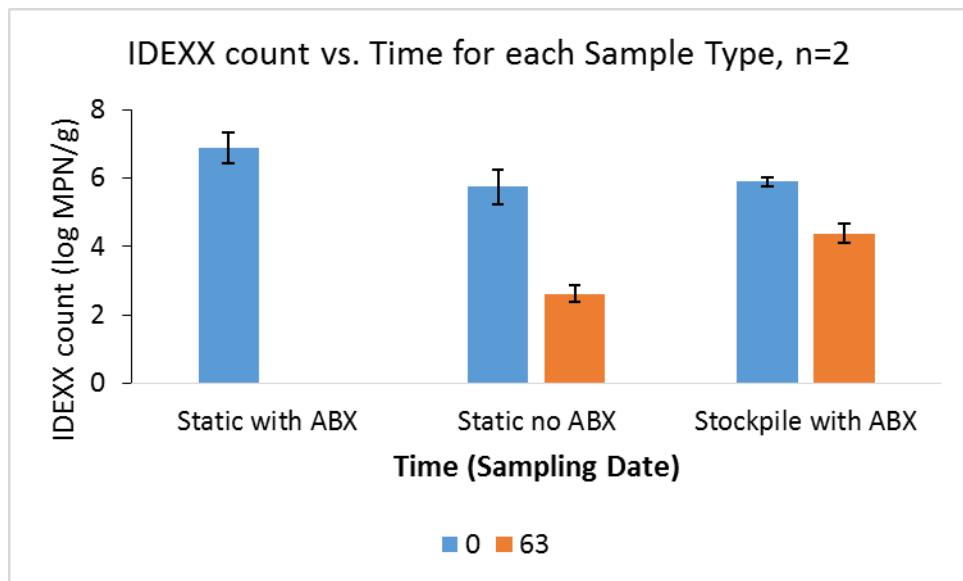
Figure 3.17. Abundance of *E. coli* measured using IDEXX with Colilert. Time was a significant factor impacting change in abundance (p=0.0201; Kruskal-Wallis). Neither composting method nor prior antibiotic administration significantly impacted abundance on day 0 (p=0.7963 and p=0.3017, respectively; Kruskal-Wallis), but both factors had a significant impact by day 63 (p<0.0001; Kruskal-Wallis).

3.2. QUANTITATIVE POLYMERASE CHAIN REACTION (QPCR)

3.2.1. SMALL-SCALE COMPOSTING

QPCR of small scale composting was performed to quantify *sul*1 and *tet*(W) gene copies (Figures 3.18 − 3.25). Absolute *sul*1 gene abundance (Figures 3.18 and 3.19) increased significantly with time (p<0.0001; Kruskal-Wallis), but there was no impact of manure type or significant difference between static and turned composting methods. Relative abundance of *sul*1, normalized to 16S rRNA genes, varied with time, with day 42 having a significantly higher relative abundance than on day 0 (p<0.0001; Kruskal-Wallis).

Absolute *tet*(W) gene abundance decreased with time (p<0.0001; Kruskal-Wallis) and there was no significant difference among composting methods. Manure type was a significant factor in day 0 *tet*(W) abundance, with beef manures containing more *tet*(W) gene copies than dairy manures (p<0.0001; Kruskal-Wallis). Antibiotic treatment had no effect. Relative abundance of *tet*(W), normalized to 16S rRNA gene abundances, decreased rapidly with time (p<0.0001;

Kruskal-Wallis) and static composting was associated with a slightly reduced relative *tet*(W) abundance as compared to turned composting (p=0.0243; Kruskal-Wallis). Manure type significantly influenced relative abundance of *tet*(W) on day 0, with beef manure showing higher abundances than dairy (p<0.0001; Kruskal-Wallis), but no effect of antibiotic treatment.
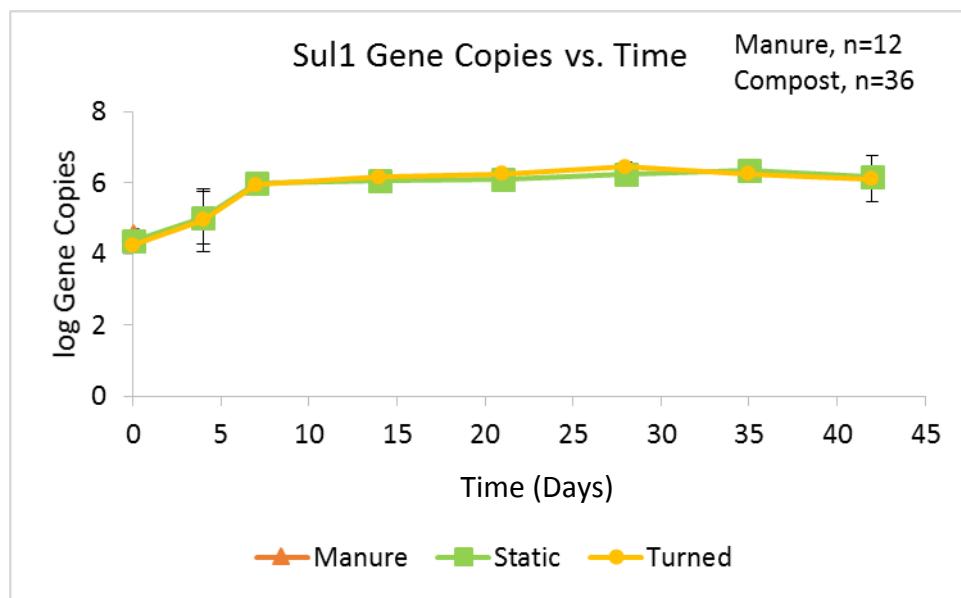

Figure 3.18. Abundance of *sul*1 genes over time, by composting method. *Sul*1 abundance increased significantly with time (p<0.0001; Kruskal-Wallis). Error bars represent standard deviation. *Sul*1 abundance on day 42 compost samples were significantly increased compared to day 0 raw manure (p<0.0001; Kruskal-Wallis); no significant difference between static and turned composting was observed (p=0.7242; Kruskal-Wallis).
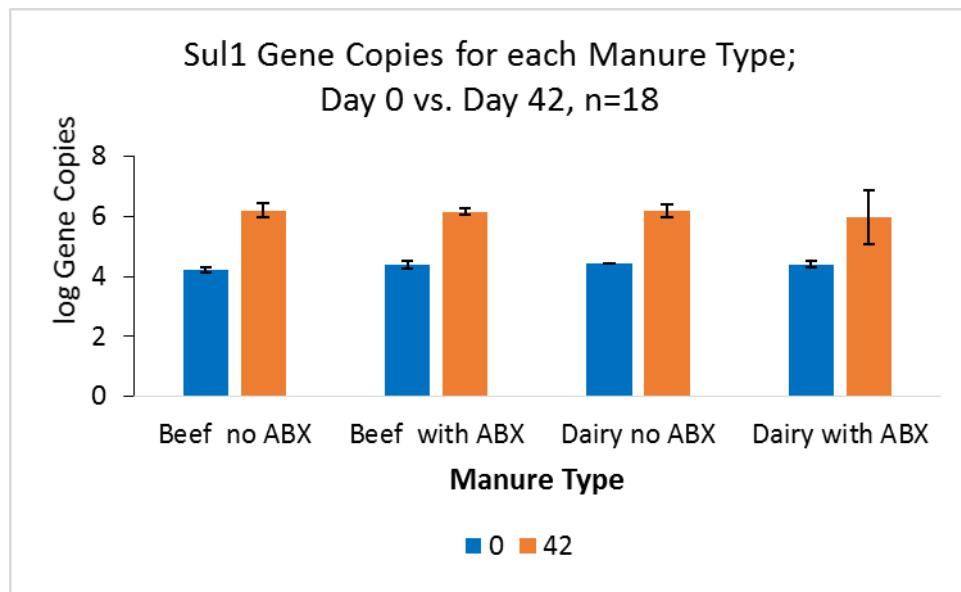
Figure 3.19. Comparison of *sul*1 gene abundance on day 0 with that on day 42, by manure type subject to composting. Manure type did not significantly influence *sul*1 gene abundance on day 0 or day 42 (p=0.1507 and p=0.8948, respectively; Kruskal-Wallis). *Sul*1 gene abundance increased significantly for each manure type by day 42 relative to day 0 (p<0.0001; Kruskal-Wallis).
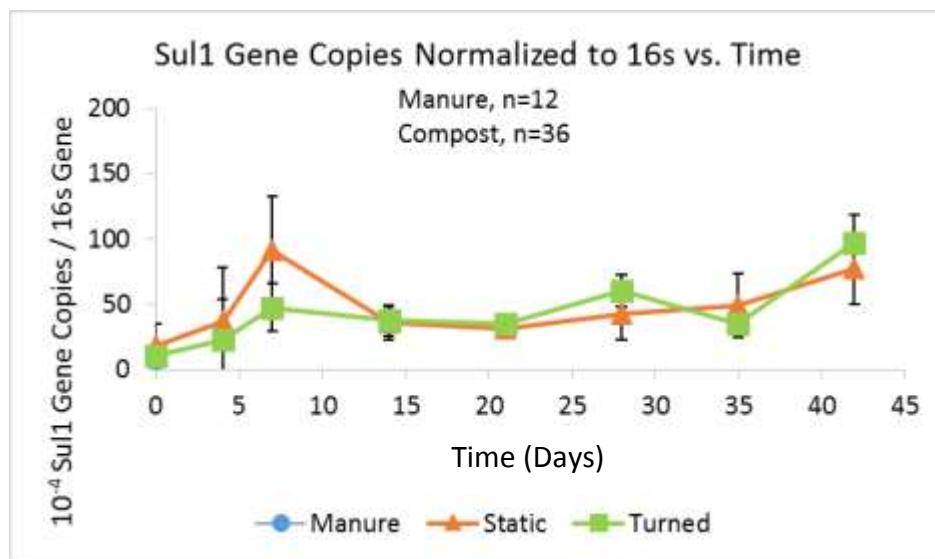

Figure 3.20. Relative abundance of *sul*1 genes to 16S genes over time, by composting method. Relative abundance varies significantly with respect to time (p<0.0001; Kruskal-Wallis). Composting was associated with a significant increase in relative abundance on day 42 with

respect to raw manure on day 0 (p<0.0001; Kruskal-Wallis). There is no significant difference between static and turned composting methods on day 42 (p=0.5657; Kruskal-Wallis).
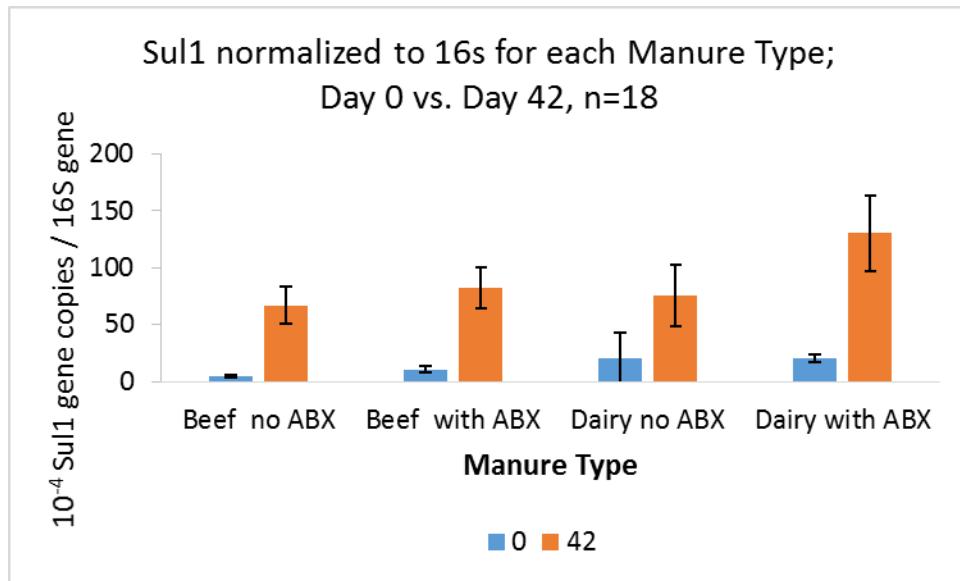

Figure 3.21. Comparison of relative abundance of *sul*1 to 16S rRNA genes on day 0 with that on day 42, by manure type subject to composting. On day 0, manure type significantly impacts relative *sul*1 abundance with dairy manures having a higher relative abundance than beef manures (p<0.0001; Kruskal-Wallis). On day 42, there is no significant impact of manure type on relative abundance (p=0.1443; Kruskal-Wallis). Relative *sul*1 abundance is significantly increased on day 42 with respect to day 0 for all manure types (p<0.0001; Kruskal-Wallis).
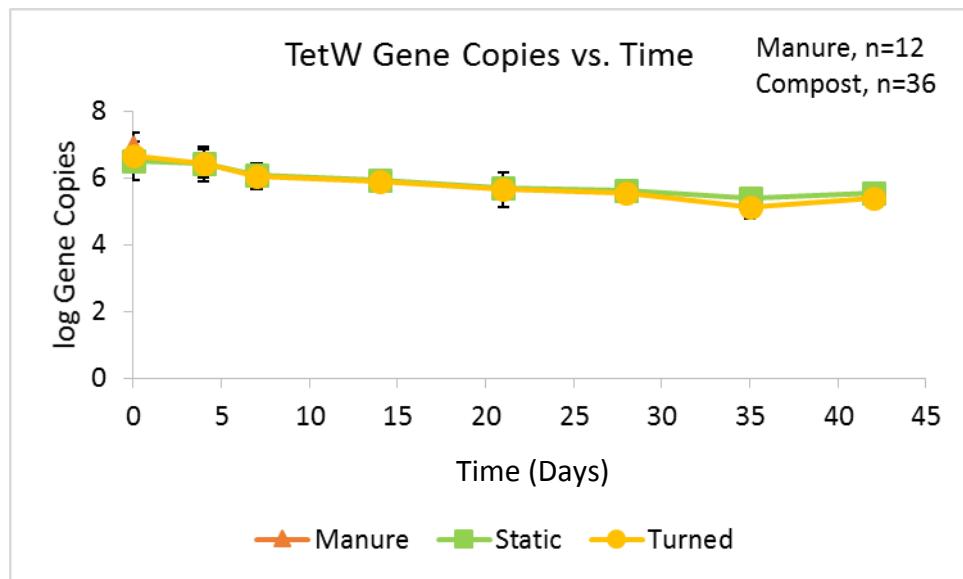
Figure 3.22. Abundance of *tet*(W) genes over time, by composting method. *Tet*(W) abundance decreased significantly with time (p<0.0001; Kruskal-Wallis). *Tet*(W) abundance in day 42 compost samples were significantly decreased compared to day 0 raw manure (p<0.0001; Kruskal-Wallis); no significant difference between static and turned composting was observed (p=0.5204; Kruskal-Wallis).
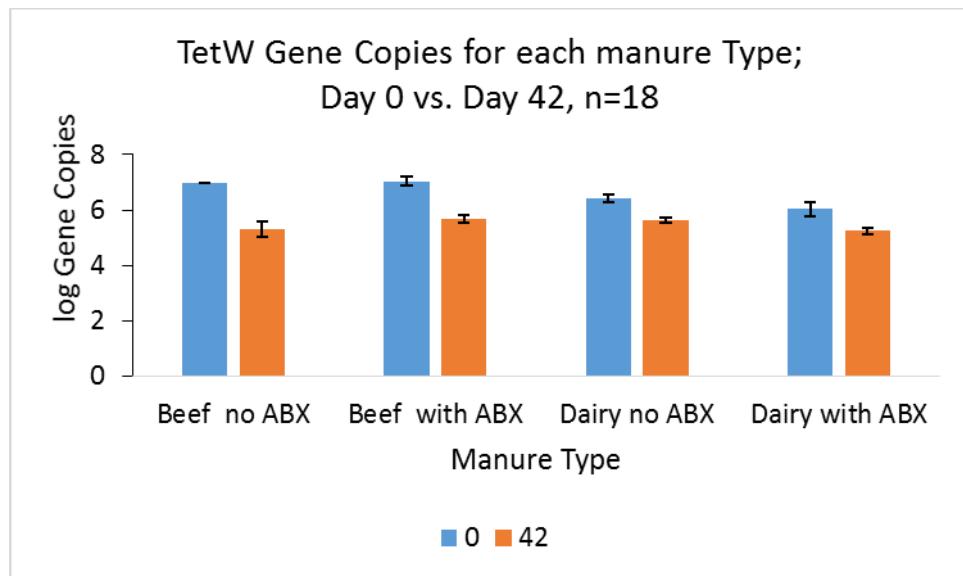

Figure 3.23. Comparison of *tet*(W) gene abundance on day 0 with that on day 42, by manure type subject to composting. Manure type had a significant impact *tet*(W) gene abundance on day 0 and

on day 42 (p<0.0001 each; Kruskal-Wallis). *Tet*(W) gene abundance decreased significantly for each manure type by day 42 relative to day 0 (p<0.0001; Kruskal-Wallis).
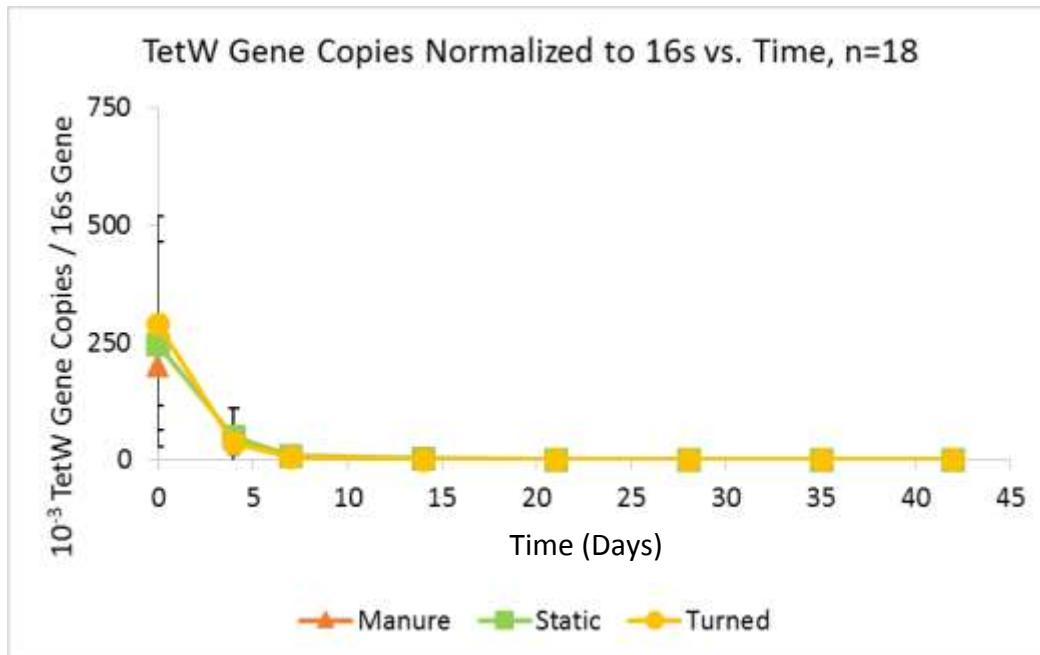

Figure 3.24. Relative abundance of *tet*(W) genes, normalized to 16S rRNA genes, over time, by composting method. Relative abundance varies significantly with respect to time (p<0.0001; Kruskal-Wallis). Composting was associated with a significant decrease in relative abundance on day 42 with respect to raw manure on day 0 (p<0.0001; Kruskal-Wallis). There was also a significant difference between static and turned composting methods on day 42 (p=0.0243; Kruskal-Wallis). Relative *tet*(W) abundance declined at rates of 0.4723 day^{-1} and 0.5321 day^{-1} for static and turned composting methods, respectively, over the first seven days of composting.
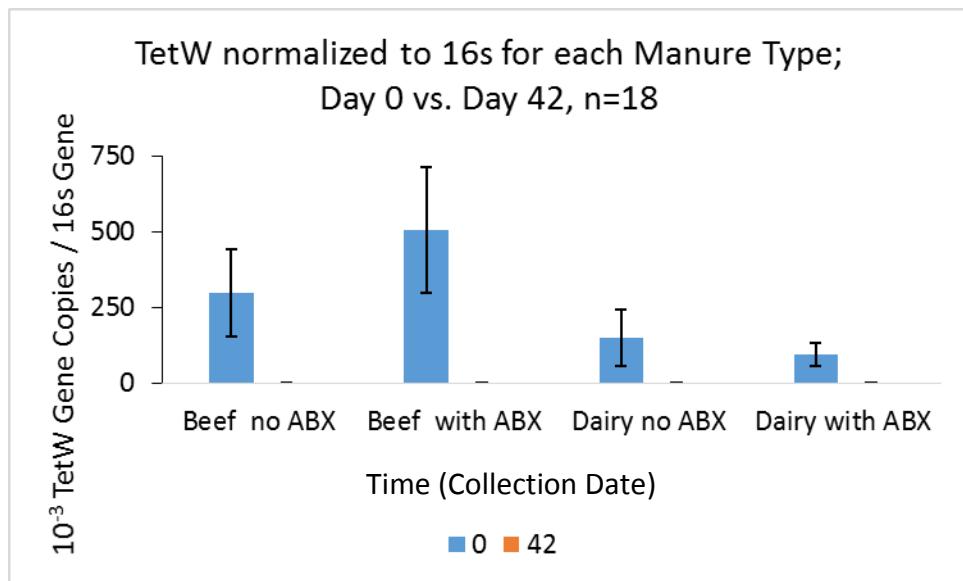
Figure 3.25. Comparison of relative abundance of *tet*(W) to 16S rRNA genes on day 0 with that on day 42, by manure type subject to composting. On day 0, manure type significantly impacted relative *tet*(W) abundance, with beef manures having a higher relative abundance than dairy manures (p<0.0001; Kruskal-Wallis). There was also a significant impact of manure type on relative *tet*(W) abundance on day 42 (p<0.001; Kruskal-Wallis). Relative *tet*(W) abundance significantly decreased by day 42 with respect to day 0 for all manure types (p<0.0001; Kruskal-Wallis).

3.2.2. HEAT CONTROLLED COMPOSTING

QPCR data for *sul*1 and *tet*(W) ARGs in heat-controlled composting samples are shown in Figures 3.26 – 3.29. Absolute *sul*1 abundance (Figure 3.26) varied significantly with time (p<0.0001; Kruskal-Wallis) and increased by 0.5 log on day 52 relative to day 0. Antibiotic treatment was not a significant factor. Relative *sul*1 abundance (Figure 3.27) also varied with time with an increase on day 42 compared to day 0 (p<0.0001; Kruskal-Wallis).

Absolute *tet*(W) abundance decreased with time (p<0.0001; Kruskal-Wallis) and antibiotic treatment resulted in a significant increase in detected *tet*(W) gene copies (p=0.0129; Kruskal-Wallis) (Figure 3.28). Relative *tet*(W) abundance decreased rapidly with time (p<0.0001; Kruskal-Wallis) and antibiotic treatment resulted in higher relative *tet*(W) abundance than manure from untreated cattle (p=0.0413; Kruskal-Wallis) (Figure 3.29).

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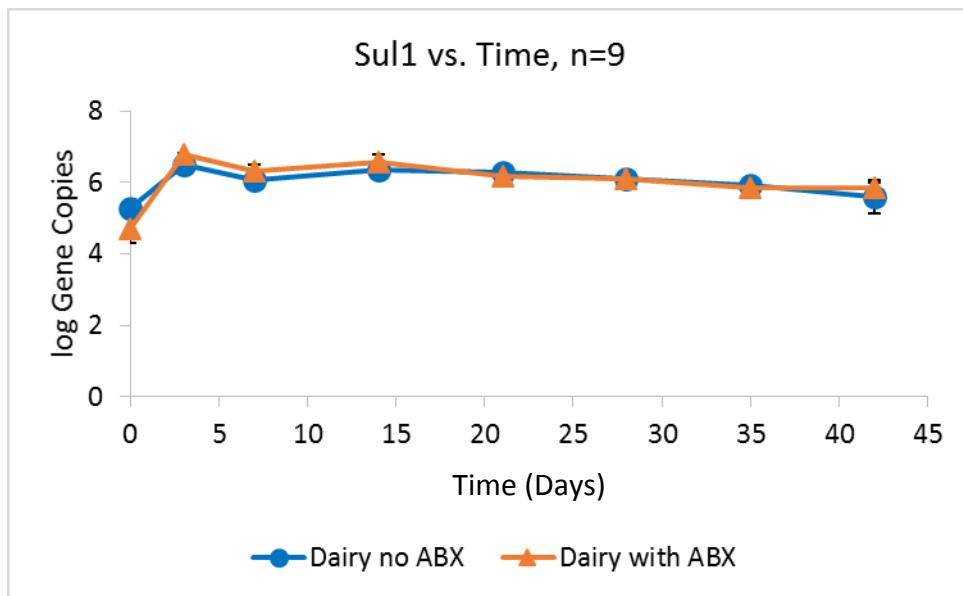
Figure 3.26. Abundance of *sul*1 genes over time by manure type. Abundance is significantly impacted by time (p<0.0001; Kruskal-Wallis). Antibiotic treatment does not have a significant impact. (p=0.4157; Kruskal-Wallis).
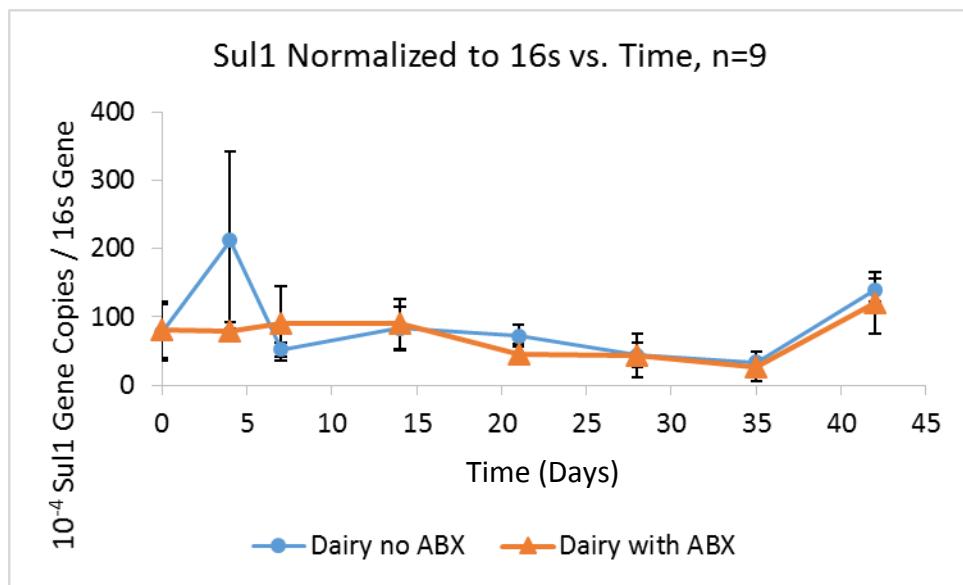
**Figure 3.27.** Relative abundance of *sul*1, normalized to 16S rRNA genes, over time by manure type subject to composting. Abundance is significantly impacted by time (p<0.0001; Kruskal-Wallis). There is no significant impact of antibiotic treatment (p=0.2952; Kruskal-Wallis).
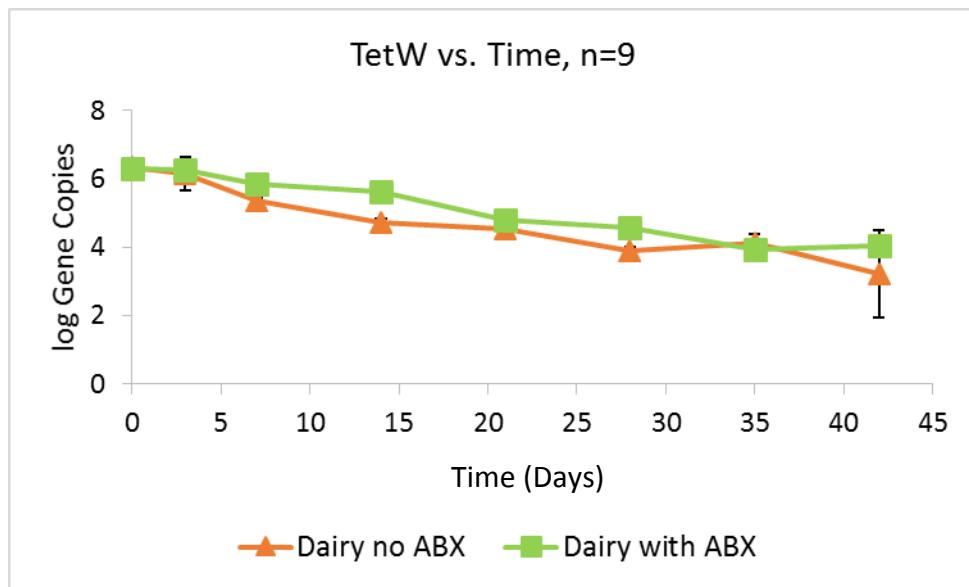
Figure 3. 28. Abundance of *tet*(W) over time by manure type subject to composting. Time had a significant effect on *tet*(W) abundance (p<0.0001; Kruskal-Wallis), as did antibiotic treatment (p=0.0129; Kruskal-Wallis).
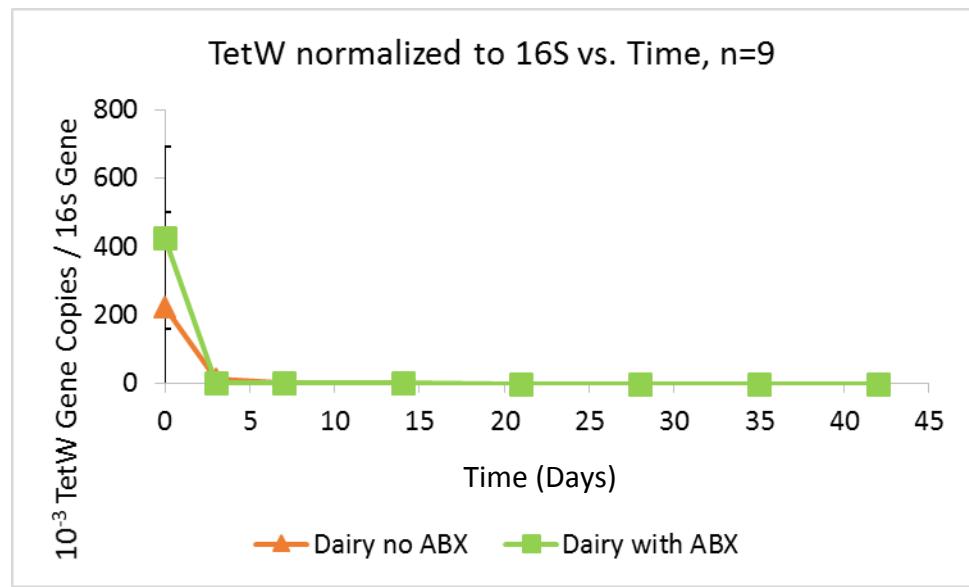
Figure 3.29. Relative abundance of *tet*(W), normalized to 16S rRNA genes, by manure type subject to composting. Time and antibiotic treatment each had a significant impact on relative *tet*(W) abundance (p<0.0001 and p=0.0413, respectively; Kruskal-Wallis). Relative *tet*(W) abundance declined at rates of 0.7725 day^{-1} and 0.7171 day^{-1} for antibiotic treated and untreated composting methods, respectively, over the first seven days of composting.

3.2.3. LARGE-SCALE COMPOSTING

QPCR data for *sul*1 and *tet*(W) gene abundances during large-scale composting are shown in Figures 3.30 – 3.33. Absolute *sul*1 abundance significantly increased when composting, as opposed to stockpiling, manure (Figure 3.30; $p < 0.0001$; Kruskal-Wallis), but there was no impact of antibiotic treatment in composted manure *sul*1 gene abundance. Relative abundance of *sul*1, normalized to 16s rRNA gene abundance, was also higher in composted samples than in stockpiled samples (Figure 3.31, $p = 0.0056$; Kruskal-Wallis).

By contrast, absolute *tet*(W) gene abundance was reduced with composting, as compared to stockpiled manure (Figure 3.32, $p < 0.0001$; Kruskal-Wallis) and unexpectedly, antibiotic treatment was associated with a decrease in absolute *tet*(W) abundance ($p = 0.0004$; Kruskal-Wallis). Relative abundance of *tet*(W) decreased rapidly with time in composted manure samples ($p < 0.0001$; Kruskal-Wallis) but did not decrease in stockpiled manure samples ($p = 0.1496$; Kruskal-Wallis) (Figure 3.33). Relative *tet*(W) abundance also decreased to a greater extent when cattle had previously been administered antibiotics ($p = 0.0011$; Kruskal-Wallis).
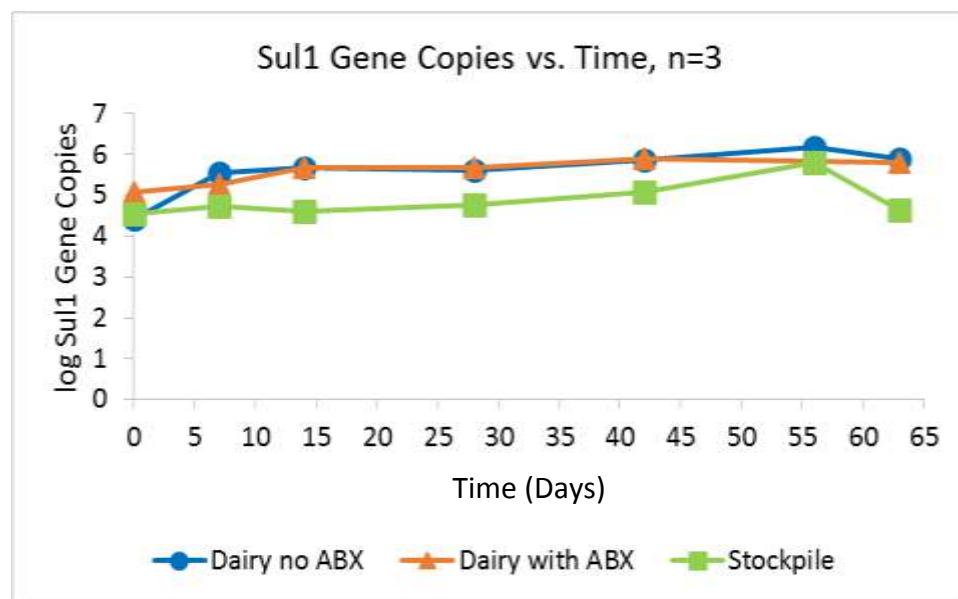
Figure 3.30. Effect of treatment type on abundance of *sul*1 genes with time. There was a significant difference between composted and stockpiled treatments ($p < 0.0001$; Kruskal-Wallis). There was no significant impact of antibiotic treatment on *sul*1 abundance in composted manures ($p = 0.0809$; Kruskal-Wallis). Time had a significant impact ($p = 0.0002$; Kruskal-Wallis).
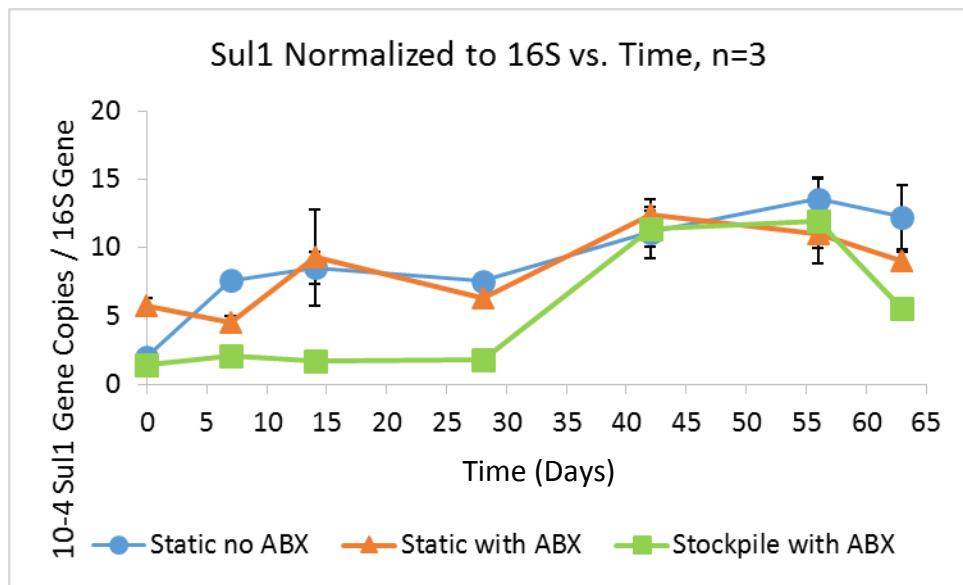
Figure 3.31. Relative abundance of *sul*1, normalized to 16S rRNA genes, over time by treatment type. There was a significant difference between composted and stockpiled manures (p=0.0056; Kruskal-Wallis). There was a significant difference in relative abundance based upon antibiotic treatment of composted manures (p=0.0208; Kruskal-Wallis). Time was also a significant factor in relative *sul*1 abundance (p<0.0001; Kruskal-Wallis).
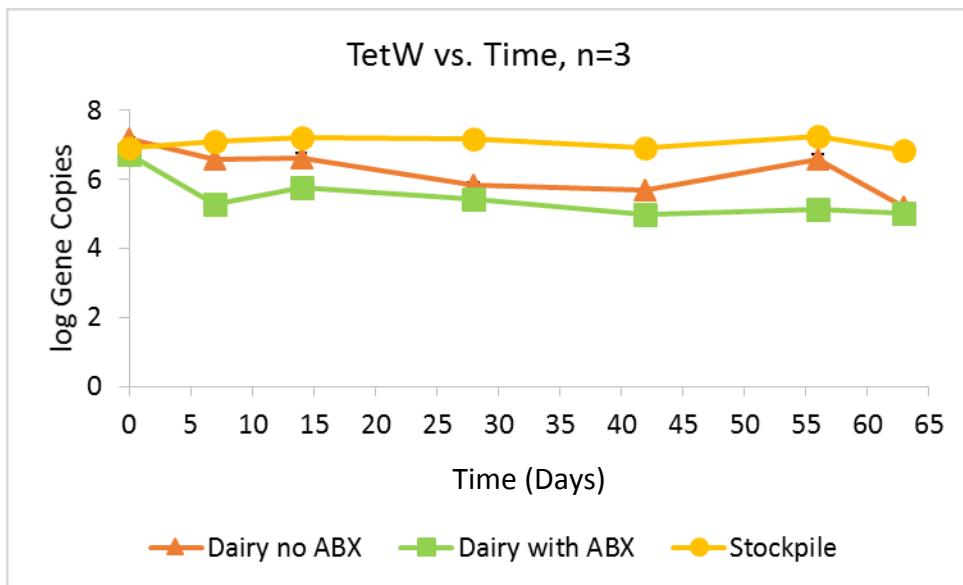
Figure 3.32. Abundance of *tet*(W) with respect to time by treatment type. *Tet*(W) abundance was significantly impacted by time (p=0.0308; Kruskal-Wallis). Composted manures had significantly reduced *tet*(W) abundance compared to stockpiled manure (p<0.0001; Kruskal-Wallis). Antibiotic

treatment of composted manures significantly impacted *tet*(W) abundance in those manures. (p=0.0004; Kruskal-Wallis).
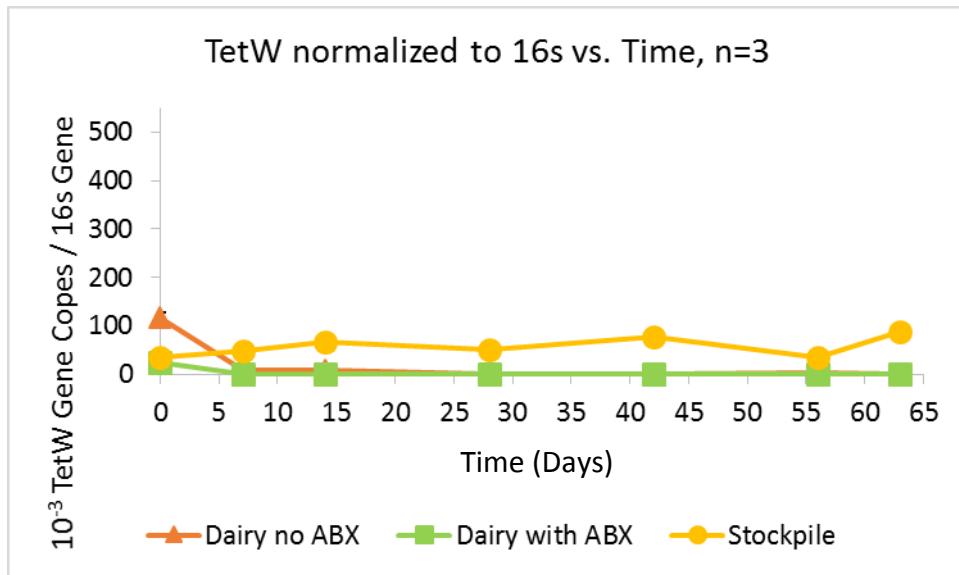
Figure 3.33. Relative abundance of *tet*(W) to 16s genes over time by treatment type. Composting resulted in significantly reduced relative abundance relative to stockpiled manure (p<0.0001; Kruskal-Wallis). Antibiotic treatment significantly impacted relative *tet*(W) abundance in composted manures (p=0.0011; Kruskal-Wallis). Time had a significant impact on relative abundance in composted manures, but not in stockpiled manure (p<0.0001 and p=0.1496, respectively; Kruskal-Wallis).

3.3. METAGENOMIC SEQUENCING

3.3.1 SMALL-SCALE COMPOSTING

Metagenomic sequencing permits the analysis of all genes in an environmental sample without requiring specific targeted primers, providing much more information than qPCR. Results of metagenomic sequencing on small-scale compost samples are shown in Figures 3.34 – 3.40. Figures 3.34 – 3.37 represent metagenomic ARG profiles as stacked bar charts with the relative proportion of each category of ARG shown. On day 0, there is no significant difference between raw manure and the compost mixtures (p=0.7560; ANOSIM) (Figure 3.34), but there was a difference in ARG profiles of beef and dairy manures (p=0.042; ANOSIM). By day 42, the trend

reversed, with composting method significantly impacting ARG profile similarity (p<0.0001; ANOSIM) (Figure 3.36) while manure type no longer had an effect (p=0.385; ANOSIM) (Figure 3.37).

Figures 3.38 – 3.40 display MDS plots based on the Bray-Curtis similarity matrix of ARG abundance profiles for each sample. Samples cluster significantly with respect to time (p<0.001; ANOSIM) (Figure3.38), with day 42 samples clustering with respect to composting method (p=0.012; ANOSIM) (Figure 3.40). There was no apparent effect of manure type by day 42 (p=0.714; ANOSIM) (Figure 3.39).

Figure 3.41 displays the relative abundance of ARGs coding resistance to the four antibiotics considered to be the "highest priority critically important antimicrobials" by the WHO, organized by composting method (58). This data was taken from the metagenomic sequencing results and are based on comparison with the CARD database. Results show that composting increases relative abundance of genes coding resistance to fluoroquinolones (p<0.0001; Kruskal-Wallis) and glycopeptides (p<0.0001; Kruskal-Wallis) by day 42 of composting in both static and turned composts, and a reduction in relative abundance of macrolide resistance genes (p=0.0164; Kruskal-Wallis). Day 42 turned compost had higher abundance of β-lactam resistance genes than static compost (p=0.0102; Kruskal-Wallis).
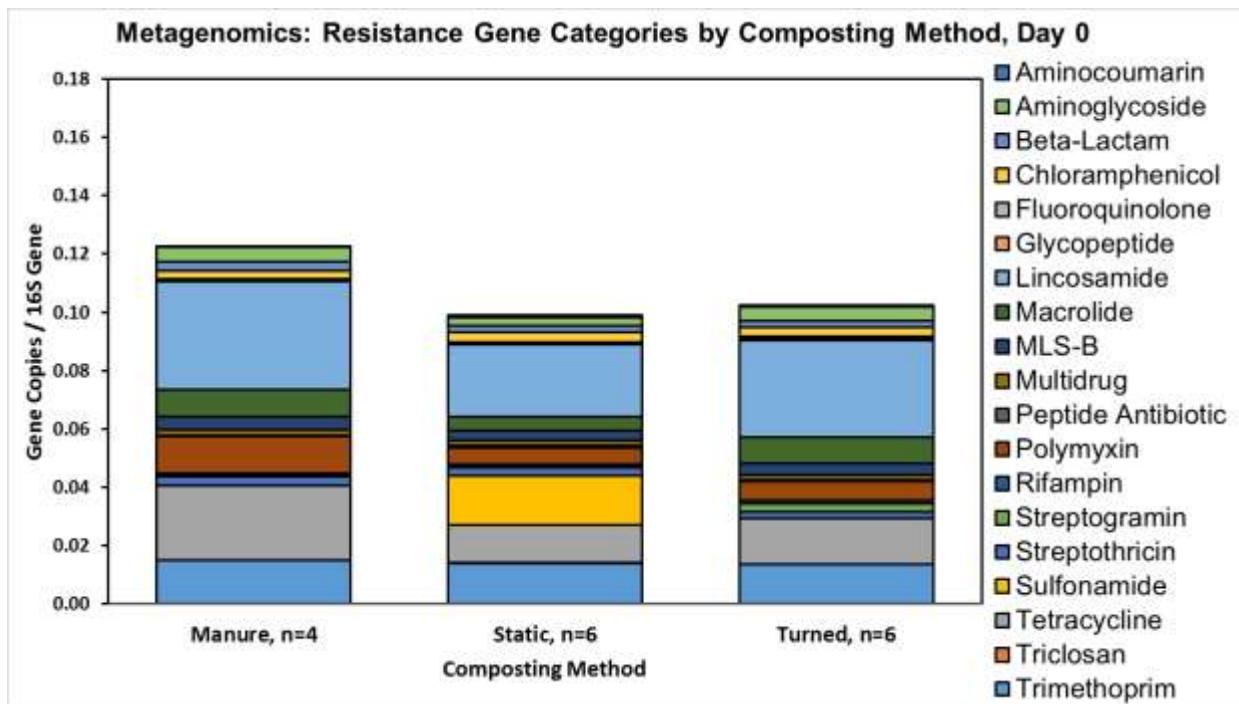
Figure 3.34. Comparison of total resistance gene category abundance by composting method on day 0. Based on metagenomic sequencing and comparison with the CARD database. There is no significant effect of composting method (p=0.7560; ANOSIM).
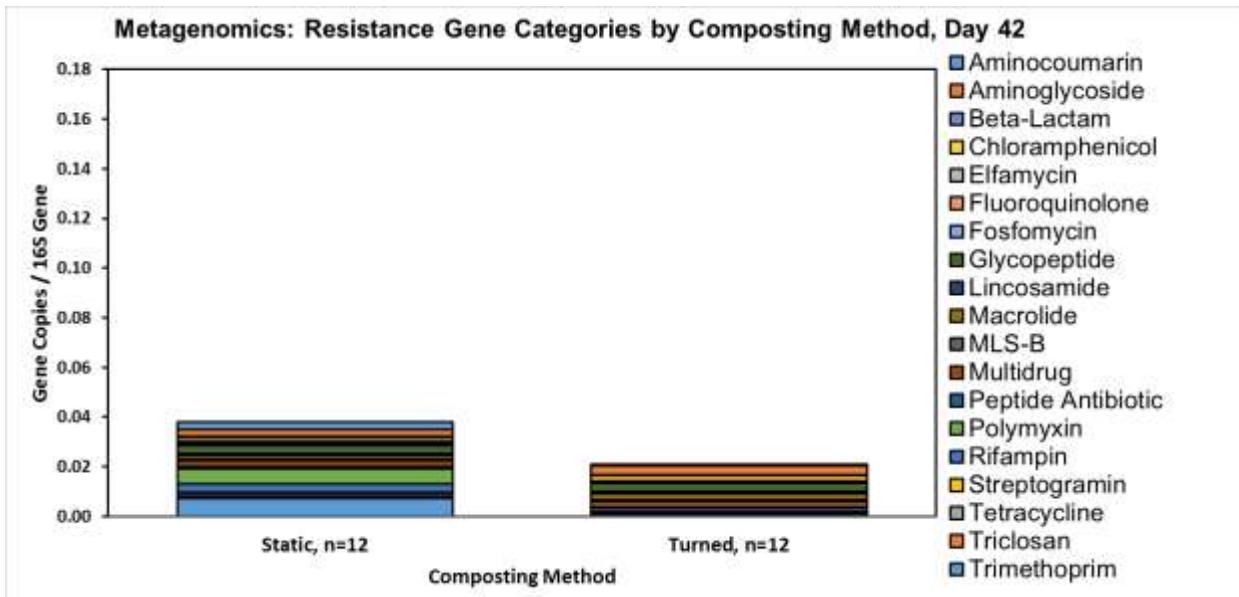

Figure 3.35. Comparison of total resistance gene category abundance by composting method on day 42. Based on metagenomic sequencing and comparison with the CARD database. There is a

significant difference in resistance profile similarity between static and turned composting methods (p<0.0001; ANOSIM).
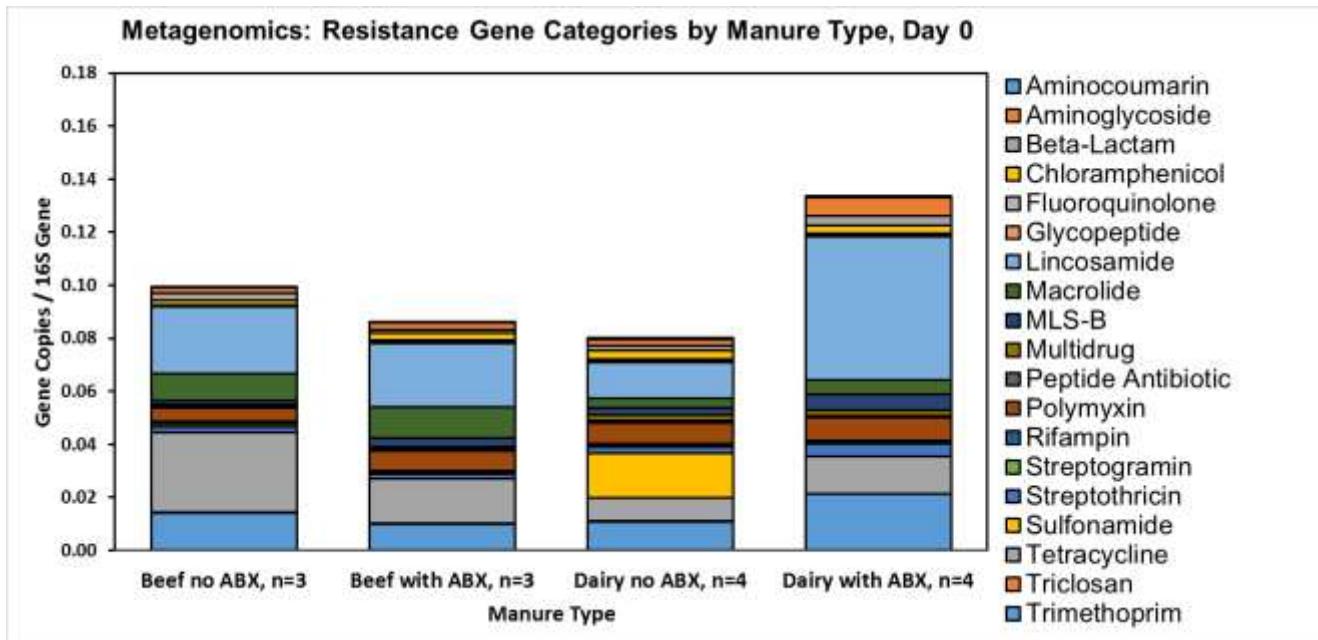
Figure 3.36. Comparison of total resistance gene category abundance by manure type on day 0. Based on metagenomic sequencing and comparison with the CARD database. There is a significant difference between beef and dairy manures (p=0.042; ANOSIM). There is no significant difference between beef with and without antibiotics (p=0.301; ANOSIM) or between dairy with and without antibiotics (p=0.171; ANOSIM).
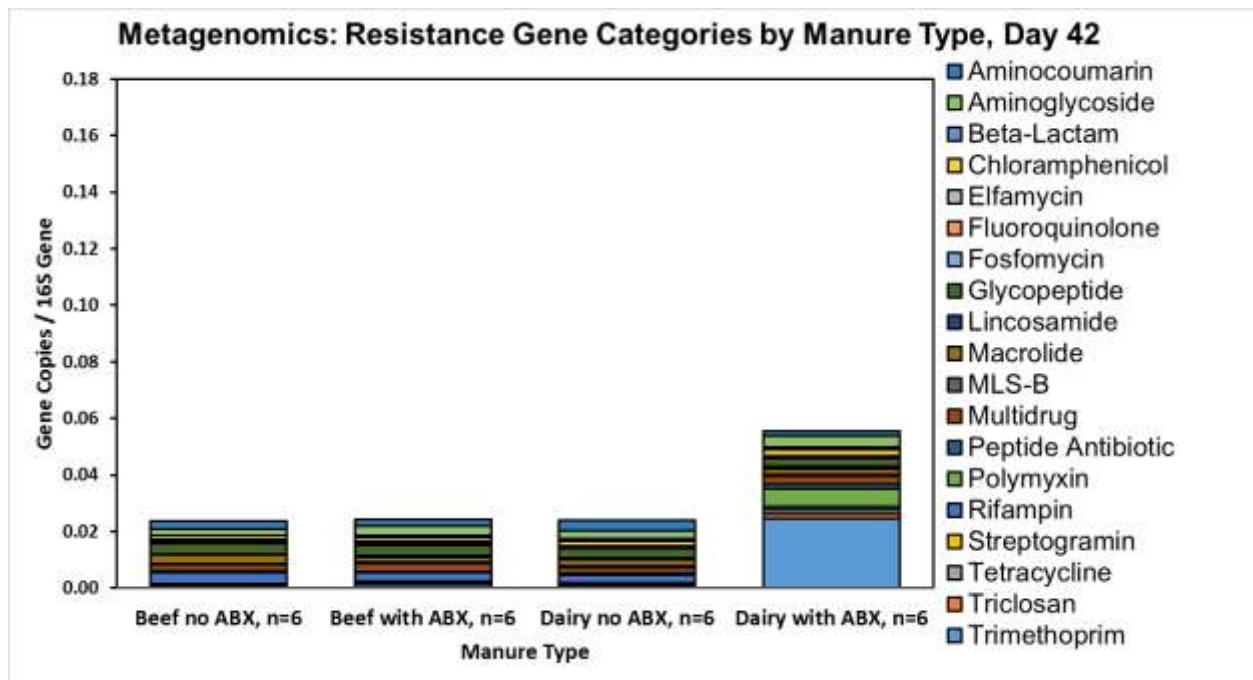
Figure 3.37. Comparison of resistance gene category abundance by manure type on day 42. Based on metagenomic sequencing and comparison with the CARD database. Despite the increase in trimethoprim resistance in antibiotic treated dairy manure composts, there is no significant difference between resistance gene category profiles based upon manure type (p=0.385; ANOSIM). There is no significant difference between dairy and beef (p=0.125; ANOSIM), between dairy with and without antibiotics (p=0.714; ANOSIM), or between beef with and without antibiotics (p=0.136; ANOSIM).
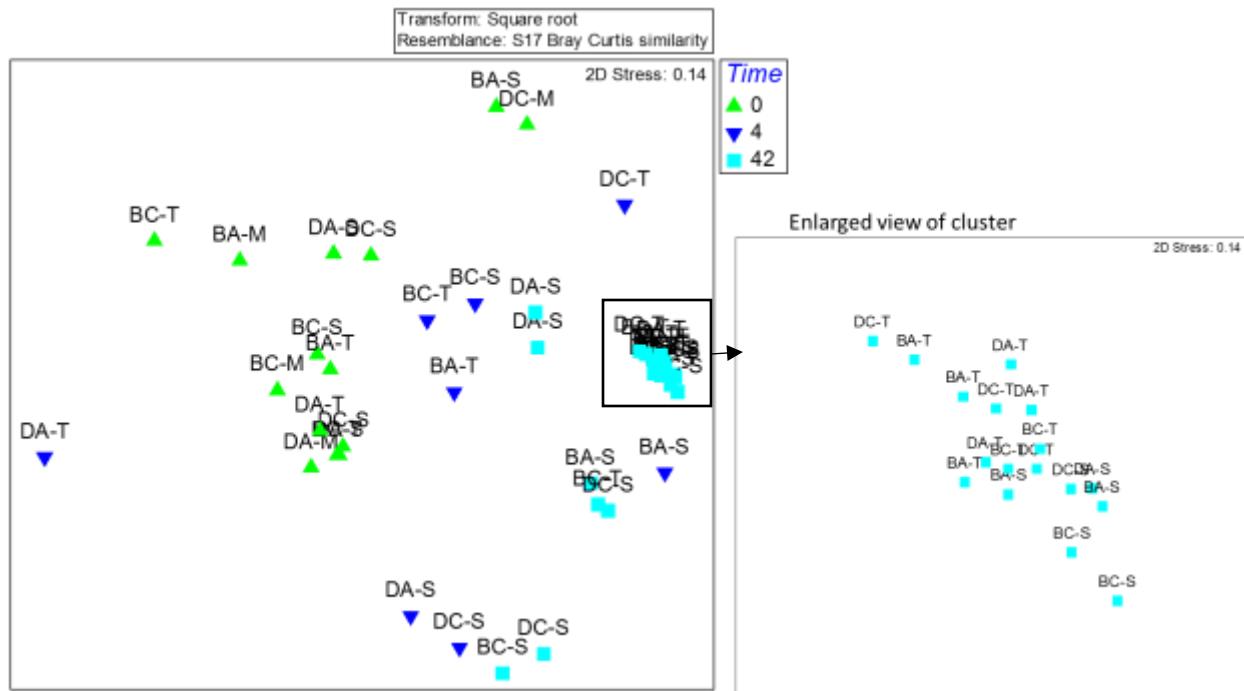
Figure 3.38. MDS plot of ARG profile similarity with respect to time. D=Dairy, B=Beef, A=with ABX, C=no ABX, S=Static, T=Turned, M=Manure (Raw). There is a significant clustering with respect to time (p<0.001; ANOSIM).
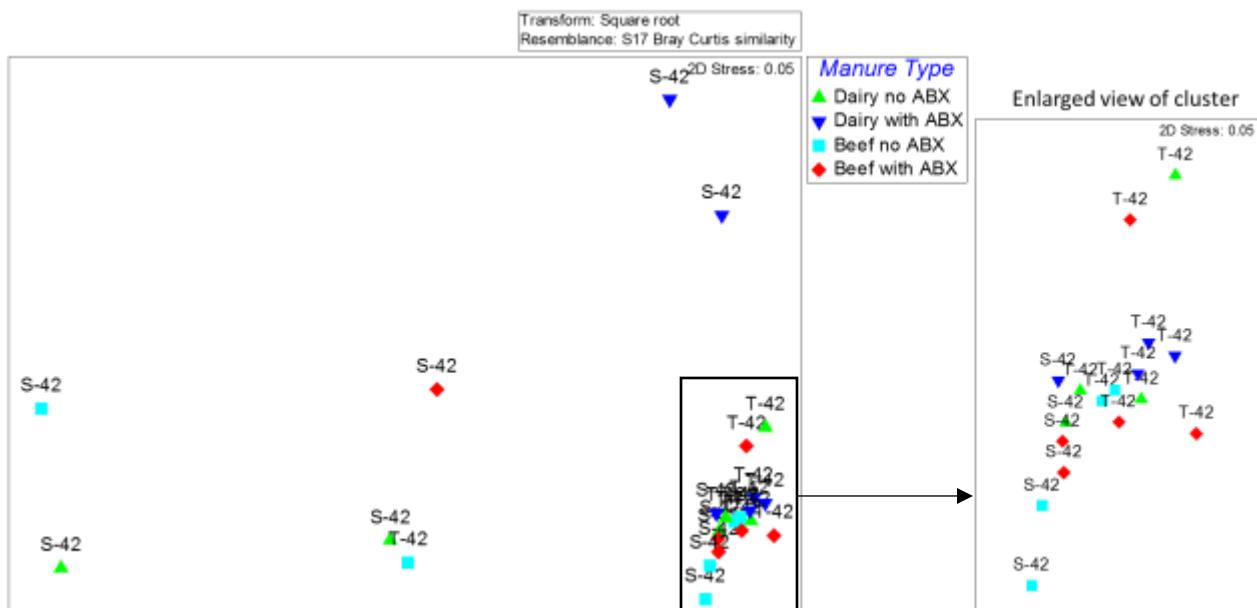
Figure 3.39. MDS plot of ARG profile similarity by manure type, day 42 only. S=Static, T=Turned. There is no significant separation of ARG profiles of dairy cattle manure with or

without antibiotics (p=0.714; ANOSIM), no significant separation of beef with and without antibiotics (p=0.136; ANOSIM), and no significant separation of dairy and beef cattle manure compost ARG similarity profiles overall (p=0.125; ANOSIM).
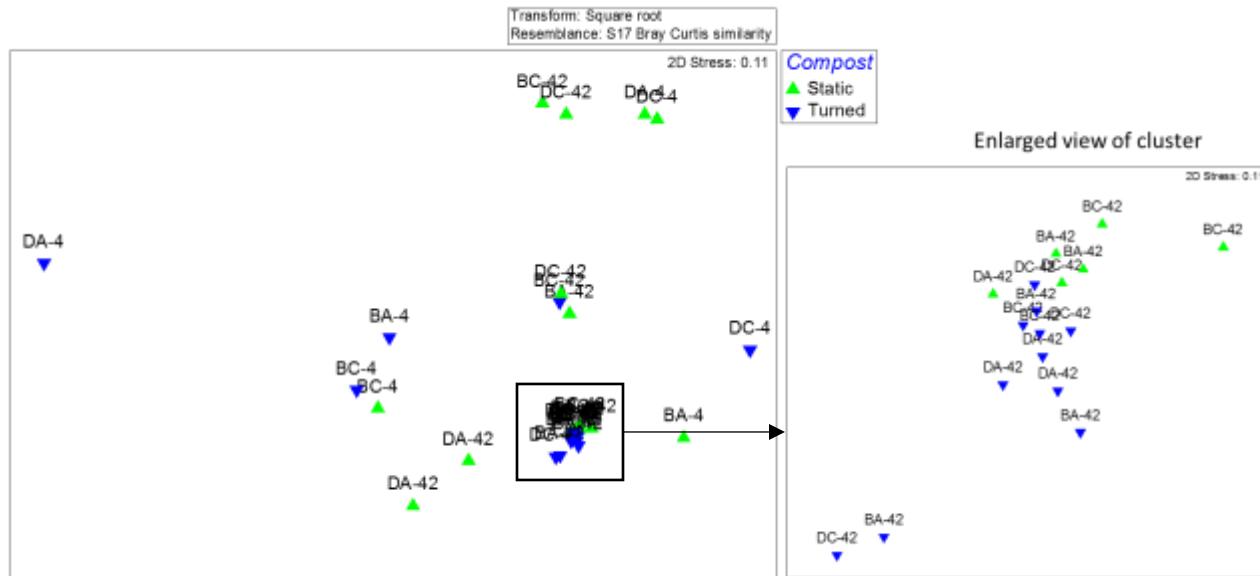
Figure 3.40. MDS plot of ARG profile similarity for day 4 and day 42 labelled by composting method. D=Dairy, B=Beef A=with ABX, C=no ABX. There is significant separation in clustering of statically composted manures and turned composting manures (p=0.021; ANOSIM).
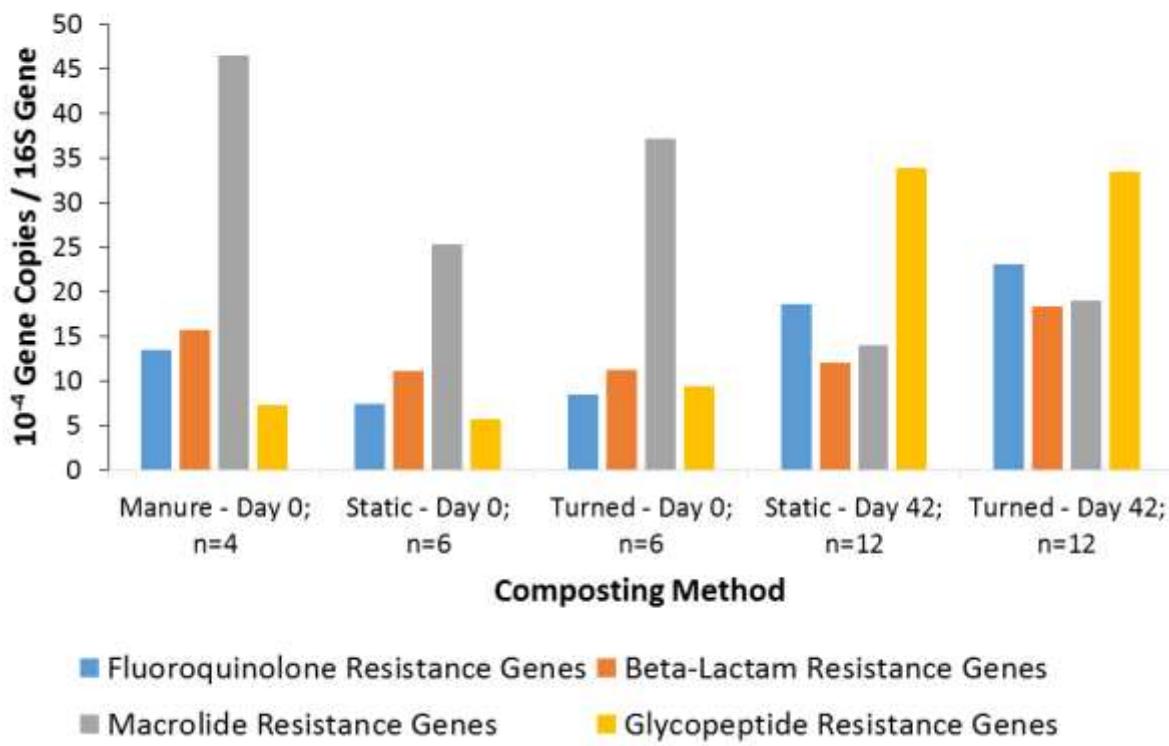
Figure 3.41: Comparison among composting methods of total ARGs of clinical concern based on metagenomic sequencing and comparison with the CARD database, day 0 versus day 42. Here ARGs of clinical concern are those which confer resistance to the antibiotics considered to be the "highest priority critically important antimicrobials" by the WHO (58). Composting significantly increased relative abundance of fluoroquinolone resistance genes ($p<0.0001$; Kruskal-Wallis) and glycopeptide resistance genes ($p<0.0001$; Kruskal-Wallis, but decreased relative abundance of macrolide resistance genes ($p=0.0164$; Kruskal-Wallis) while β-lactam resistance genes were not significantly affected ($p=0.1650$; Kruskal-Wallis). Day 42 turned compost had significantly higher β-lactam relative abundance than day 42 static compost ($p=0.0102$; Kruskal-Wallis).

3.3.2 HEAT CONTROLLED COMPOSTING

Results of metagenomic sequencing on heat controlled compost samples are shown in Figures 3.42 – 3.45. Figures 3.42 and 3.43 show metagenomic ARG profiles as stacked column

charts with the relative proportion of each category of ARG shown; there is no significant effect of antibiotic treatment on either day 0 or day 42.

Figures 3.44 and 3.45 display MDS plots on the Bray-Curtis similarity matrix of ARG abundance profiles for each sample. Samples cluster significantly with respect to time (p<0.001; ANOSIM) (Figure3.44). There is no significant clustering with respect to antibiotic treatment of day 42 manure samples (Figure 3.45).

Figure 3.46 displays the relative abundance of ARGs coding resistance to the four antibiotics considered to be the "highest priority critically important antimicrobials" by the WHO, organized by composting method (58). This data was taken from the metagenomic sequencing results and are based on comparison with the CARD database. Results show that composting increases relative abundance of genes coding resistance to glycopeptides (p<0.0330; Kruskal-Wallis) by day 42 of composting in both manures with and without prior antibiotic administration.
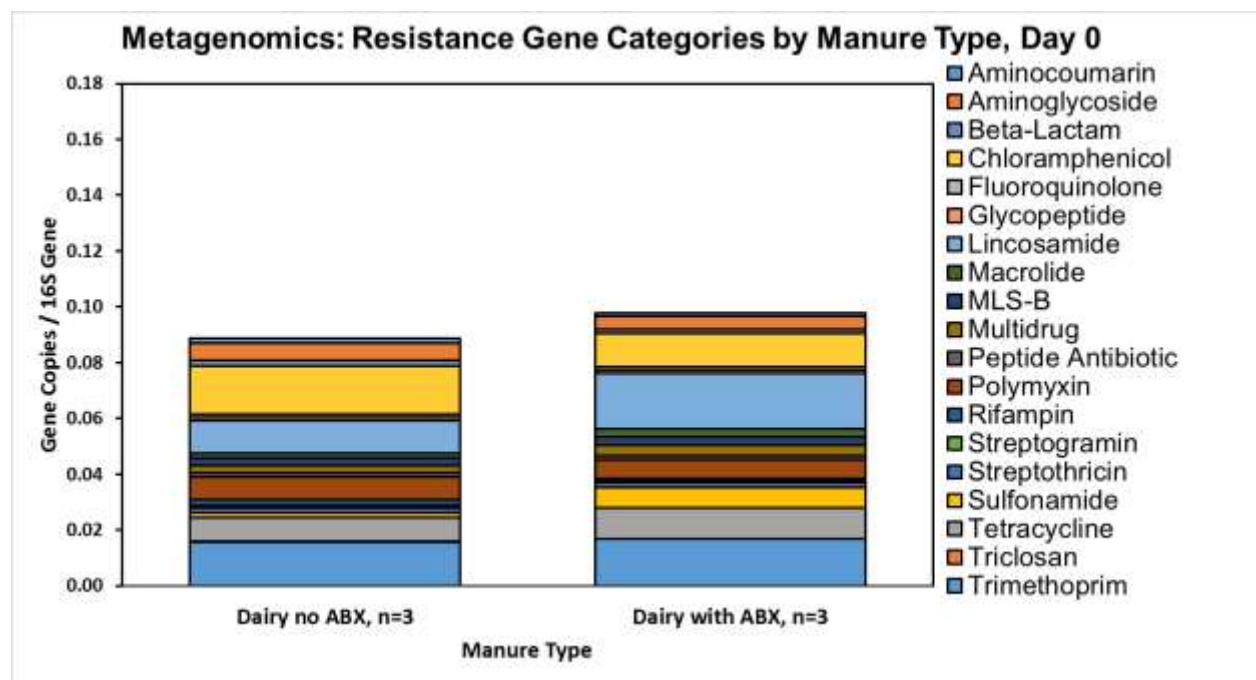
Figure 3.42. Comparison of total resistance gene category abundance by manure type on day 0. Based on metagenomic sequencing and comparison with the CARD database. There is no significant effect of antibiotic treatment (p=1.000; ANOSIM).
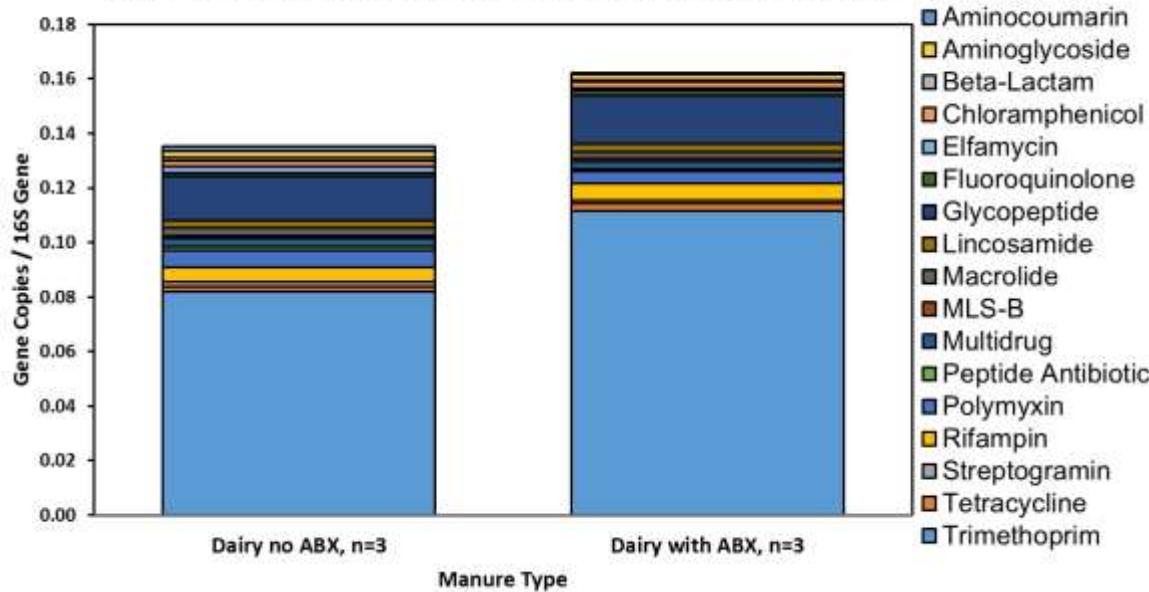
Figure 3.43. Comparison of total resistance gene category abundance by manure type on day 42. Based on metagenomic sequencing and comparison with the CARD database. There is no significant effect of antibiotic treatment (p=0.500; ANOSIM).
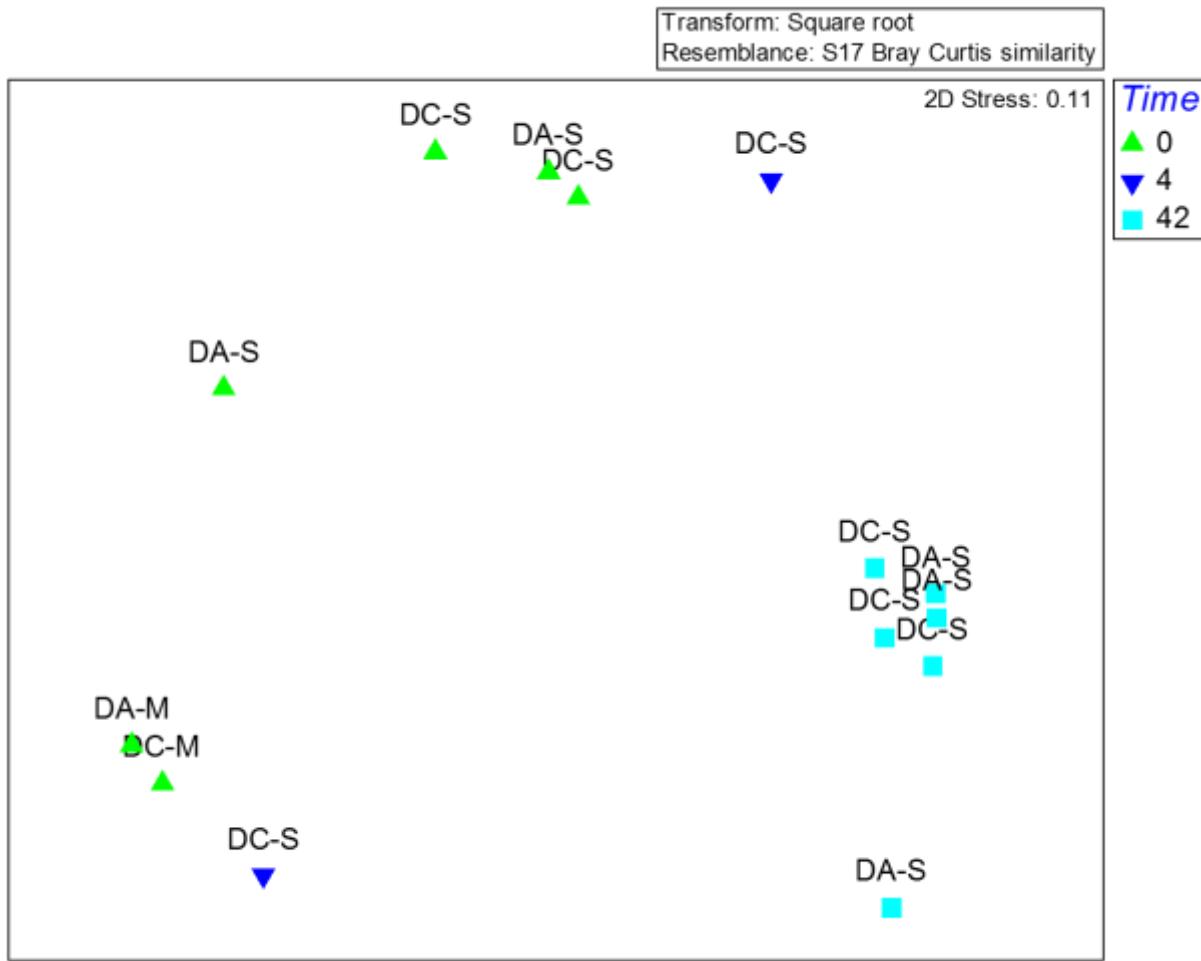
Figure 3.44. MDS plot comparing ARG profile similarity by time. D=Dairy, A=with ABX, C=no ABX, S= Static, M=Manure (Raw). There is significant separation of profile clusters with respect to time (p<0.001; ANOSIM).
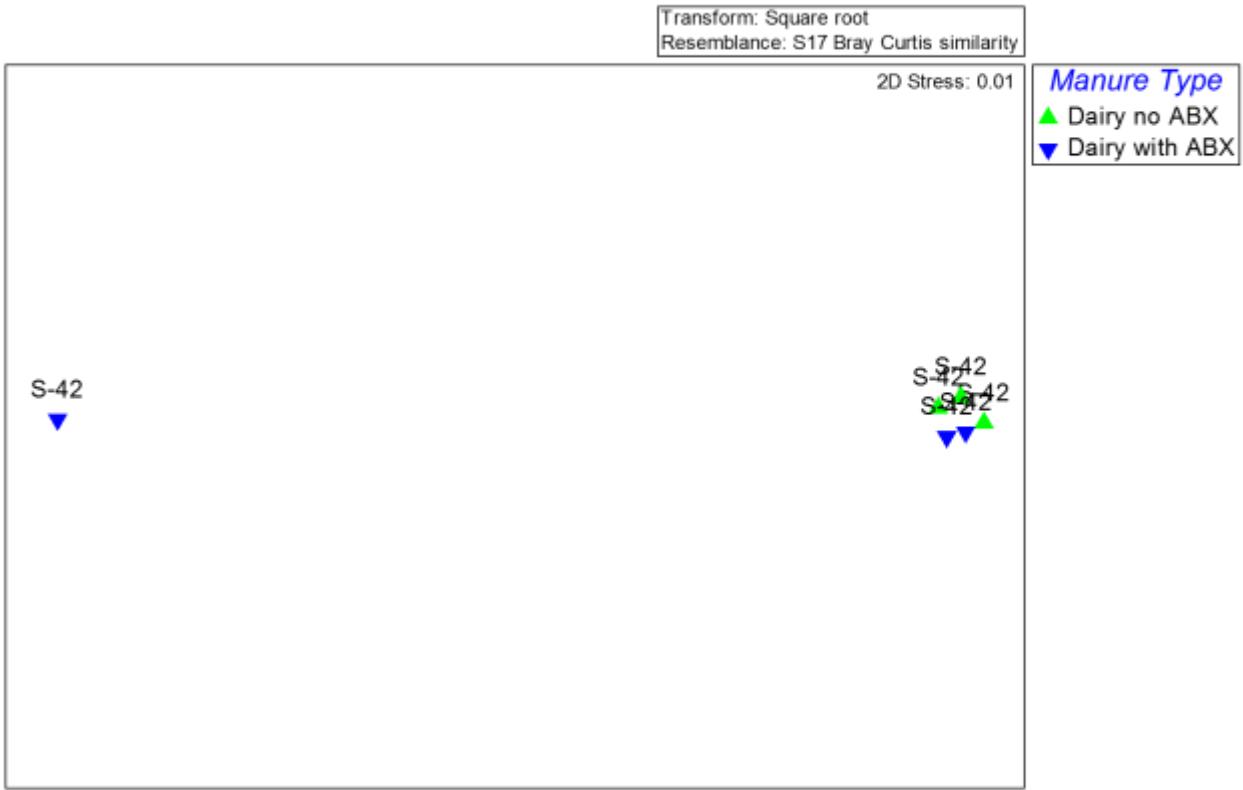
Figure 3.45. MDS plot comparing ARG profile similarity with respect to manure type, day 42 only. S=Static. There is no significant separation of ARG profile clusters with respect to antibiotic treatment (p=0.500; ANOSIM).
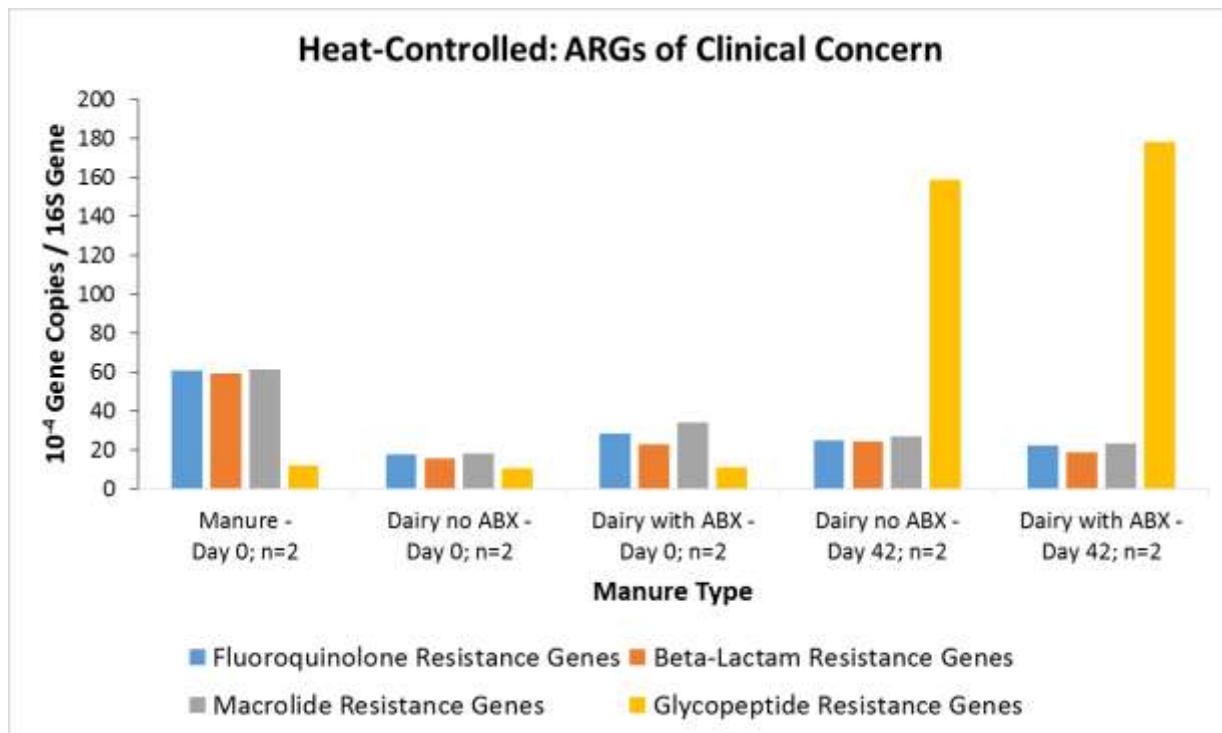
Figure 3.46: Comparison among manure types of total ARGs of clinical concern based on metagenomic sequencing and comparison with the CARD database, day 0 versus day 42. Here ARGs of clinical concern are those which confer resistance to the antibiotics considered to be the "highest priority critically important antimicrobials" by the WHO (58). Composting significantly increased the relative abundance of glycopeptide resistance genes on day 42 compared to day 0 (p=0.0330; Kruskal-Wallis). There was no significant effect of antibiotic administration.

3.4. 16S rRNA GENE AMPLICON SEQUENCING

3.4.1. SMALL-SCALE COMPOSTING

The results of 16S rRNA gene amplicon sequencing and resulting taxonomic analyses for small scale composting are displayed in Figures 3.47 – 3.54. Figures 3.47 – 3.50 show stacked column charts comparing the relative abundance of each of the 9 most common bacterial classes present in the compost and manure samples, with the remaining, less abundant classes combined into the "other" category. There were significant differences in taxonomic composition with respect to time (p<0.001; ANOSIM) (Figure 3.47); there were also differences between day 42 composted manures as compared to day 0 non-composted raw manure (p<0.001; ANOSIM) as

well as differences between taxonomic composition of static and turned composts at day 42 (p<0.001; ANOSIM) (Figure 3.48). Beef and dairy manure composts on day 42 differ significantly in taxonomic composition similarity (p=0.03; ANOSIM) (Figure 3.49). There was also a significant difference in taxonomic composition between the two DNA extraction kits used (p<0.001; ANOSIM) (Figure 3.50).

Figures 3.51 – 3.54 show MDS plots on the unweighted UniFrac distances comparing the taxonomic similarity between samples. Samples cluster significantly with respect to time (p<0.001; ANOSIM) (Figure 3.51). Day 42 compost samples clustered significantly with respect to composting method (p<0.001; ANOSIM) (Figure 3.52). Neither beef nor dairy manure samples clustered significantly with respect to antibiotic treatment (Figure 3.53 and 3.54 for beef and dairy, respectively).
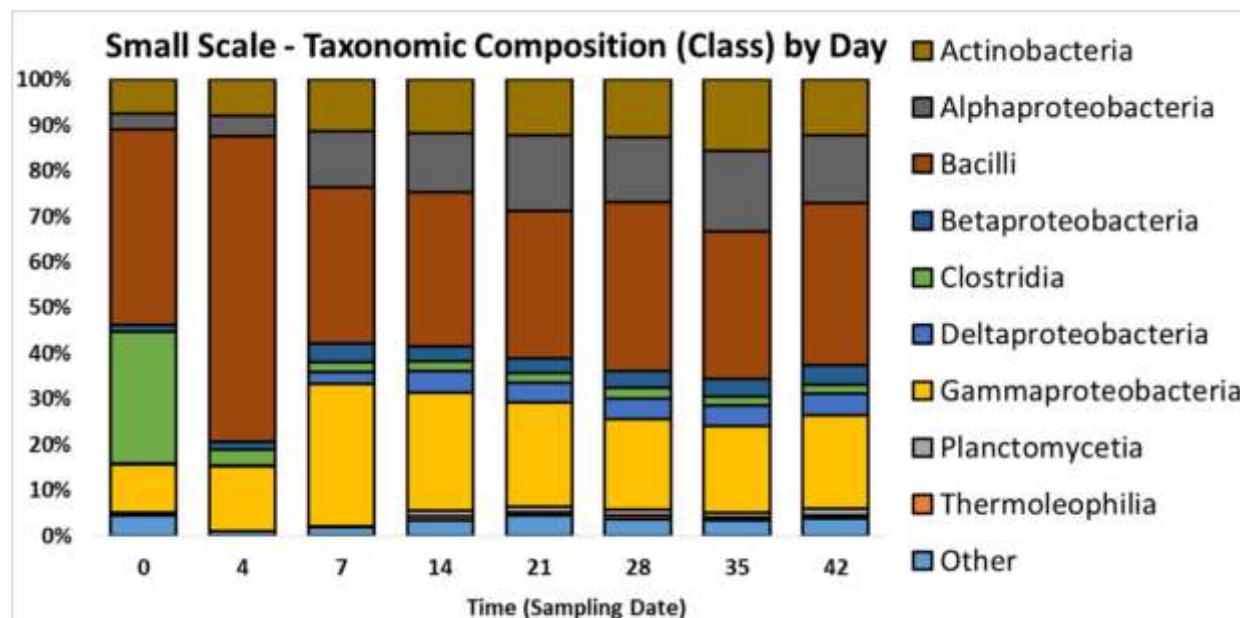
Figure 3.47. Comparison of a class-level taxonomic composition with respect to time. Time has a significant impact on taxonomic composition similarity. (p<0.001; ANOSIM).
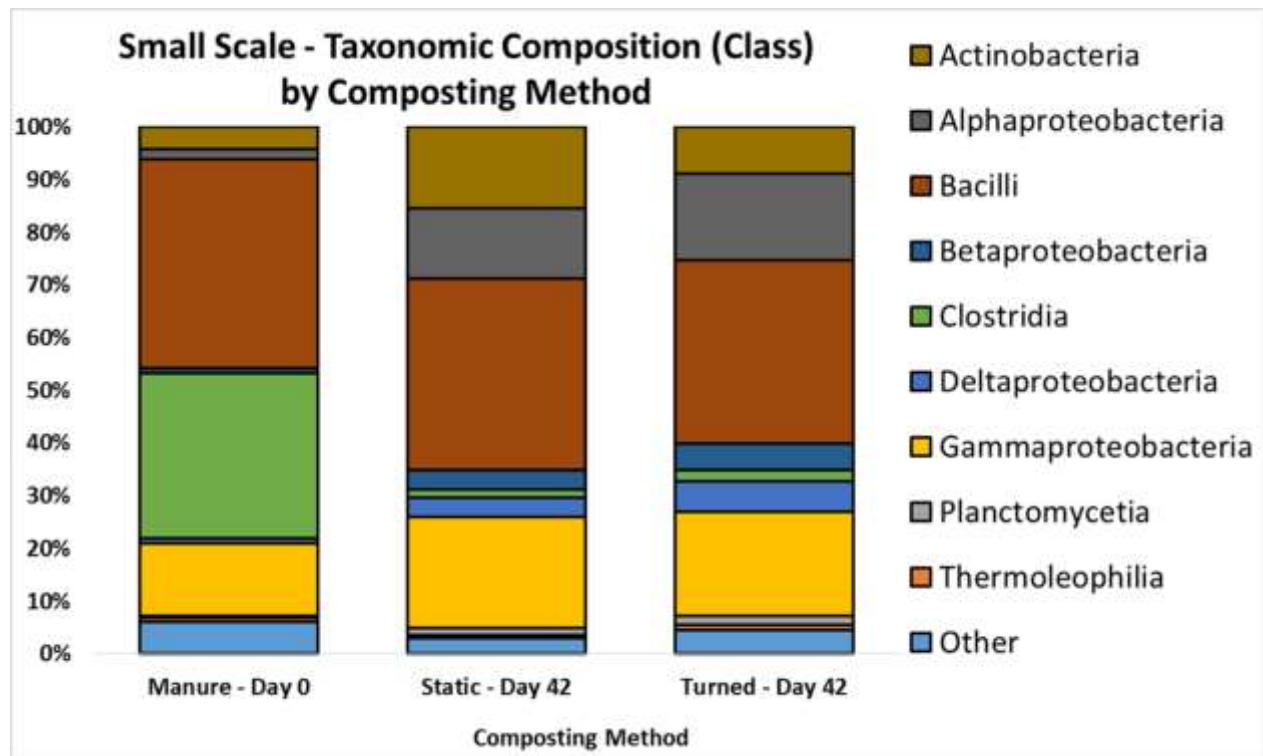
Figure 3.48. Comparison of a class-level taxonomic composition with respect to composting method. Both static and turned composting at day 42 differ significantly with respect to taxonomic composition similarity as compared to raw manure at day 0 (p<0.001; ANOSIM). Taxonomy of finished day 42 static compost also differs significantly from that of finished day 42 turned compost (p<0.001; ANOSIM).
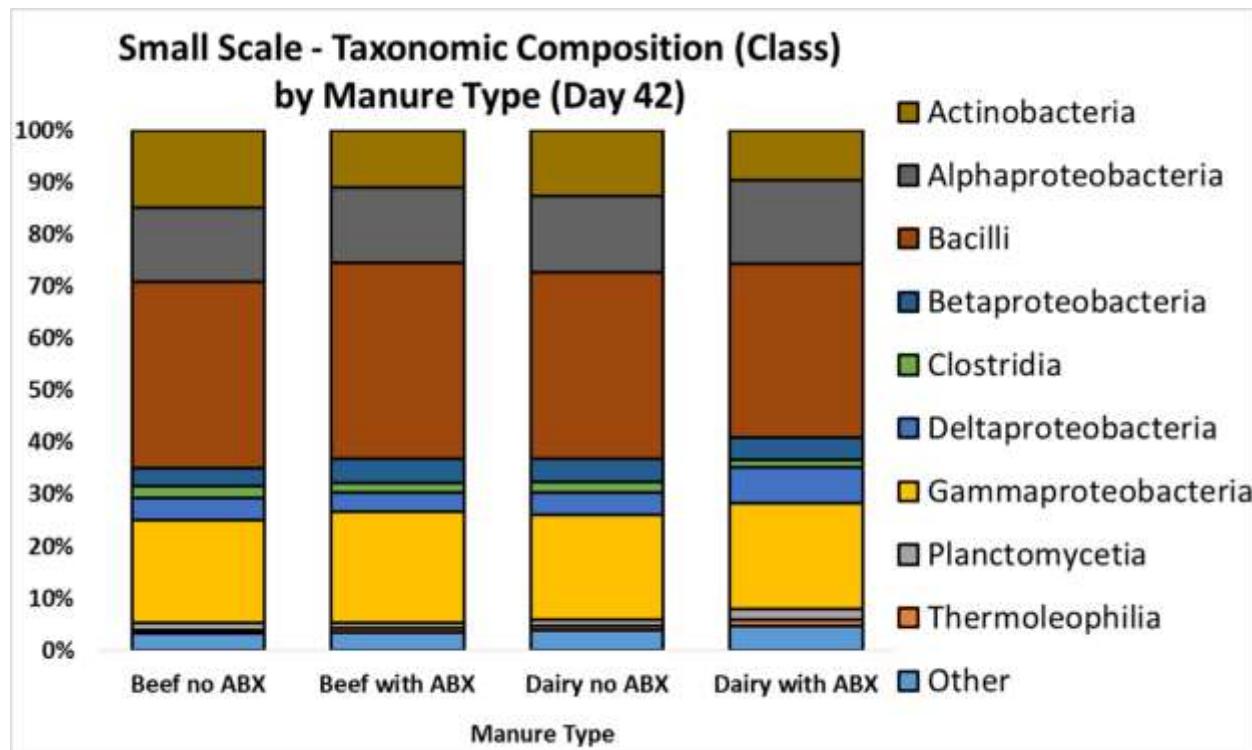
Figure 3.49. Comparison of a class-level taxonomic composition with respect to manure type. There is a significant difference in taxonomic composition similarity between beef and dairy manure composts (p=0.03; ANOSIM) but no difference between beef with or without antibiotic treatment (p=0.513; ANOSIM) or between dairy with or without antibiotic treatment (p=0.221; ANOSIM).
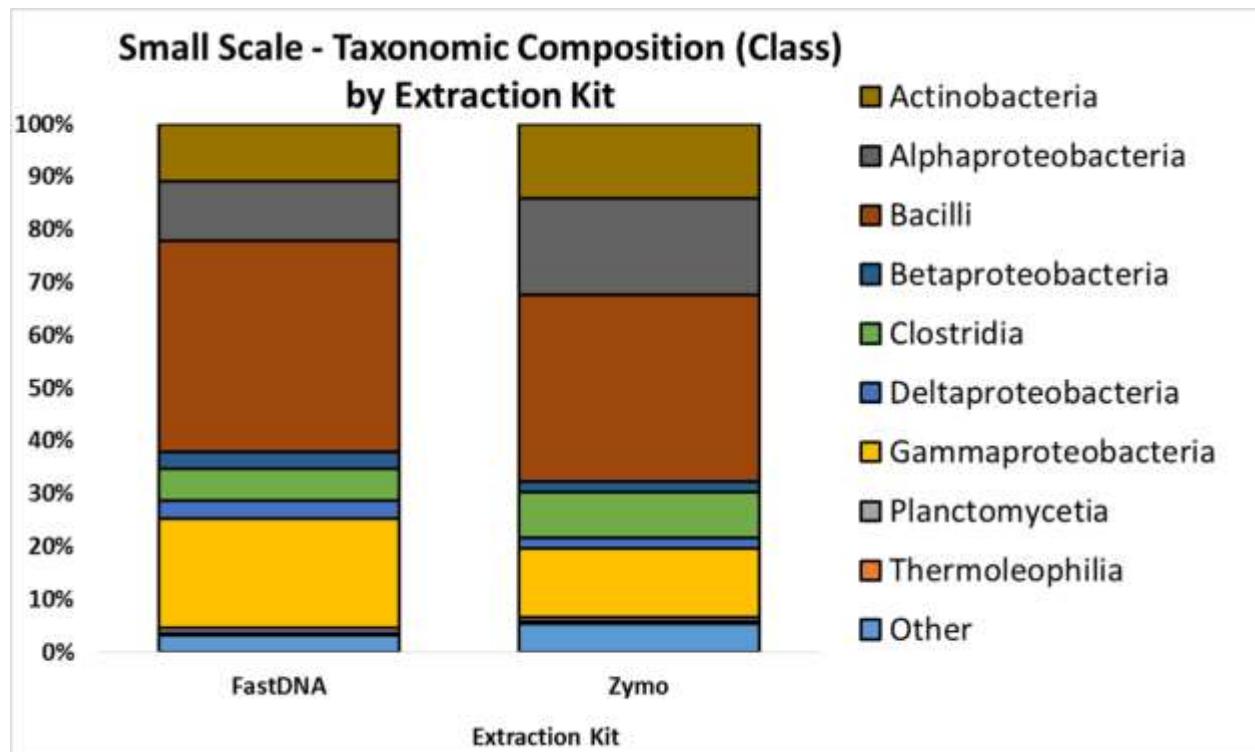
Figure 3.50. Comparison of a class-level taxonomic composition with respect to DNA extraction kit used. There is a significant difference in taxonomic composition similarity between those samples extracted using the FastDNA Spin Kit for Soil, and those extracted using the Zymo Fecal kit. (p<0.001; ANOSIM)**.**
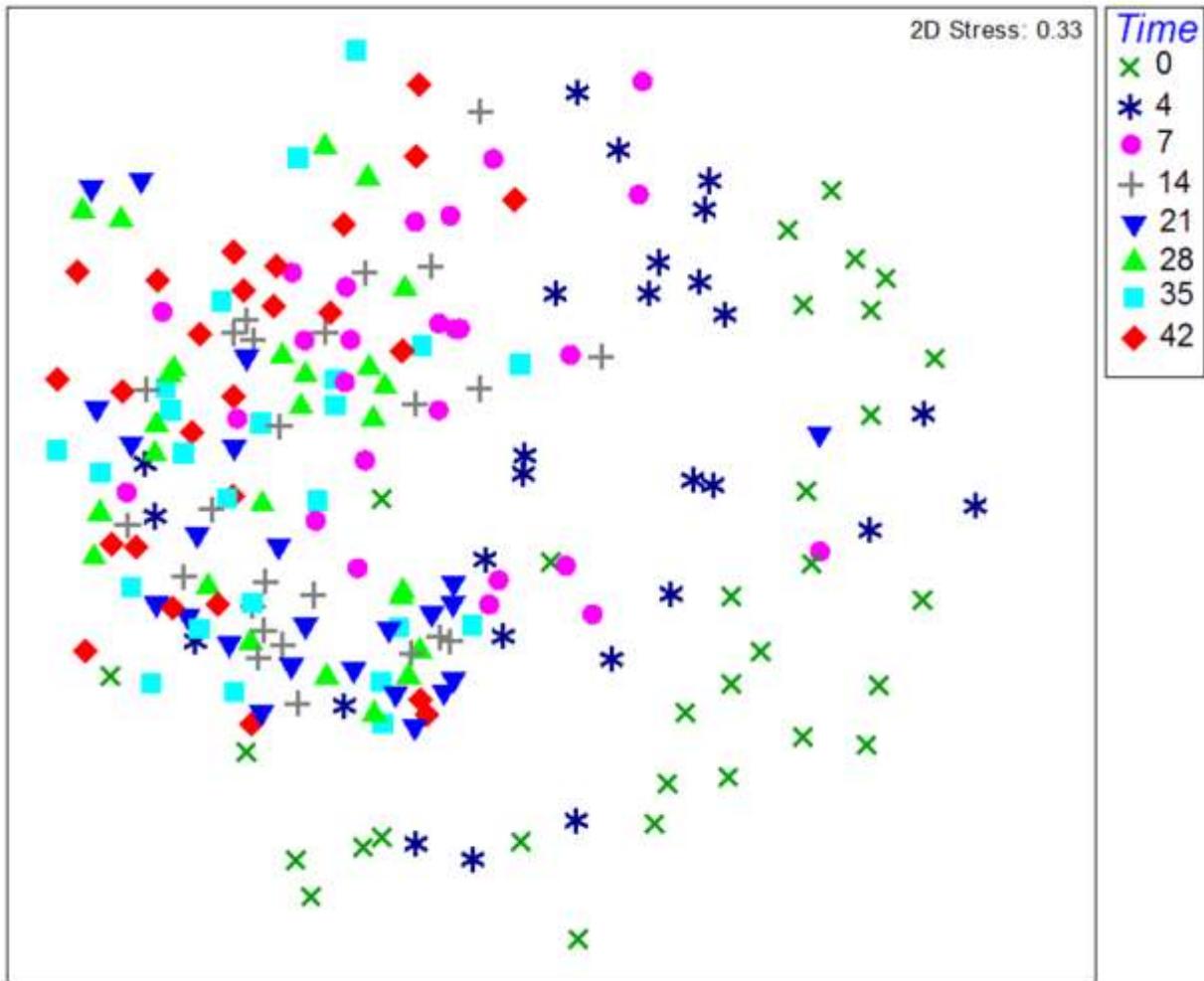
Figure 3.51. MDS plot based on the unweighted UniFrac distance matrix comparing taxonomic similarity, labelled with respect to time. There is significant separation of taxonomic similarity clusters with respect to time. (p<0.001; ANOSIM).
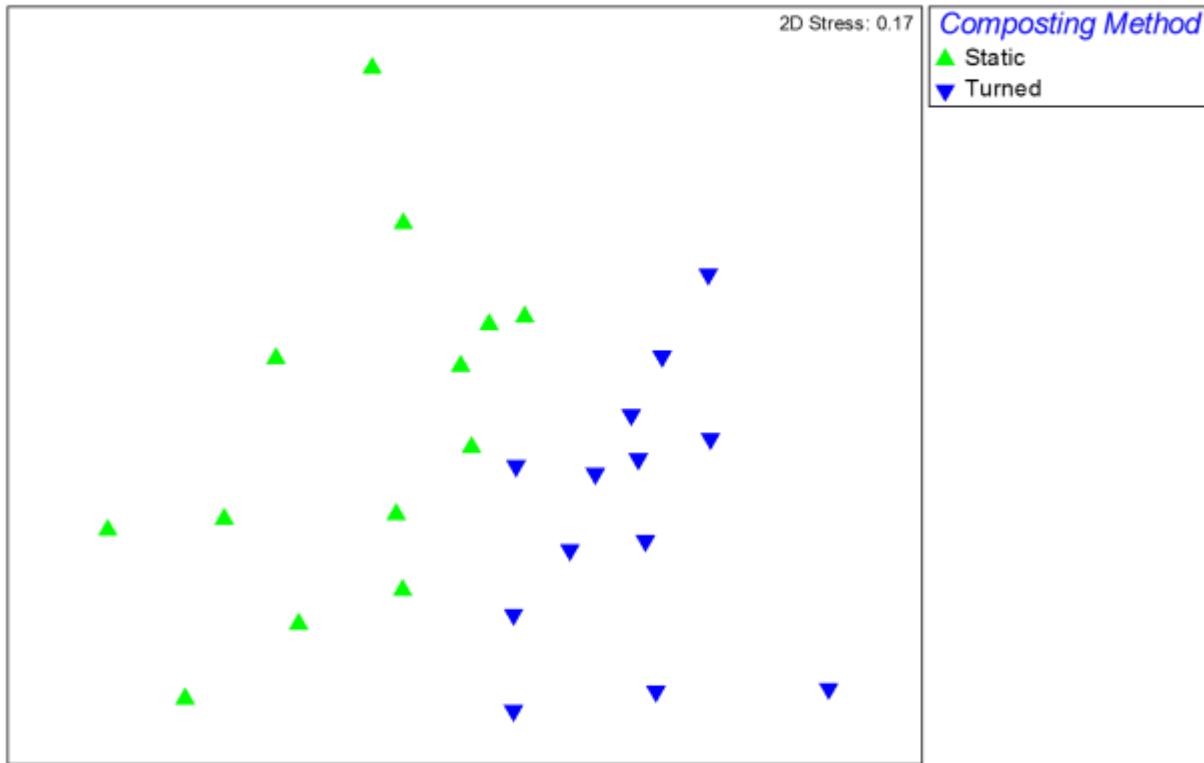
Figure 3.52. MDS plot on the unweighted UniFrac distance matrix comparing taxonomic similarity of day 42 samples only, labelled with respect to composting method. There is significant separation of sample clusters based upon composting method ($p<0.001$; ANOSIM).
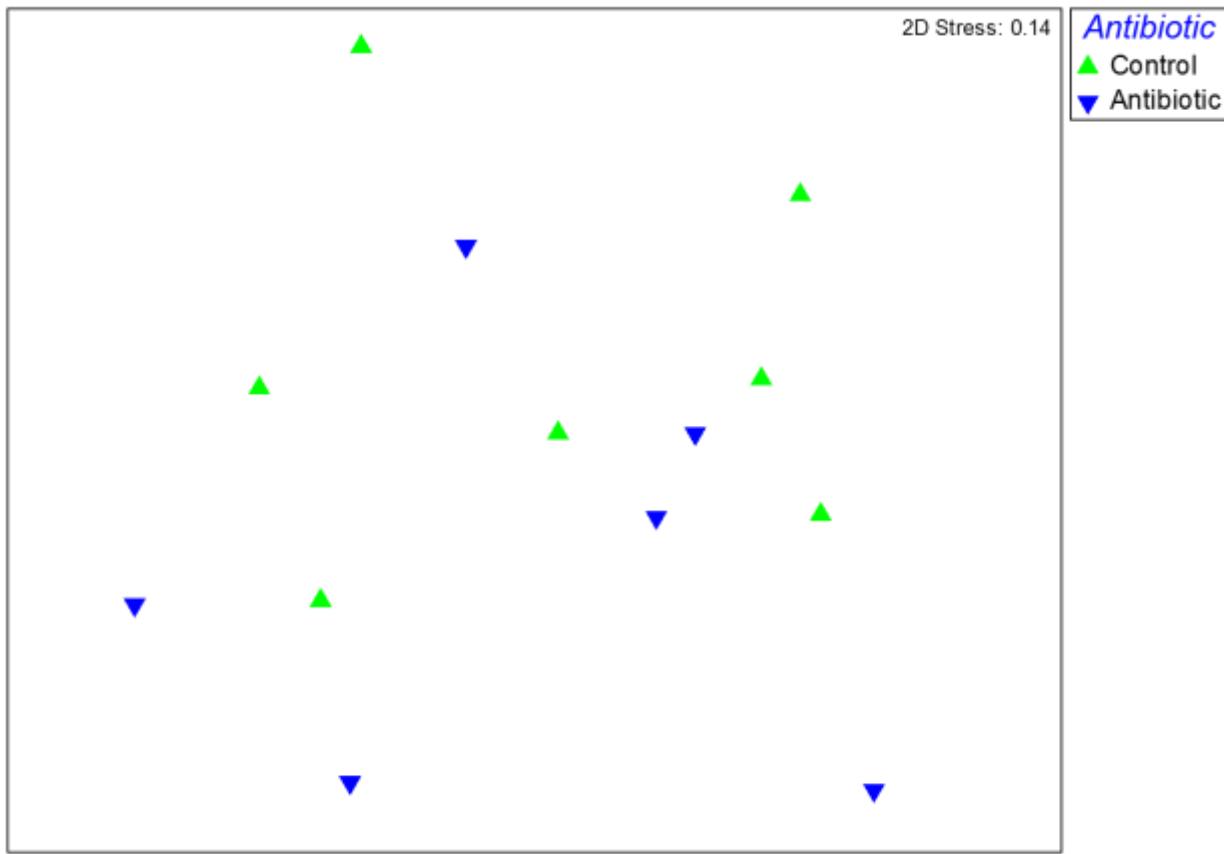
Figure 3.53. MDS plot on the unweighted UniFrac distance matrix comparing taxonomic similarity of day 42 beef compost samples only, labelled with respect to antibiotic treatment. There is no significant separation of sample clusters based upon antibiotic treatment of beef manure compost (p=0.269; ANOSIM).
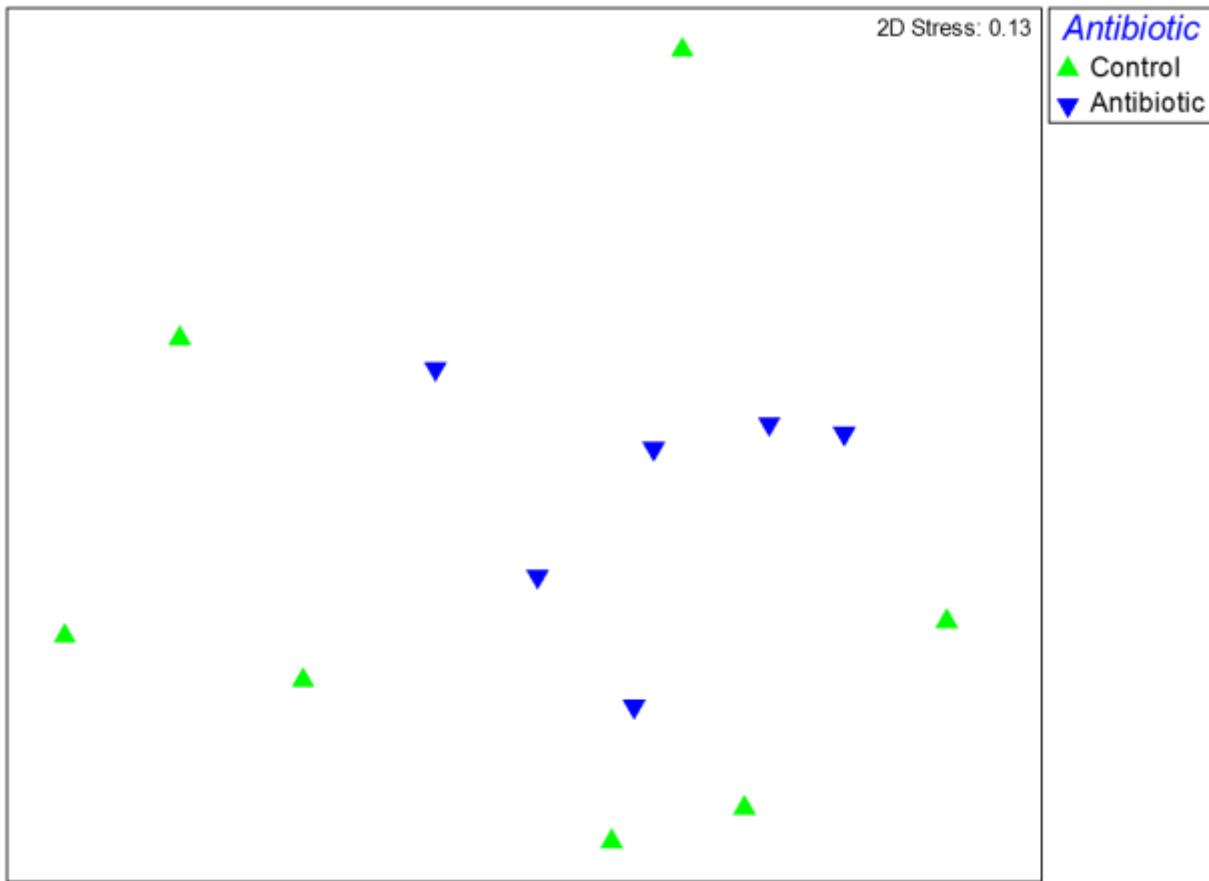
Figure 3.54. MDS plot on the unweighted UniFrac distance matrix comparing taxonomic similarity of day 42 dairy compost samples only, labelled with respect to antibiotic treatment. There is no significant separation of sample clusters based upon antibiotic treatment of dairy manure compost (p=0.206; ANOSIM).

3.4.2. HEAT-CONTROLLED COMPOSTING

The results of 16S amplicon sequencing and resulting taxonomic analyses for heat controlled composting are displayed in Figures 3.55 − 3.60. Figures 3.55 − 3.58 show stacked column charts comparing the relative abundance of each of the 9 most common bacterial classes present in the compost and manure samples, with the remaining, less abundant classes combined into the "other" category. There are significant differences in taxonomic composition with respect to time (p<0.001; ANOSIM) (Figure 3.55); there are also differences between day 42 composted manure as compared to day 0 non-composted raw manure (p=0.022; ANOSIM) (Figure 3.56).

There is no significant impact of antibiotic treatment on taxonomic composition similarity (p=0.413; ANOSIM) (Figure 3.57). There was however a significant difference in taxonomic composition between the two DNA extraction kits used for all treatments (p<0.001; ANOSIM) (Figure 3.58).

Figures 3.59 and 3.60 show MDS plots on the unweighted UniFrac distances comparing the taxonomic similarity between samples. Samples cluster significantly with respect to time (p<0.001; ANOSIM) (Figure 3.59). Day 42 compost samples did not cluster significantly with respect to antibiotic treatment (Figure 3.60).
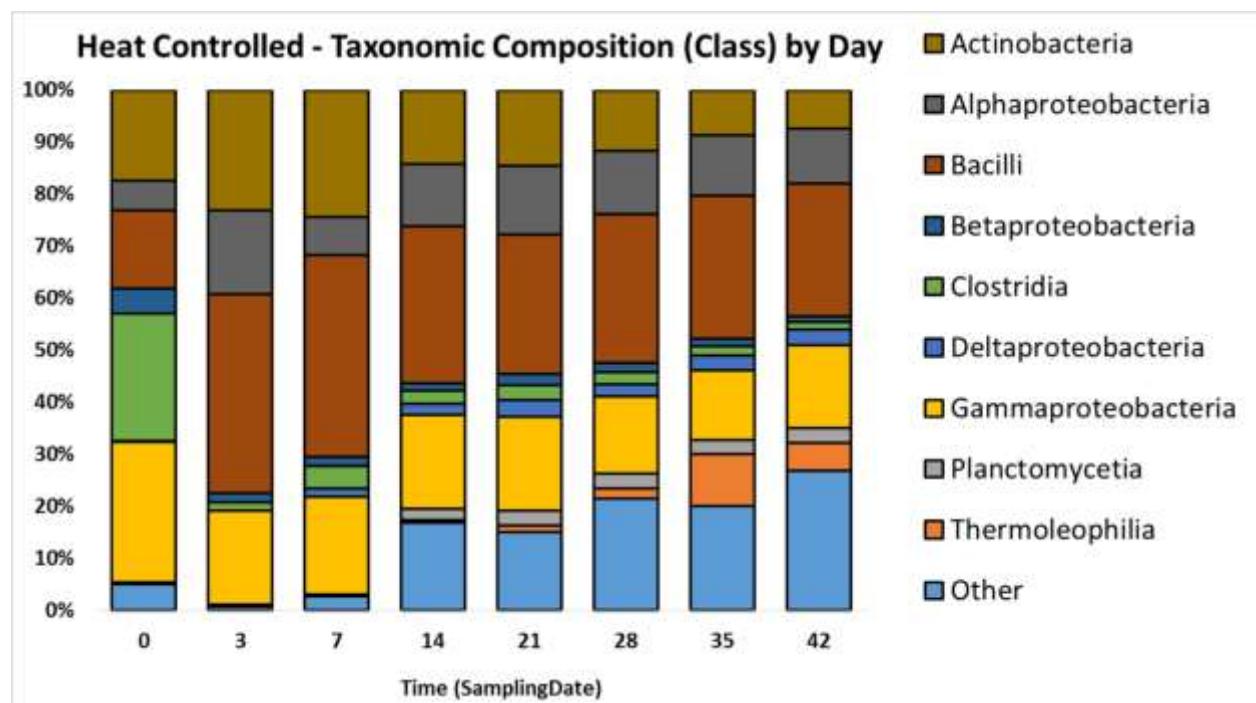
Figure 3.55. Comparison of a class-level taxonomic composition with respect to time. Time has a significant impact on taxonomic composition similarity. (p<0.001; ANOSIM).
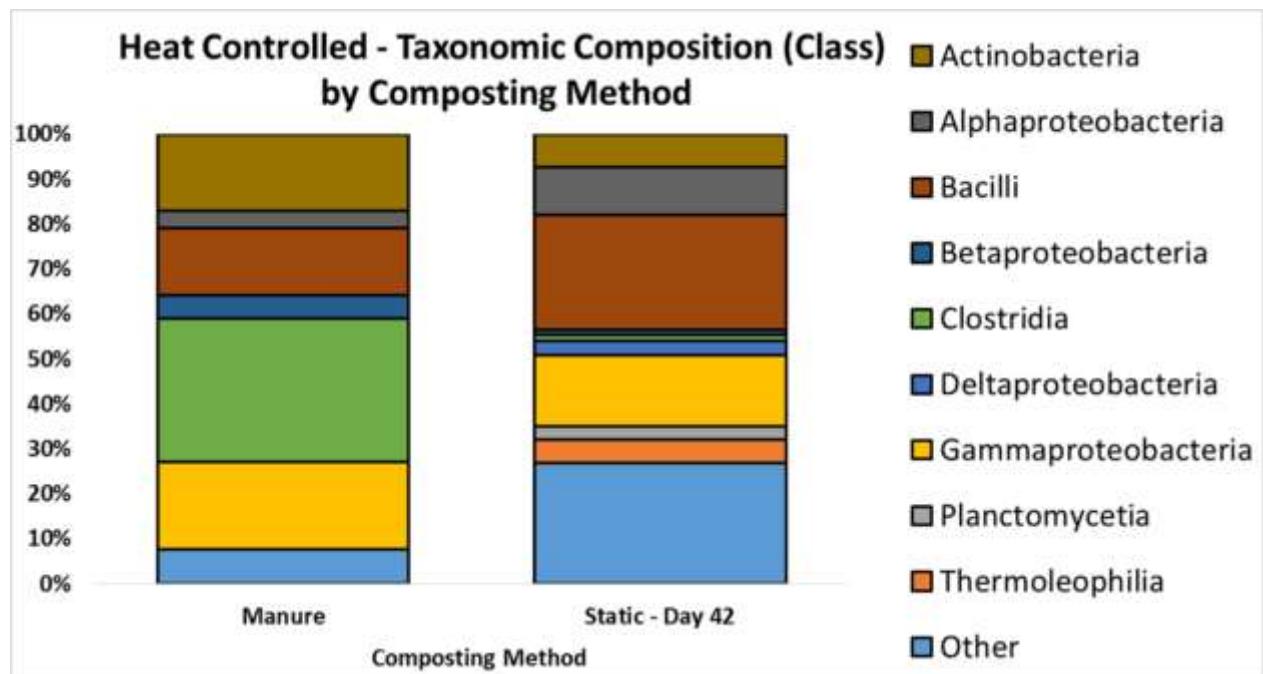
Figure 3.56. Comparison of a class-level taxonomic composition with respect to composting method. Composted manure on day 42 has a significantly different taxonomic composition similarity compared to raw manure on day 0 (p<0.022; ANOSIM).
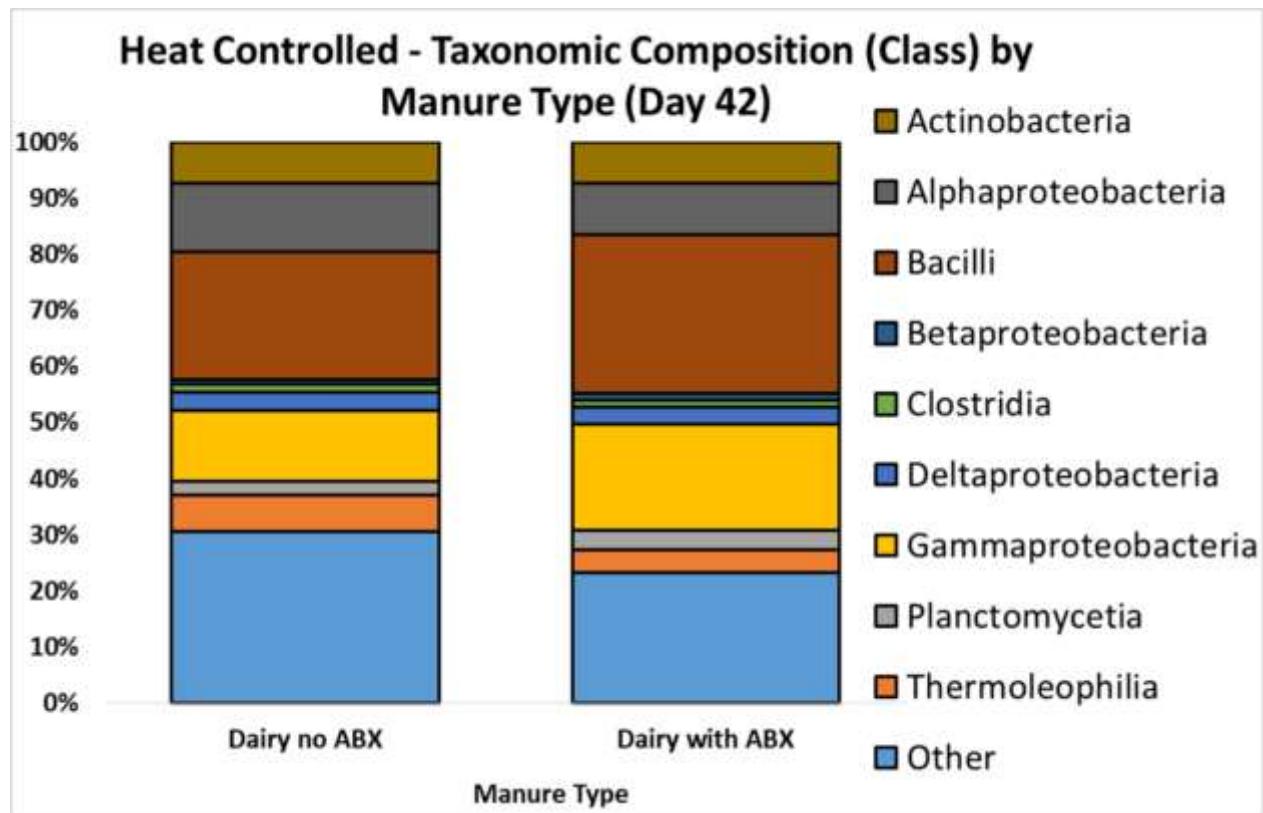
Figure 3.57. Comparison of a class-level taxonomic composition with respect to manure type on day 42. Antibiotic treatment does not have a significant impact on taxonomic composition similarity of day 42 composts (p=0.413; ANOSIM).
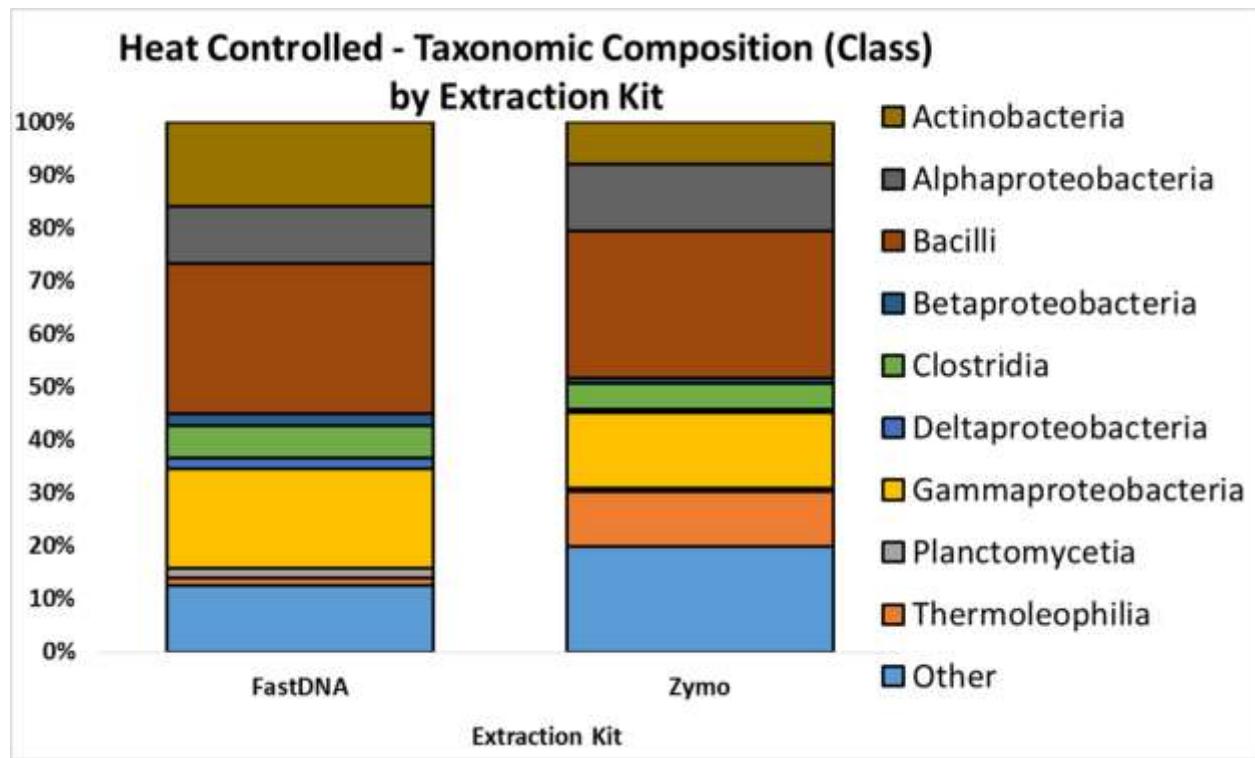
Figure 3.58. Comparison of a class-level taxonomic composition with respect to the DNA extraction kit used. The type of extraction kit used had a significant impact on taxonomic composition similarity profiles across both treatments (p<0.001; ANOSIM).
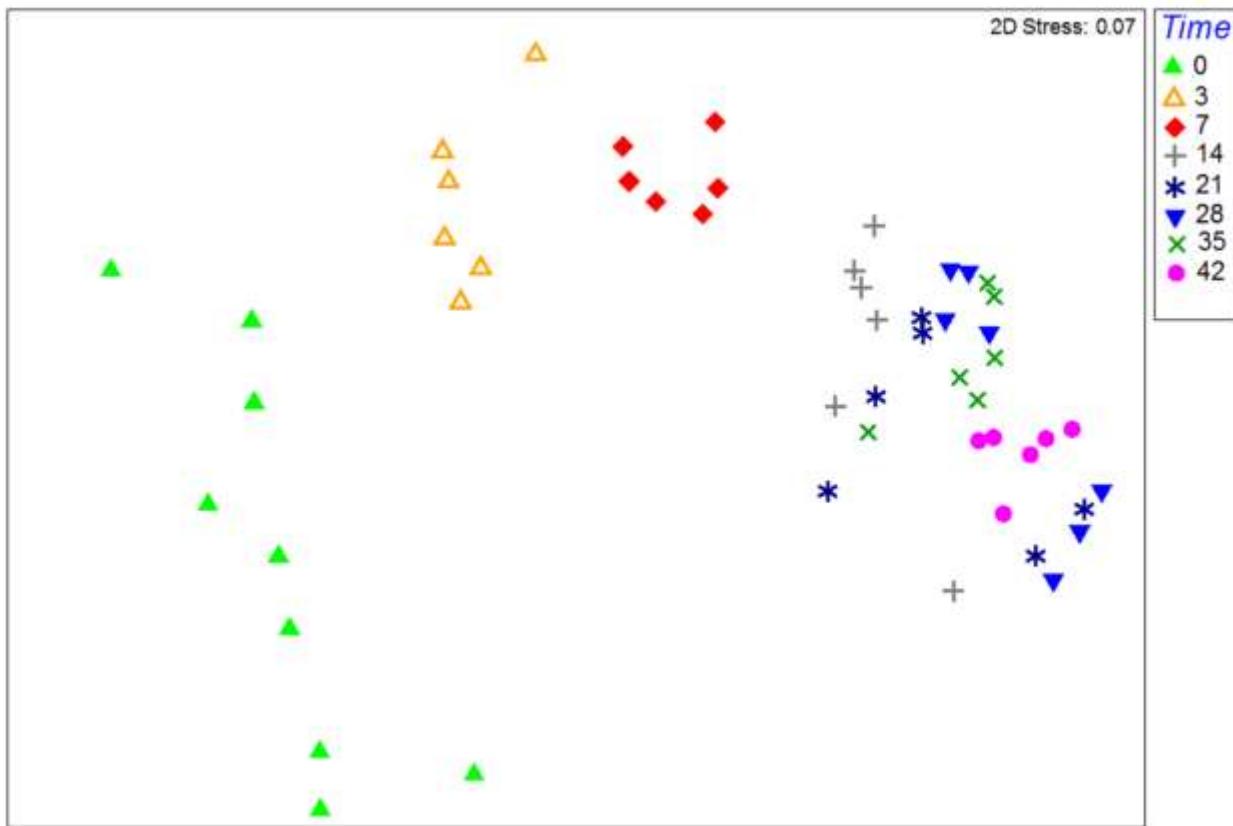
Figure 3.59. MDS plot based on the unweighted UniFrac distance matrix comparing taxonomic similarity, labelled with respect to time. There is significant separation of taxonomic similarity clusters with respect to time. (p<0.001; ANOSIM).
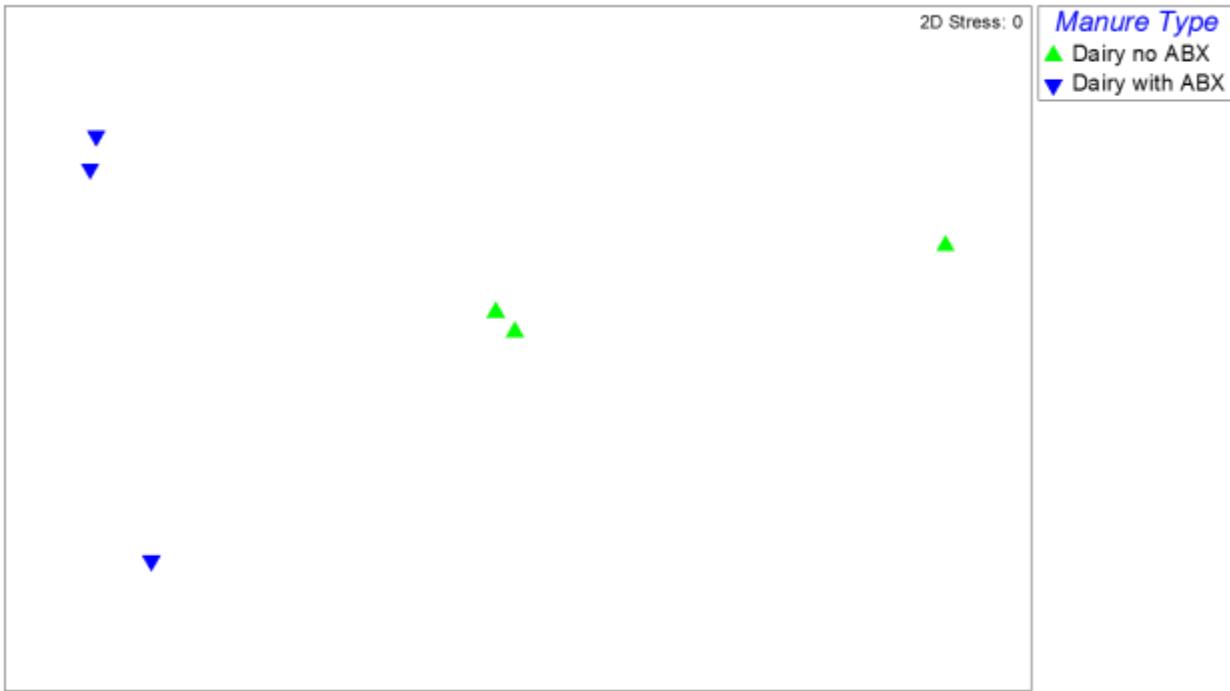
Figure 3.60. MDS plot based on the unweighted UniFrac distance matrix comparing taxonomic similarity, day 42 samples only, labelled with respect to manure type. There is no significant separation of taxonomic similarity clusters with respect to antibiotic treatment. (p<0.100; ANOSIM).

3.4.3. LARGE SCALE COMPOSTING

The results of 16S amplicon sequencing and resulting taxonomic analyses for large scale composting are displayed in Figures 3.61 – 3.67. Figures 3.61 – 3.64 show stacked bar charts comparing the relative abundance of each of the nine most common bacterial classes present in the compost and manure samples, with the remaining, less abundant classes combined into the "other" category. There is no significant difference in taxonomic composition with respect to time (p=0.891; ANOSIM) (Figure 3.61); but there is a difference between composted and stockpiled manures (p<0.001; ANOSIM) (Figure 3.62). Antibiotic treatment had no significant effect of taxonomic composition similarity (Figure 3.63), but there was a significant difference based on the type of DNA extraction kit used (p=0.004; ANOSIM)(Figure 3.64).

Figures 3.65 – 3.67 show MDS plots on the unweighted UniFrac distances comparing the taxonomic similarity between large scale composting samples. Samples do not cluster significantly

with respect to time (p=0.688; ANOSIM) (Figure 3.65), nor did they cluster significantly with respect to antibiotic treatment (p=0.748; ANOSIM) (Figure 3.66), but composting did cluster separately from stockpiled manure samples (p<0.001; ANOSIM)(Figure 3.67).
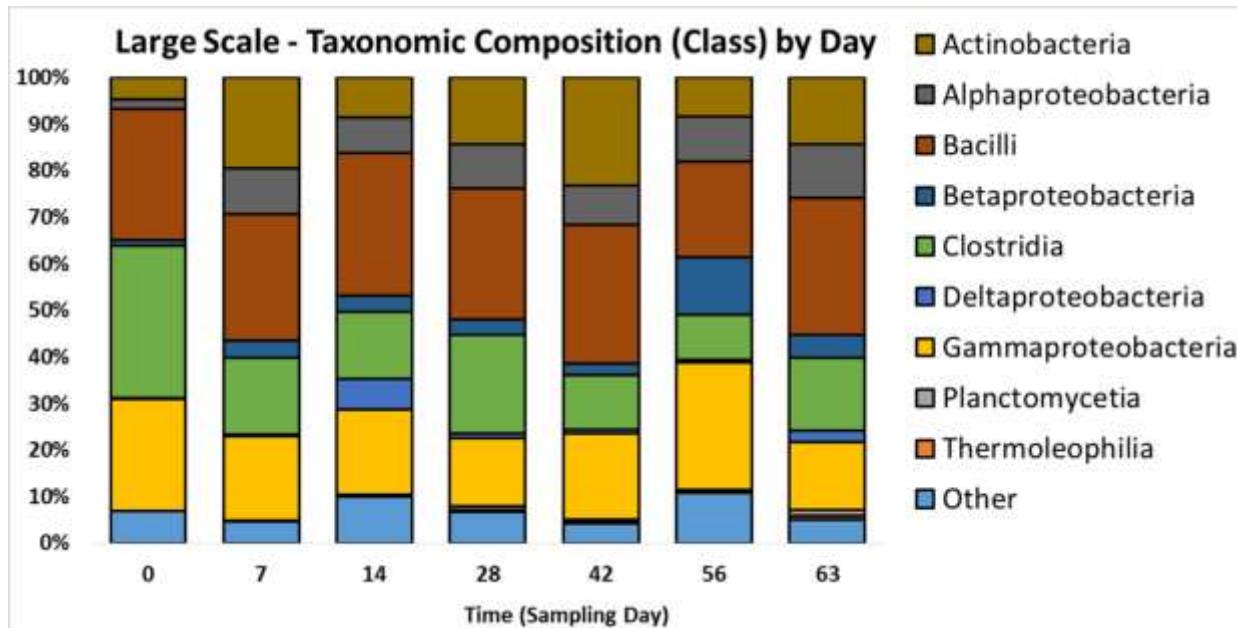

Figure 3.61. Comparison of a class-level taxonomic composition with respect to time. There is no significant impact of time on taxonomic composition similarity profiles (p=0.891; ANOSIM).
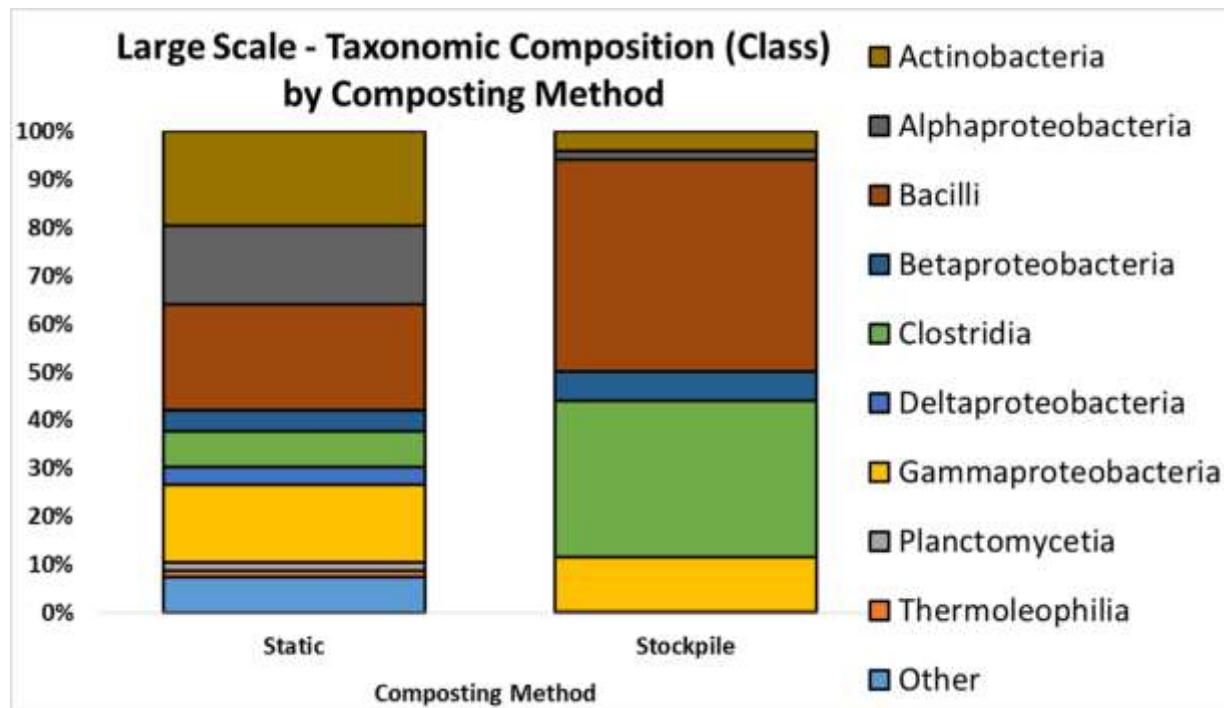
Figure 3.62. Comparison of a class-level taxonomic composition with respect to composting method. There is a significant difference in taxonomic composition similarity between composted and stockpiled manures. (p<0.001; ANOSIM).
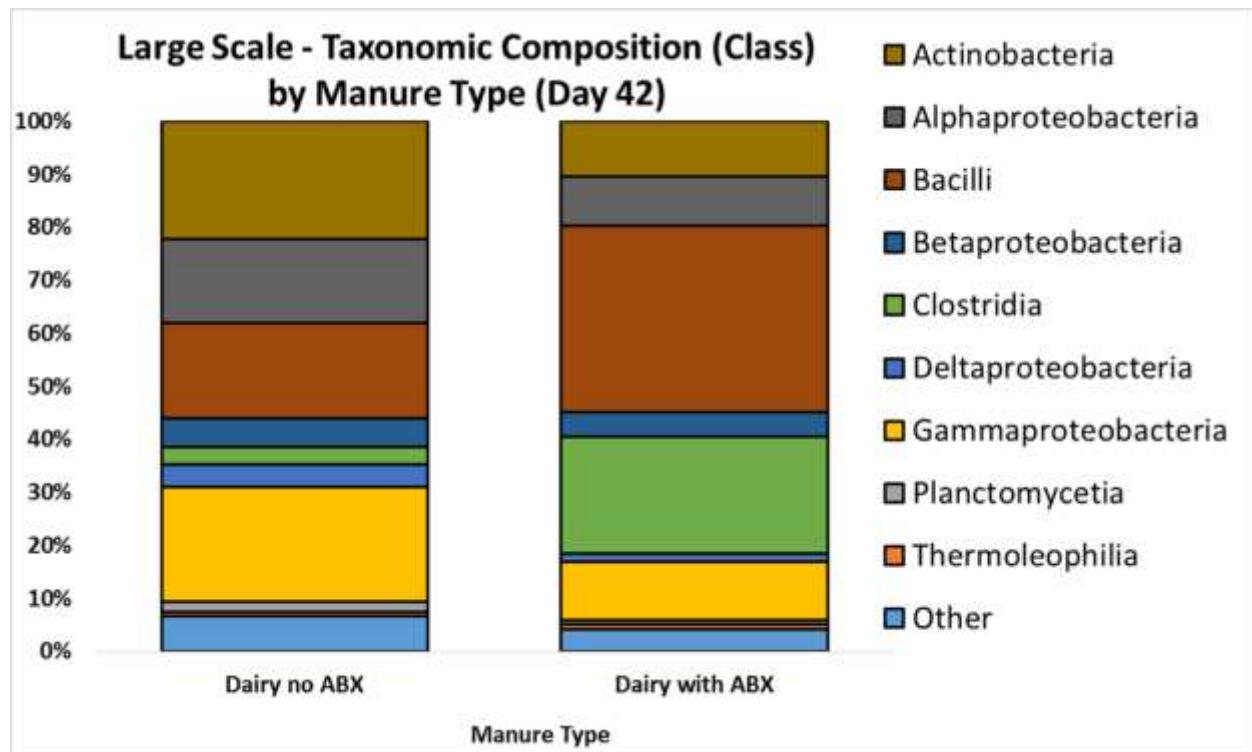
Figure 3.63. Comparison of a class-level taxonomic composition with respect to manure type, day 42 only. Antibiotic treatment does not have a significant impact on taxonomic composition similarity. (p=0.665; ANOSIM).
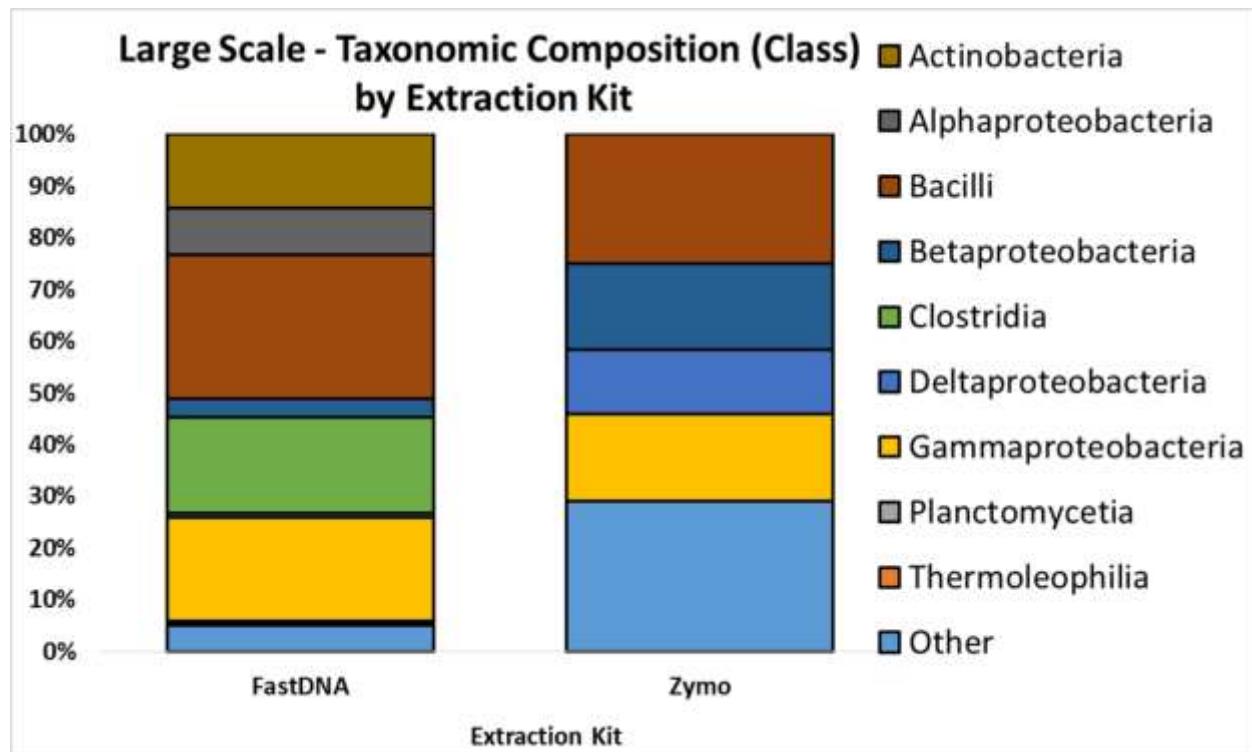
Figure 3.64. Comparison of a class-level taxonomic composition with respect to the DNA extraction kit used. The type of extraction kit used has a significant impact on taxonomic composition similarity. (p=0.004; ANOSIM). The Zymo Fecal kit resulted in a lower relative abundance of the classes actinobacteria, alphaproteobacteria, and clostridia, while having increased relative abundances of the many less abundant classes which are combined into the "other" classes category.
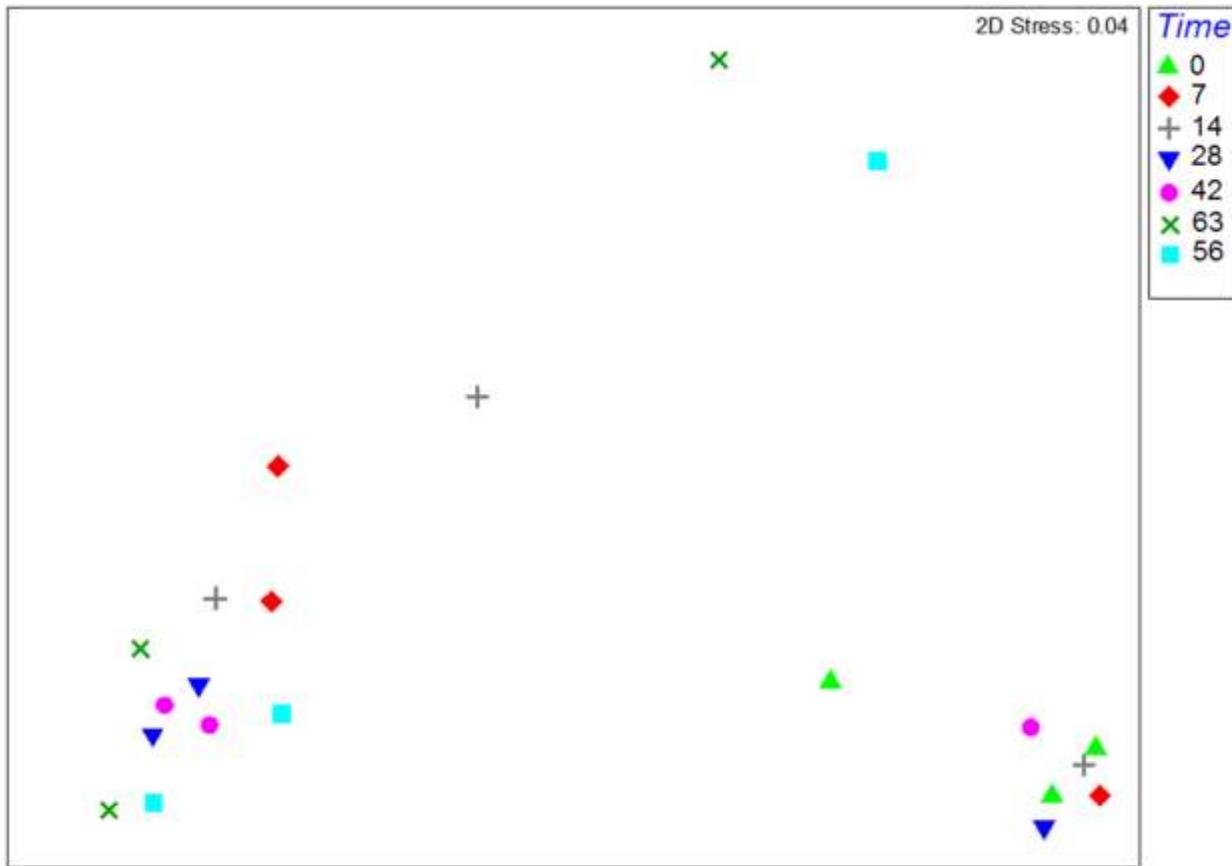
Figure 3.65. MDS plot based on the unweighted UniFrac distance matrix comparing taxonomic similarity, labelled with respect to time. There is no significant separation of taxonomic similarity clusters with respect to time. (p=0.688; ANOSIM).
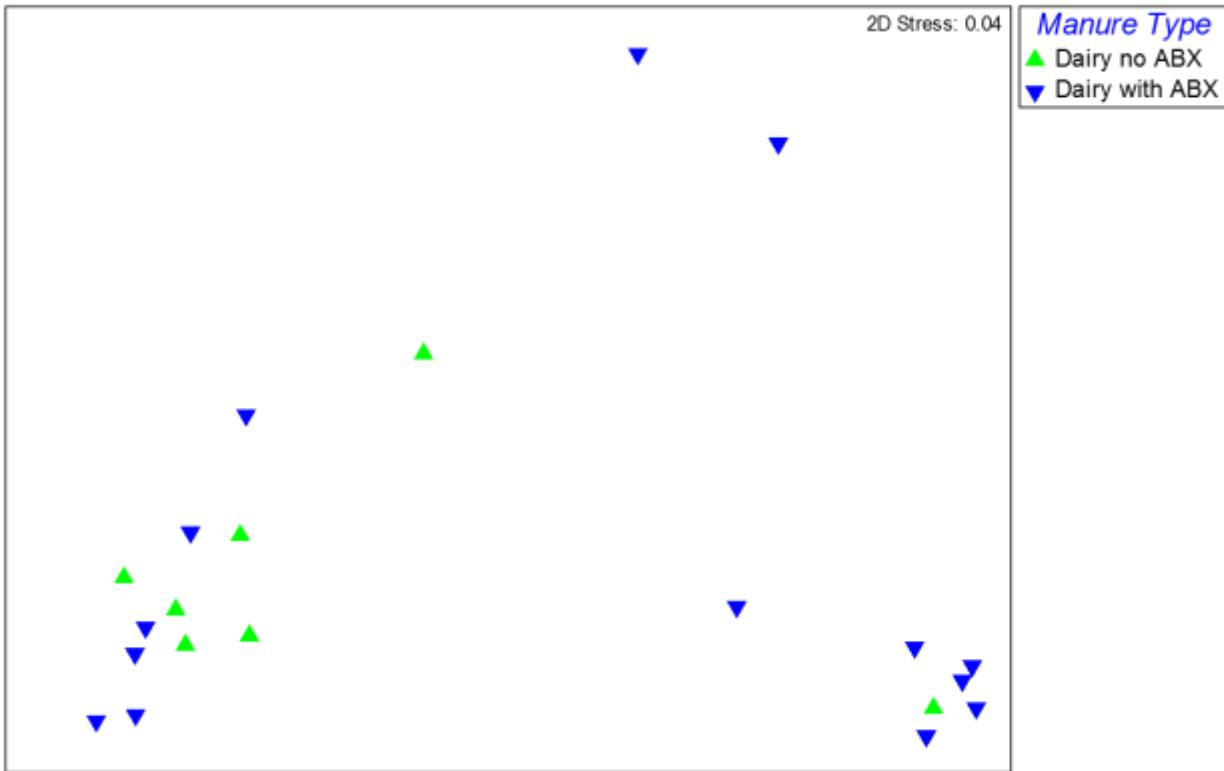
Figure 3.66. MDS plot based on the unweighted UniFrac distance matrix comparing taxonomic similarity, labelled with respect to manure type. There is no significant separation of taxonomic similarity clusters with respect to antibiotic treatment. (p<0.748; ANOSIM).
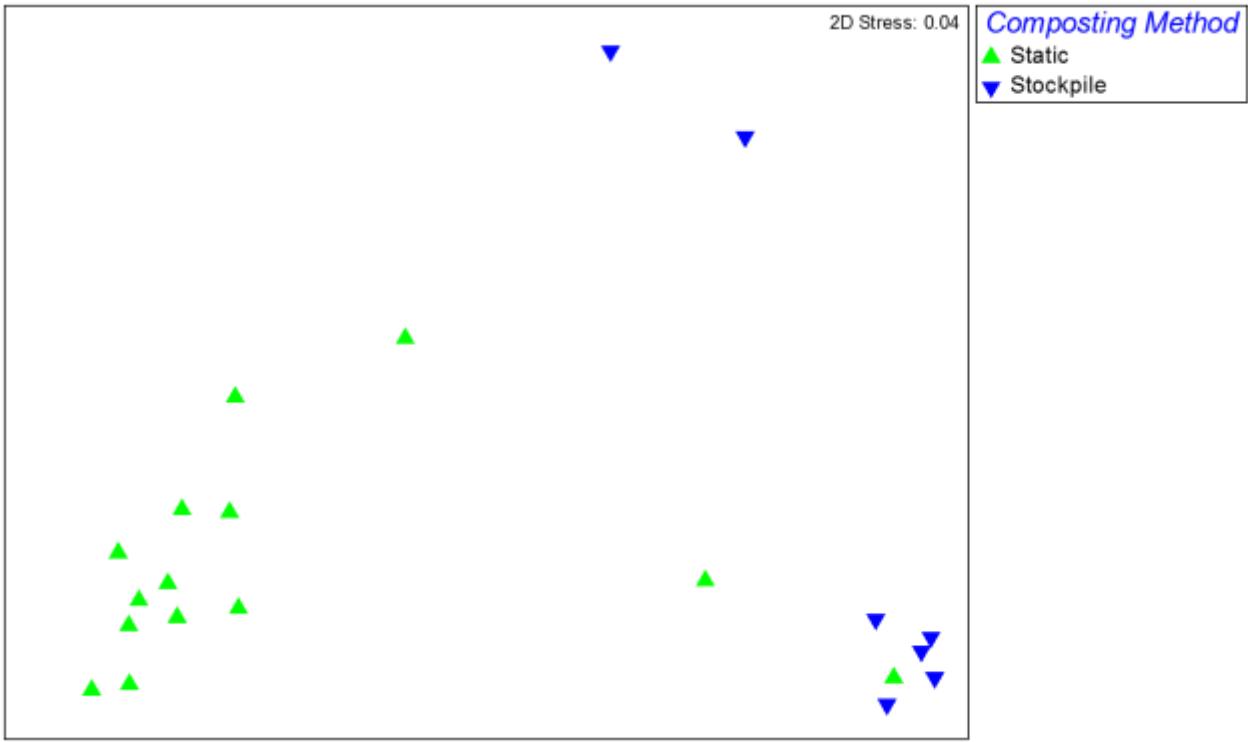
Figure 3.67. MDS plot based on the unweighted UniFrac distance matrix comparing taxonomic similarity, labelled with respect to composting method. There is significant separation of taxonomic similarity clusters with respect to composting method (p<0.001; ANOSIM).

4. EFFECT OF COMPOSTING ON THE PREVALENCE
OF ARBs AND ARGs IN CATTLE MANURE:
DISCUSSION AND CONCLUSIONS

Antibiotic resistance presents a growing human health risk and agriculture plays a significant potential role as a reservoir and source of ARB and ARG dissemination. This study examined the effect of several parameters on the abundance of ARBs and ARGs in cattle manure and cattle manure composts. The following sections summarize the overall findings of this study with respect to the study objectives.

4.1. EFFECT OF COMPOSTING METHOD

Composting has been explored as a potential method of reducing abundance of antibiotic resistance, however the results of this study show that the potential outcomes are complex. On the one hand, it seems that composting can increase phenotypic resistance in bacteria when they survive composting. Despite following FSMA guidelines for composting, *E. coli* was not eliminated during initial small-scale composting. The FSMA guidelines for temperature were maintained for small-scale static composting, but not for small-scale turned composting. Despite this discrepancy, neither treatment resulted in reducing fecal coliform counts below the FSMA target of 1000 MPN/g as indicated by the high IDEXX *E.* coli counts of 9 log MPN/g. The resulting isolates collected on day 42 and tested for resistance using the Kirby Bauer disk diffusion method showed an increase in the number of isolates testing as resistant to 3 of 6 antibiotics when composted as compared to raw manures on day 0 (Figure 3.7). The mean number of antibiotics that each isolate tested as resistant to increased from 0.10 resistances per isolate for raw manure to 2.55 and 2.10 resistances per isolate for static and turned composting, respectively. Similarly, relative resistance to ceftazidime, cefotaxime, and tetracycline antibiotics increased by an average of 0.5 antibiotic plate CFU per control plate CFU in composted manures on day 3 of the heat-controlled composting trial (Figures 3.11 – 3.13). Yet when target bacteria are successfully eradicated during the composting process then composting could result in a decrease in ARBs, as it did by day 7 of the heat-controlled composting trial when fecal coliforms fell below the detection limit of plating methods (Figures 3.11 – 3.15). It could be that resistant bacteria are more fit for

surviving high composting temperatures than non-resistant bacteria of the same species resulting in a higher relative proportion of resistant bacteria.

QPCR data suggests that the effect of composting on specific ARGs depends on the gene of interest. In general, absolute *sul*1 abundance increased by 2 logs during composting while relative abundance increased by a factor of 5 from day 0 to day 42 (Figures 3.18, 3.20, 3.27, and 3.31) while *tet*(W) absolute abundance decreased by 1 to 2 logs during composting and relative abundance rapidly approached zero (Figures 3.22, 3.24, 3.28, 3.29, and 3.33). These results agree with the findings of Selvam et al. with respect to reduction of *tet*(W) during 42 days of composting, although a complete reduction to below detection levels was not observed as it was in that study (18). However, results contradict those of Selvam et al. with respect to *sul*1 and those of Storteboom et al. with respect to *tet*(W) (18,55). The small-scale metagenomic data suggests that the total abundance of ARGs decreases throughout composting by (Figures 3.34 and 3.35), while the heat controlled trial shows a decrease in the abundance of most classes of ARGs, but a significant increase in trimethoprim resistance genes which increases ARG abundance overall (Figures 3.42 and 3.43). These metagenomic results contradict the high-throughput qPCR results of Su et al. which showed an overall increase in ARG abundance and diversity (19). This discrepancy is possibly due to the difference in the bacterial community composition of municipal sewage sludge as compared to that of animal manure.

There was no difference between static and turned composting methods on fecal coliform counts (Figure 3.3) or on resistant Kirby Bauer isolates (Figure 3.7). However, there were differences in the total ARG profiles based on metagenomics profiling (Figures 3.35 and 3.40) and in clustering based upon taxonomic similarity (Figures 3.48 and 3.52).

On the whole, there is evidence that composting attenuates most ARGs, but select ARGs may behave in radically different ways. ARBs can either be enriched or eliminated depending on the bacteria of interest and the ability of the compost to maintain adequate time at thermophilic temperatures. Because of the evidence that it can greatly reduce the abundance of most ARGs, composting is definitely promising as a tool for preventing the spread of antibiotic resistance. However, due to the fact that some of the ARGs that increase during composting confer resistance to antibiotics that are critically important for human medicine, and because composting must be carried out correctly in order to eliminate pathogens and achieve the desired results, it is not

recommended that composting be promoted for the purpose of attenuating ARGs until further study can be done. It is worth noting that composting in accordance with FSMA guidelines can be challenging to perform and it may be difficult for the average farmer to meet these standards in practice. As far as composting method, metagenomic results indicate that turned composting better reduces total ARG abundance than does static composting, even when the required time and temperature guidelines are not met. For this reason turned composting may be the preferred method, though a follow up study evaluating the effects of turned composting when the time and temperature guidelines are met is needed.

Interestingly, the effect of composting was varied with respect to the four classes of ARGs of clinical concern analyzed from the metagenomic sequencing data. In the small-scale trial, composting increased the relative abundance of genes coding for resistance to fluoroquinolones, and glycopeptides, but reduced the relative abundance of genes coding for macrolide resistance (Figure 3.41). In the heat-controlled trial, the only statistically significant effect was a sharp increase in glycopeptide resistance gene relative abundance (Figure 3.46).

4.2. EFFECT OF ANTIBIOTIC TREATMENT OF CATTLE

Widespread antibiotic usage in cattle and other livestock is one reason why antibiotic resistance in agriculture is such a major concern. This study assessed the impact of administration of antibiotics to cattle on the resulting patterns of resistance in manure and manure composts. Antibiotic treatment of cattle increased phenotypic bacterial resistance to certain antibiotics such as relative tetracycline, erythromycin, and clindamycin resistances measured in the heat controlled composting experiment (Figures 3.13, 3.14, and 3.15), but had no effect on any of the resistances to antibiotics measured during the Kirby Bauer testing of small scale composting isolates (Figure 3.8), or on resistance to either cephalosporin antibiotic measured during the heat controlled trial (Figures 3.11 and 3.12), which is interesting because these dairy cattle had been administered cephapirin, yet this did not enrich resistance to cephalosporins.

There was no significant effect of antibiotic treatment on most sul1 qPCR abundances (Figures 3.19, 3.26, 3.27, and 3.30) or on any of the metagenomic ARG similarity profiles or any of the taxonomic composition profile similarities of samples. This suggests that antibiotic administration is not a major driver of community composition or ARG profiles overall. In

contrast, antibiotic administration did selectively enrich for increased tetracycline resistance in both heat controlled and large scale composting trials (Figures 3.28, 3.29, 3.31, and 3.32). Since tetracyclines were not administered to the dairy cattle in either of these trials it is especially interesting that *tet*(W) abundance is positively correlated with antibiotic treatment. These results suggest that antibiotic administration does have a significant impact on phenotypic resistance to certain antibiotics but that this is not as important a driver of resistance as is the composting process itself.

4.3. EFFECT OF DAIRY OR BEEF CATTLE

There are differences between the types of antibiotics administered to dairy and beef cattle and their diets also vary, which results in gut microbiomes which have adapted to different conditions and may therefore be significantly different. This study examined the difference in ARG and ARB abundance between beef and cattle manure after composting. For the most part, few significant differences were observed between the two cattle types. Those that were observed were greater *sul*1 abundance on day 0 of small scale composting in dairy manures as compared with beef manures (Figure 3.23), greater absolute and relative *tet*(W) abundance in beef manure on day 0 as compared with dairy manure (Figures 3.23 and 3.25), and differences in metagenomic ARG profile similarity between the two cattle types on day 0 (Figure 3.36). In all of these cases the difference is only an initial one which vanishes after composting as the composts made from the two cattle manure types become more similar. This suggests that the difference in dairy and beef manure is not a significant factor in altering the resistance profiles of finished composts. In practice, composting of dairy manure is more common than beef because dairy cows are typically confined to smaller areas, allowing for easier manure collection.

4.4. EFFECT OF DNA EXTRACTION KIT

All extraction kits use varied protocols that are optimized for different purposes. This study compared the taxonomic composition of DNA extracted using two different kits, the FastDNA Spin Kit for Soil by MP Biomedicals, and the Zymo Fecal kit. In all three trials, there was a significant difference in the similarity of taxonomic composition between DNA extracted from the two kits (Figures 3.50, 3.58, and 3.64). On the whole, the Zymo fecal kit resulted in a lower

relative abundance of the classes actinobacteria, alphaproteobacteria, and clostridia, while having increased relative abundances of the many less abundant classes which are combined into the "other" classes category. This may indicate that the Zymo kit is less able to fully extract DNA from these more abundant classes and that the less abundant species therefore make up a larger proportion of the classes that remain.

4.5 SUMMARY AND CONCLUSIONS

The results of this experiment show many complex interactions among bacteria and antibiotics throughout the composting process. Because of these complexities, composting may either increase or decrease phenotypic or genetic resistance to any specific antibiotic, but on the whole the metagenomics data suggests that composting helps to attenuate total ARG abundance more so than increase it. Still, further research should be conducted to allow for better understanding. High priority research questions include: 1) examining the spatial variation in ARB and ARG abundance at various locations within a large composting heap and 2) exploring different time and temperature combinations to optimize ARG attenuation and especially focus on reduction of ARGs of clinical importance. Antibiotic administration has an effect only on select ARGs, increasing their abundance, but has no effect on most. There is no significant impact on the choice of beef or dairy manure on the final ARG profiles of finished compost. Different DNA extraction kits may be better at extracting DNA from some species and not others.

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