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A MICROBIOLOGICAL SURVEY AND CHARACTERIZATION

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A MICROBIOLOGICAL SURVEY AND CHARACTERIZATION OF ENTEROCOCCI AND VANCOMYCIN RESISTANT ENTEROCOCCI IN COMPOST

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ABSTRACT

Vancomycin resistant enterococci (VRE) have played a major part in causing nosocomial, or hospital borne illnesses. VRE have been found with resistance levels in excess of 256 µg/ml for vancomycin, which makes it very difficult to treat patients infected with VRE. Therefore, it is very important to identify the sources of this organism to control its' transmission into the environment. Composting is a process that has been often utilized to inactivate pathogens present in animal waste. The objectives of this study were to 1) enumerate enterococci and VRE from three different composting trials with various compost mixtures, 2) characterize the antibiotic resistance profiles of the VRE isolates obtained from composting trials phenotypically and genotypically, and 3) determine the VRE isolates' changes in heat resistance and persistence during the 4-month composting trial.

In this study, two dairy compost heaps and one vegetable scrap compost heap were constructed on two research farms in Clemson, SC. Samples were taken from each heap from different locations at 8 elected intervals and analyzed for enterococci and VRE counts by spread plating on Bile Esculin agar (BEA) and BEA containing 6 µg/ml of vancomycin, respectively. After 30 days of active composting the average VRE populations declined ca. 4.6, 4.07, 3.97 and 1.6 logs at the top, center, bottom and surface locations of the heaps, respectively, whereas the enterococci populations declined for 4.36, 3.54, 3.10, and 2.28 logs, respectively. Our results revealed that the genus of all 88 VRE isolates was enterococci and that no VRE were *Enterococcus faecalis* or

Enterococcus faecium. However, 9 and 6% of enterococci isolates from Trial 1 were tested as *E. faecalis* and *E. faecium,* respectively. For those VRE isolates taken from composting, minimum inhibitory concentration (MIC) testing revealed that 85 out of 88 suspected VRE isolates were resistant to > 256 µg/ml of vancomycin and all 88 isolates were resistant to > 64 µg/ml of teicoplanin. Eighty-three (94%) of VRE isolates were confirmed to contain the *vanA* gene for vancomycin resistance. The D-values of selected VRE isolates (n=8) from Trial 1 compost heaps were in the range of 9.7-17.73 min at 60°C, 4.73-12.57 min at 65°C, and 1.59-4.44 min at 70°C. The z-values for those VRE isolates ranged from 11.92°C-17.87°C. Box-PCR analysis of VRE isolates (n=12) from Trial 1 revealed that 42% were identical and appeared on compost days 7, 14, and 60, suggesting that this specific strain was able to adapt to the elevated temperatures in compost by developing heat resistance.

Our studies demonstrated that VRE may become inactivated in compost if conditions are optimal and that some enterococci may develop heat resistance during the thermophilic phase of composting. This is important because it suggests that improperly composted manures may serve as a means for the spread of VRE on food products, especially fresh produce, intended for human consumption.

DEDICATION

I would like to dedicate this work to my mother, Pamela Shea; my father, Arthur Daane; my step father, Nicholas Shea; and my brother, Alex Daane. Without their love, support, and encouragement this could not have been possible.

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CHAPTER ONE

LITERATURE REVIEW

Enterococci

Enterococci are gram positive, facultativly anaerobic bacteria. The enterococci

genus can tolerate a wide range of temperatures ranging from 10ºC to 50ºC and survive in

hypertonic, hypotonic, alkaline, and acidic environments (Laport et al., 2002; Willet,

1992; Nakajo et al., 2006). *Enterococcus faecalis* can survive in a pH ranging from 2.5-

12, which is attributed to their membrane durability and the presence of potassium and

hydrogen ion pumps (Nakajo et al., 2006; Kakinuma and Igarashi, 1995). Enterococci

are usually considered as fermenters due to the lack of a Kreb's cycle and respiratory

chain, but they grow in oxygenated and reduced oxygen environments (Willett, 1992).

Enterococci are normally found in intestinal tracts of many different types of animals or

in vegetation and surface water, likely because of contamination by untreated sewage or

animal waste (Jett et al., 1994).

Enterococci are a major cause of nosocomial infections around the world, being

responsible for approximately 110,000 urinary tract infections, 40,000 wound infections,

25,000 cases of bacteremia, and 1,100 cases of endocarditis annually in the United States

(Emori et al., 1993; Haley et al., 1985; Harris, 1992). Deaths attributed solely to

enterococcal infections have been difficult to ascertain because other severe illnesses

often infect the patient concurrently. Many different infections are common, however

enterococcal sepsis is implicated in 7 to 50% of fatal cases (Rice et al., 1995). There are

two species of enterococci that are found in most enterococcal infections: *E. faecalis*

causing about 80% of infections and *Enterococcus faecium* for most of the remaining

20% of infections (Ritchey, 1974). There are two types of enterococci that cause

infection: those that originate from the normal flora of the patient and those that are able

to transmit in a nosocomial environment. The bacteria normally found internally, or

normal flora, are unlikely to possess antibiotic resistance beyond what the species is

known to be intrinsically resistant to. Normal flora is also unlikely to be able to spread

from one hospital bed to another. Enterococci that are capable of spreading from patient

to patient usually possess resistance to multiple antibiotics (Huycke et al., 1998). Several

cases and historical studies show that the death risk associated with antibiotic resistant

enterococcal bacteremia is several fold higher than the death risk associated with

susceptible enterococcal bacteremia (Edmond et al., 1996).

Antibiotic Resistance of Enterococci

A major health issue concerning enterococci is that the organism has inducible

resistance to many different antibiotics, which makes treating an infection extremely

difficult. Enterococci are intrinsically resistant to many different antibiotics including β-

lactam-based antibiotics and aminoglycosides (Clark et al., 1993). β-lactam-based

antibiotics including cephalosporin, carbapenems, monobactams, and penicillin are the

most widely used type of antibiotics (Clark et al., 1993). These antibiotics act by inhibiting the synthesis of the peptidoglycan layer of the cell wall, which is very important in the structural integrity of gram-positive bacteria like enterococci (Clark et al., 1993). Penicillin binding proteins (PBPs) assist in the final step of making the peptidoglycan. β-lactam antibiotics mimics the D-alanyl-D-alanine binding site on the PBPs, and the antibiotic is able to irreversibly bind to this active site and prevent the production of peptidoglycan (Clark et al., 1993). By preventing the production of peptidoglycan the antibiotic successfully inhibits cell wall synthesis. These drugs have been administered in the past but due to the emergence of antibiotic resistance they aren't nearly as effective as they have been in the past. In the past 20 years new drugs, like vancomycin, have been administered as a last resort treatment in an attempt to treat multiple drug resistant enterococcal infections.

Multiple drug resistant (MDR) enterococci are resistant to at least two different antibiotics (Ike, 1999). MDR enterococci have become a major threat concerning human health in the last 20 years. The most notable resistance is enterococci's glycopeptide resistance, which includes the antibiotic vancomycin. Enterococci's acquired resistance to glycopeptides is due to the production of modified glycopeptide precursors ending in D-alanine-D-lactate. These D-alanine-D-lactate precursors are products of the vancomycin resistant genes A, B, and D. The production of the D-alanine-D-serine precursor ending is a product of the vancomycin genes C, E, and G (Mulvey et al., 2003). Expression of the resistance gene clusters is controlled by a two component regulatory system that is composed of VanR-type response regulators acting as transcriptional activators and VanS-type histidine kinases that are associated with the membrane (Clark et al., 1993).

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The regulatory and resistance genes are transcribed from distinct promoters that are coordinately regulated (Woodford, 2001).

Different vancomycin resistance genes convey varying levels of vancomycin resistance. Therefore, it would be beneficial to know which gene an isolate possesses when attempting to treat an infection. Among the different vancomycin resistant genes, *vanA* and *vanB* are the most common (Clark et al., 2005). *VanA* is transferred among enterococci on the transposon Tn1546, which is located on a conjugative plasmid. There are two types of the *vanB* operon: *vanB1* and *vanB2*. *VanB1* is generally carried by large (90- to 250-kb) elements that are transferable by conjugation between chromosomes (Quintiliani, 1994). The more common operon, *vanB2*, is associated with Tn1549 and Tn5382 located on two different plasmids (Garnier et al., 2000). Both plasmids have not been shown to promote conjugative transposition but they can be transferred as an integral part of variably sized chromosome fragments or of plasmids (Dahl et al., 2003). *VanB2* and Tn1549 have been found in many different genera including *Enterococcus*, *Streptococcus*, *Clostridium*, *Eggerthella,* and *Ruminococcus*, which suggests that the spread of resistance may be due to transposition (Dahl, 2003; Dahl, 1999; Ballard et al., 2005). Table 1 summarizes the most common gene's characteristics of vancomycin resistance.

Table 1 Glycopeptide resistance in enterococci. (Moellering, 1998)

	VanA	VanB	VanC	VanD
Phenotype Vanco MIC (μg/mL)	64 to >1000	4 to 1000	2 to 32	64
Teico MIC (μg/mL)	16 to 512	0.5 to 1	0.5 to 1	4
Expression	Inducible	Inducible	Inducible	Inducible
Location of R genes	Plasmids	Chromosome (plasmids)	Chromosome	?
Transfer by conjugation	+	+	−	−
Mobile element	Tn 1546	Tn 1547	−	?
Modified target	D-Ala-D-Lac	D-Ala-D-Lac	D-Ala-D-Ser	D-Ala-D-Lac
Species	E. faecalis E. mundtii E. faecium E. raffinosus E. avium E. gallinarum E. durans E. casseliflavus	E. faecalis E. faecium	E. fallinarum E. casseliflavus E. flavescens	E. faecium

VanA and *vanB* genes account for almost 60% and 40% of all isolated VRE, respectively (Clark et. al., 1993). This includes samples from clinical, veterinary, and animal specimens. Enterococci carrying the *vanA* gene are characteristically resistant to high levels of vancomycin (>256 μg/ml) as well as teicoplanin (> 256 μg/ml) (Clark, 1993). *VanB*, which has only been found in *E. faecalis* and *E. faecium* (Moellering, 1998), conveys resistance to vancomycin in excess of 256 μg/ml but it is more common to observe a minimal inhibitory concentration (MIC) of vancomycin of > 64 μg/ml. *VanB* does not, however, convey resistance to teicoplanin like *vanA* (Clark, 1993). Therefore, testing isolates that are highly resistant to vancomycin with teicoplanin would help distinguish between *vanA* and *vanB* phenotypes. The *vanC* gene is associated with a lower MIC for vancomycin (< 32 μg/ml), susceptibility to teicoplanin and usually occurs

in *E. gallinarium* and *E. casseliflavus* (Clark, 1993). One study found *vanD* in a few

isolates of *E. faecium*, *vanE* in two strains of *E. faecalis*, and *vanG* in four isolates of *E.

faecalis* from Australia, however these genes occur infrequently (Emori and Gaynes,

1993). Thus, the minimal inhibitory concentration values of vancomycin can help to

phenotypically categorize isolates obtained from environmental samples. Susceptibility

to teicoplanin can also be used to phenotypically categorize *vanA* and *vanB* isolates.

Prevalence of VRE

From 1989 to 1993 there was a 20-fold increase in the number of VRE isolated

from infected patients (CDC, 1993). In the United States there was about 5 times the

amount of vancomycin used in hospitals in 1996 compared to France, Germany, and the

UK combined (Van den Boogard et al., 2005). The widespread use of vancomycin, along

with other antibiotics, could account in part for the rapid increase in VRE in the United

States.   Consequently, enterococci's resistance genes would become more prevalent

because of the antibiotics administered by the hospital (Van den Boogard et al., 2005).

In Europe, vancomycin wasn't administered to patients as frequently as it is in the

United States, however VRE is still widespread. Avoparcin has often been presumed to

be responsible for the elevated levels of VRE found in European livestock as well as food

from the farm areas (Van den Boogard et al., 2005). Avoparcin was used as an additive

for livestock and poultry feed to increase animal growth. It was used widely throughout

Europe until 1997 when it was banned as a feed additive because of the rapid increase in VRE. Farms where avoparcin was never used, such as in Sweden, have low levels of VRE (Torrell et al., 1999). The increased prevalence of VRE in animals subsequently caused an increase in the prevalence of VRE in food and in the communities of the areas surrounding the farms. The Netherlands had as much as 79% of their poultry products contaminated with VRE mostly due to the 80,000 kg of avoparcin used annually until 1997 (Van den Boogard et al., 2005). VRE have also been detected in turkeys raised on farms that used avoparcin (Stobberingh et al., 1999). Stobberingh et al. (1999) reported that similar strains of VRE were found in humans and turkeys in southern Netherlands, suggesting the possibility of food borne contamination. Along with the rest of Europe, the Netherlands banned avoparcin in 1997 and within 2 years the levels of VRE decreased significantly in both food animal sources as well as normal flora from healthy humans (Van den Boogard et al., 2005). However, 81% of poultry samples taken from a Norwegian poultry farm were contaminated with VRE 3 years after the ban of avoparcin (Borgen et al., 2001). Similar to Europe, Australia had widespread use of avoparcin but didn't use vancomycin frequently to treat hospital infections. VRE have been found in hospitals in smaller towns where widespread vancomycin use is unlikely (Van den Boogard et al., 2005). This suggests that the VRE in Australia is spreading through the food chain, like in Europe. Although avoparcin was never administered in the United States, research on VRE in cattle would be beneficial to evaluate the prevalence of VRE in a farm environment.

Enterococci in soil

Most food-borne pathogens are enteric in nature, but are able to survive for extended periods of time in the environment. Lau and Ingram (2001) demonstrated that enterococci were inactivated quicker than *E. coli* in loamy soils when combined with bovine manure. In that study, the soils were exposed to environmental conditions similar to those in Wisconsin during late spring or early summer. Another study was conducted in the laboratory to investigate the survival of *Enterococcus* spp. and *E. coli* (Cools et al., 2001) in unautoclaved soils including: sandy, loamy, and loamy sand. In the study, contaminated pig slurry was mixed with soils at various ratios and stored at 5, 15, and 25ºC. At 5ºC, numbers of *Enterococcus* spp. remained constant in all soil textures, while numbers of *E. coli* dropped below the detection limit at day 68. At 25ºC, both species declined rapidly below the detection limit at day 54 for *Enterococcus* spp. and 28 for *E. coli* after inoculation. Among the soil types the sandy soil was the best for *E. coli* survival whereas being the worst for *Enterococcus* spp. survival. However, *Enterococcus* spp. survived best in loamy soil.

Researchers have performed field-based studies to determine how enterococci survive in soil. Andrews et al. (2004) inoculated enterococci into native soil microcosms as well as autoclaved soil and quantified the reduction in enterococci for 5 weeks. The native soil microcosms and autoclaved soil reduced enterococci cell numbers by 2 logs, and 5 logs respectively, suggesting that the native organisms found in soil may produce various compounds that aren't present in autoclaved soil that promote enterococci survival.

These studies described above illustrate the extended survival of enterococci in soil. The type of soil and resident microbial populations can affect *Enterococcus* spp. survivability. Knowing the effect of these factors could further the understanding of what supports the persistence or contributes to the inactivation of enterococci in soils. Treatment of animal waste before incorporation into the soil through proper composting will reduce or eliminate the likelihood of introducing problematic bacteria into the environment where survival can occur for extended periods of time.

Enterococci Contamination of Plants

A study was performed to assess the occurrence of enterococci on plants, which could be an indication of the amount of enterococci in the surrounding environment. Mundt (1961) found enterococci in corn enclosed tassels and silks on 22 out of 60 ears of corn tested. Enterococci were also detected on 10.4% and 27.5% of grass and flowers tested, respectively. There are major differences in the *Enterococcus* species present on plants versus those in animal waste. *E. faecium*, *E. faecalis*, *E. gallinarium*, and *E. hirae* make up almost all of *Enterococcus* population in animal waste while plants harbor almost exclusively *E. cassiliflavus*, *E. mundtii*, and *E. sulfurous* (Moore et al., 2007). In an attempt to elucidate if the presence of enterococci signifies fecal contamination, a study by Moore et al. (2007) was performed in Orange County, CA by examining the different species of enterococci from different locations in the county including bays, harbors, and wetlands. The results indicated that there was a mixture of fecal material-

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specific species as well as species exclusively found on plants, suggesting that contamination was caused by both fecal material and plants.

These studies demonstrate that contamination can originate from plant material and that incorporating contaminated material for use as a fertilizer can pose a risk to the safety of food supply. Therefore, waste material from produce must undergo treatment to inactivate potentially hazardous pathogens to prevent the contamination of crops.

Methods of Composting

The inactivation of pathogens in manure by composting is important to decrease their transmission into the environment and also to decrease the risk of contaminating fresh produce. This is extremely important for organic farming where manure based fertilizers are utilized in place of synthetic fertilizers. As the demand for organic produce increases, so must the ability to produce pathogen-free produce with the use of manure-based fertilizers.

The most common methods for composting include passive heaps, static heaps, windrow, and in-vessel composting systems (Sherman, 2005). Passive and static heaps both involve the stacking of composting materials into piles, referred to as heaps. The difference between static and passive composting is that static heaps are mechanically turned frequently to force aeration while passive compost heaps are not (Sherman, 2005). In windrow composting, composted materials are mixed and formed into long, narrow heaps, which are subjected to regular agitation (Sherman, 2005). In-vessel composting

uses a container or an enclosed staging area to perform composting and can involve

forced aeration (Cochran and Carney, 2006). Table 2 summarizes several different

substrates that have been used in compost.

Table 2. Time required for composting depending on the method and substrates used.
(Rynk, 1992)

Method	Materials	Active composting time		Curing time
		Range	Typical	
Passive composting	Leaves	2-3 years	2 years	-
	Well-bedded manure	6 months to 2 years	1 year	-
Windrow-infrequent[a] turning	Leaves	6 months to 1 year	9 months	4 months
	Manure + amendments	4-8 months	6 months	1-2 months
Windrow-frequent[b] turning	Manure + amendments	1-4 months	2 months	1-2 months
Passively aerated windrow	Manure + bedding	10-12 weeks	-	1-2 months
	Fish wastes + peat moss	8-10 weeks	-	1-2 months
Aerated static pile	Sludge + wood chips	3-5 weeks	4 weeks	1-2 months
Rectangular agitated bed	Sludge + yard waste or Manure + sawdust	2-4 weeks	3 weeks	months
Rotating drums	Sludge and/or solid wastes	3-8 days	-	2 months[c]
Vertical silos	Sludge and/or solid wastes	1-2 weeks	-	2 months[c]

[a] For example with bucket loader.

[b] For example, with special windrow turner.

[c] Often involves a second composting stage (for example windrows or aerated piles).

Government agencies of the United States have guidelines for composting to

prevent contamination of the environment and to reduce the risk of pathogens

contaminating crops. The Environmental Protection Agency (EPA) states that biosolid

compost's high temperatures help destroy pathogens under certain conditions (EPA,

2004). For Class A compost designation, which indicates pathogens are below

detectable levels, static aerated compost heaps containing biosolids should maintain

temperatures of 55ºC for a minimum of 3 days (EPA, 1994). Windrow composting

systems require temperatures of 55-77ºC for a minimum of 15 days with 5 turns

(Cekmecelioglu et al., 2005). The USDA recommends organic growers maintain their

compost at a temperature in the range of 55-70°C for 3 days for a static, aerated pile

system (NOSB, 2002). If all guidelines are followed the end product of composting can

be a pathogen-free soil amendment, which can provide nutrients to the soil.

Microbiology of Composting

Composting relies on micro-organisms to break down organic material to produce

water, carbon dioxide, humus, and heat (Glanville and Trampel, 1997).   Humus consists

of micro-organisms, non-biodegradable inorganics, and organic compounds that are

resistance to biodegradation. If done properly, composting is also an effective way of

inactivating potentially hazardous pathogens.

Many different substrates can be biodegraded through composting, but there are

some basic guidelines that will help ensure efficient composting. The carbon to nitrogen

(C:N) ratio is one of the most important parameters to monitor. A C:N ratio of 20:1 –

40:1 is acceptable for composting, but a 25:1 – 30:1 is ideal (Sherman, 2005). If there is

insufficient carbon it will hinder the organism's growth because of their reliance on

carbon for an energy source. The availability of carbon can also be an issue because of the difficulty to break down some materials that are rich in carbon and in some cases they may require fungi to convert the carbon source into a more usable form. Nitrogen is essential for protein synthesis and reproduction but if there is more nitrogen than can be effectively utilized it will be given off as foul smelling ammonia. Figure 1 summarizes the three distinct phases of composting.
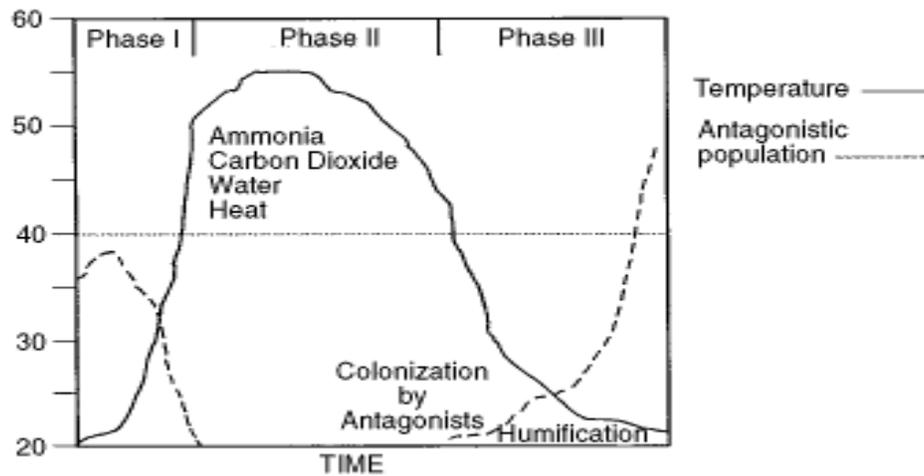
Figure 1. Phase changes during the composting process (Smith, 1992)

The moisture content of compost is an important parameter that can ultimately affect pathogen inactivation. For composting, a moisture content of 40-60% is desired for microbial activity, with 50-60% being ideal for optimum microbial activity (Imam, 2004). If the moisture percentage is too low the microbial activity will be slowed due to the lack of available water for microorganisms. However, if the moisture content is too high it will cause the heap to become anaerobic due to high amount of water and lack of oxygen, which will lead to foul odors and inefficient degradation.

Two other factors that influence composting are the pH and the size of the heap. The ideal pH range for composting is 6.5 – 8.0. The pH can become too high if there is more nitrogen in the compost heap than can be utilized by the microorganisms. If the pH falls too low the microorganisms won't have sufficient nitrogen and therefore biodegradation will begin to slow down. The size of the heap is also a factor in the efficiency of composting. If the heap is too small it won't be able to retain heat and therefore won't reach the thermophilic phase required to inactive potentially hazardous pathogens.

Enterococci in Compost

Compost is used as a growth medium for plants by providing them with nutrients. The raw ingredients of compost consist of biodegradable ingredients such as: manure, hay, sawdust bedding, feed waste, vegetable scraps, and other agricultural wastes. With the right conditions, micro-organisms present begin to break down the organic material in the compost heap which causes a rapid increase in temperature, especially in the first few days after mixing. Due to the cone shape of the heap, heat will naturally accumulate towards the top of the heap. A recent study by Venglovsky et al. (2005) observed the change in temperature in the center of two compost heaps consisting of pig waste and natural zeolite. By day 3 of the experiment the center temperature exceeded 55ºC and remained above this level for 15 days. The highest temperature recorded for pile 1 and pile 2 was 62.1ºC and 61.8ºC, respectively. By day 35 the internal temperature decreased

to an ambient temperature (20ºC to 35ºC) where it remained for the remainder of the experiment. The study reported that over the 3 month compost trial the enterococci population dropped by 4 logs.

A few studies have investigated the fate of enterococci in a compost heap and the rate at which the enterococci population declines over time. A study by Bhamidimarri (1996) observed the decline of the enterococci population in a compost mixture consisting of piggery solid wastes. The researchers added sawdust as a bulking agent to the heap to provide additional carbon and increase the porosity of the heap. The enterococci population decreased by 4 log values over the 3 month composting trial (Bhamidimarri, 1996). In another study, Pourcher et al. (2005) reported that enterococci declined by 4 log values over the course of a 7-month composting trial using a sewage sludge and straw mixture. A third study, performed by Tonner-Klank et al. (2005), reported a 4 log reduction in enterococci cell numbers through composting over 3 months using compost toilets. Collectively, these studies suggests that the compost was able to reach temperatures that could effectively inactivate enterococci, however the study did not test temperatures or enterococci cell numbers at various locations inside the compost heap, which could have helped elucidate how different heap locations inactivate enterococci differently. These studies also suggest that compost can be a reliable means to inactivate enterococci. However, there has not been any work done on the presence of VRE in different locations in a compost heap.

Inactivation of other Pathogens, Protozoans, and Viruses through Composting

Several pathogens such as *E. coli* O157:H7, *Bacillus subtilus*, *Listeria* spp. and *Salmonella* spp have been shown to become inactivated in compost. Kudva et al.(1998) reported that *E. coli* O157:H7 survived in a manure mixture for 21 months. While the study did not provide the temperatures of the heap it did suggest that *E. coli* O157:H7 may be able to persist in dairy waste composting. In another study, Shepherd et al. (2007) reported that *E. coli* O157:H7, at an initial cell concentration of ca. 10^7 CFU/g in dairy waste, became inactivated at all internal heap locations after 2 weeks of composting while the organism survived in surface samples for up to 4 months.

Several studies have also investigated the survival of various gram-positive bacteria in compost (Droffner and Brinton, 1999; McDonald et al., 1998). Droffner and Brinton (1999) inoculated compost with *Listeria* spp. at an initial concentration of 10^7 cells per gram. The researchers reported that *Listeria* spp. were undetectable after 1 week of composting, during which the temperatures reached 64°C. In addition, McDonald et al. (1998) reported that *Bacillus subtilis* was inactivated after 30 days in the mushroom compost mixture, which notably reached 65°C. Another study by Amner et al. (1991) showed that *B. subtilis* endospores were present after 28 days in mushroom compost. These studies demonstrate that composting is capable of inactivating potentially pathogenic organisms, but for the organisms that are capable of producing protective endospores, they may be able to persist in a compost ecosystem for extended periods of time.

In addition to bacterial pathogens, animal wastes can contain high levels of

parasites and viruses that may cause illnesses in humans. Composting can be an effective

means to inactivate viruses and parasites. Pourcher et al. (2005) reported that infectious

enteroviruses were inactivated after one month of composting using a sewege sludge-

straw mixture. Virus inactivation was determined after a virus-loaded solution was

inoculated into buffalo green monkey cells. The study also stated that other enteroviruses

were detected after 3 months of composting using PCR. However, these viruses were

determined to be noninfectious since they failed to be detected after culturing in an active

cell line. Ghaley et al. (2006) investigated the ability of the tobacco mosaic virus (TMV)

to survive in a compost mixture consisting of tomato plant residues-wood shavings-

municipal solid waste. The heaps had a C:N ratio and moisture content of 30:1 and 60%,

respectively. Following 96 hours of active thermophilic compost, the TMV was unable

to infect the leaves of susceptible hosts, including tobacco and tomato plants. The above

studies suggest that infectious enteroviruses and plant viruses can be effectively

inactivated through composting.

Rimhanen-Finne et al. (2004) examined the inactivation of *Cryptosporidium*

parvum and *Giardia intestinalis,* two parasitic protozoans, remaining after sewege sludge

disinfection at wastewater treatment facilities in a Finland. Wastewater sludge was

treated using the following composting techniques: windrow composting, mesophilic

anaerobic digestion, drum composting, and tunnel composting. The researchers reported

that after 30 weeks, the protozoan pathogens were not detected in sludge treated by any

one of the above mentioned composting methods.

The studies presented above demonstrated that potentially hazardous bacterial

pathogens including *Salmonella* spp., *Listeria* spp., *B. subtilis* and *E. coli* O157:H7 can

be inactivated through composting containing animal or human waste and that infectious viruses and parasites can be inactivated by composting wastewater sludge. However, further studies need to be performed in order to confirm that viruses and parasites present in animal wastes may be inactivated through composting.

Persistence of Resistance Genes During Composting

Vancomycin resistance can be spread among micro-organisms in several different ways. It would be beneficial to know the persistence of resistance genes in a compost heap and their ability to survive the composting process. The study by Andrews et al. (2004) analyzed the survival of enterococci and the Tn916 transposon in soil microcosms. The content of the Tn916 plasmid decreased by about 20% in both the autoclaved and non-autoclaved microcosms based on PCR of DNA extracts, whereas *E. faecalis* cell numbers decreased by 5 logs after 5 weeks. Similar results were also obtained when the source of fecal enterococci and Tn916-like elements was from swine waste. These authors concluded that the Tn916 stability in the soil results from a transfer of the transposon to the normal soil microbial flora and not reliant on the survival of *E. faecalis* DNA in the soil, suggesting that the soil is able to act as a reservoir for transposable elements involved in conveying antibiotic resistance. This study is pertinent to our research because the *vanA* gene is carried on the Tn1546 transposon and its presence in compost could have an effect on the presence of VRE.

Another concern over the persistence of VRE in the environment is whether the temperature of the compost heap has an effect on the presence of plasmids and therefore the spread of resistance to other micro-organisms. Guan et al. (2007) determined if temperature had an effect on the efficiency of plasmids present in chicken manure being able to infect uninfected chicken. At 23ºC both self transmissible RP4 and mobile pIE723 plasmids were successfully transferred to *E. coli* in chicken manure and in compost microcosms that consisted of a mixture of chicken manure and peat. When the temperature of the compost reached 50ºC or higher neither the plasmids nor their *E. coli* hosts could be detected, suggesting that composting of chicken manure at high temperatures could help to prevent the spread of antibiotic-resistant genes located on plasmids in the environment.

Heat Resistance of Enterococci

Enterococci generally tolerate temperatures ranging from 10 to 50ºC, however several studies have revealed that enterococci can survive elevated temperatures by pre-exposure of the culture to sub-lethal heating (Benachour et al., 2005). This elevated level of heat resistance has been attributed to the presence of heat shock induced proteins that prevent protein mis-folding, thus allowing the organism to survive. These heat shock proteins, or sigma proteins, are regulated by the *sigV* gene. In an attempt to determine the role of this gene in the heat resistance of enterococci, Benachour et al. (2005) exposed

two cultures of *E. faecalis,* one wild type and one *sigV* mutant, to heating at 62ºC. After 15 min of heat exposure the *sigV* mutants were 20- to 50-fold more susceptible to the heating compared to the wild type. This study emphasizes that the *sigV* gene and sigma proteins play a major role in allowing extended survival of enterococci at elevated temperatures.

To evaluate an organism's relative heat resistance the D and z-values can be determined using the thermal death curve of a culture. The D-value is defined as the time required, at a given temperature, to reduce the bacterial population by 1 log CFU/ml, whereas the z-value is the temperature shift required to reduce the D-value by 1 log value. McAuley et al. (2005) determined the heat resistance for two *Enterococcus durans* isolates and two *Enterococcus hirae* isolates which all originated from pasteurized milk. The D-values at 63ºC for *E. durans* and *E. hirae* were 26.6 – 29.0 min and 22.2 – 26.3 min, respectively. The D-values for *E. durans* and *E. hirae* at 72ºC were 2.7 – 3.1 min and 1.3 – 5.1 min, respectively. The z-values for *E. durans* and *E. hirae* were 8.7 - 8.8ºC and 7.5 - 9.3ºC, respectively. The D and z-values are similar for *E. durans* and *E. hirae*, thus indicating that both species are resistant to pasteurization and greater temperatures would be required to inactivate these bacteria.

The following studies compared the heat resistance between *E. faecalis* and *E. faecium*. The D-values of enterococci isolates from pasteurized milk at 70ºC were 6.5 and 3.5 min for *E. faecium* and *E. faecalis*, respectively (Batish, 1988). In another study analyzing the thermal inactivation of enterococci, Sörqvist (2002) determined the z-values for *E. faecalis* and *E. faecium* to be 9.5 and 9.6ºC, respectively, in liquid matrixes. Both studies suggest that *E. faecium* may be more heat resistant than *E. faecalis*.

Differences in heat resistance between the two species are important to elucidate because of differences in population size in the intestinal tract of animals, with *E. faecalis* and *E. faecium* making up 90 - 95% and 5 – 10%, respectively (Witte et al., 1993). These intestinal species could account for the enterococci species present in animal waste and ultimately affect the heat resistance and presence of enterococci in compost.

The sources of enterococci isolates may affect the organism's heat resistance. *E. faecium,* originating from ground beef, had D-Values at 60ºC, 65ºC, and 70ºC of 22.56, 4.47, and 1.11 min, respectively (Doyle et al., 2005). When compared with the D-Value of 6.5 min at 70ºC) for *E. faecium* from pasteurized milk (Battish, 1988), the *E. faecium* isolated from ground beef is less heat resistant. These data suggest that the enterococci subjected to non-lethal elevated temperatures, like those encountered during pasteurization, could increase enterococci's heat resistance.

Summary

The occurrence of VRE infections in humans is increasing rapidly, as is the difficulty in treating such infections due to its high levels of resistance to multiple drugs. The use of growth promoters, such as avoparcin, has contributed to the increase in VRE found in animal food products in Europe and Australia. Animal and plant waste can contain this microorganism if it is treated inadequately. Farmers typically use animal

waste or compost on crops, which could potentially contaminate the crops before they are harvested. Research has not been conducted on the fate of VRE in a composting system, therefore research needs to be done in order to determine the presence of VRE in compost and the changes in thermal resistance of VRE during composting. The objectives for our study were as follows:

- Enumerating enterococci and VRE in three different composting trials with various compost mixtures.

- Characterizing the antibiotic resistance profiles of the VRE isolates obtained from composting trials phenotypically and genotypically.

- Determining the heat resistance and persistence of VRE isolates during the 4-month composting trial.

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CHAPTER TWO

MICROBIOLOGICAL SURVEY AND CHARACTERIZATION OF ENTEROCOCCI AND VANCOMYCIN RESISTANT ENTEROCOCCI IN COMPOST

Abstract

In our study, two dairy compost heaps and one vegetable scrap compost heap were constructed on two research farms in Clemson, SC. Samples were taken from each heap from different locations at 8 elected intervals and analyzed for enterococci and VRE counts by spread plating on Bile Esculin agar (BEA) and BEA containing 6 μg/ml of vancomycin, respectively. The initial populations of enterococci and VRE in compost were in the range of 6.46-7.43 and 5.36-6.70 log CFU/g, respectively. After 30 days of active composting the average VRE populations declined ca. 4.6, 4.07, 3.97 and 1.6 logs at the top, center, bottom and surface locations of the heaps, respectively, whereas the enterococci populations declined for 4.36, 3.54, 3.10, and 2.28 logs, respectively. During the two dairy compost trials, the temperatures at the top, center, and bottom locations were in excess of 55°C for 14 – 18, 12, and 8 days, respectively, whereas temperatures of the surface samples never exceeded 32°C. During thermophilic composting, microbial activity slightly reduced the pH, there after it began to rise slightly due to the production of ammonia. The moisture content inside the heaps changed very little during active composting in any of the 3 trials.

Both enterococci and VRE isolates obtained from compost trials were further characterized for genus and species determination, minimum inhibitory concentration (MIC), and heat tolerance screening. Our results revealed that the genus of all 88 VRE isolates was enterococci and that no VRE were *Enterococcus faecalis* or *Enterococcus faecium*. However, 9 and 6% of enterococci isolates from Trial 1 were tested as *E. faecalis* and *E. faecium,* respectively. For those VRE isolates taken from composting, MIC testing revealed that 85 out of 88 suspected VRE isolates were resistant to > 256 μg/ml of vancomycin and all 88 isolates were resistant to > 64 μg/ml of teicoplanin. Eighty-three (94%) of VRE isolates were confirmed to contain the *vanA* gene for vancomycin resistance as well as 4 (11%) enterococci isolates from Trial 1. The D-values of selected VRE isolates (n=8) from Trial 1 compost heaps were in the range of 9.7-17.73 min at 60°C, 4.73-12.57 min at 65°C, and 1.59-4.44 min at 70°C. The z-values for those VRE isolates ranged from 11.92-17.87ºC. The results revealed that the isolates from day 30 had the highest D-values while isolates from day 60 had the highest z-value, as compared with isolates from day 0 which had the lowest D- and z-values. Box-PCR analysis of VRE isolates (n=12) from Trial 1 revealed that 42% were identical and appeared on compost days 7, 14, and 60, suggesting that this specific strain was able to adapt to the elevated temperatures in compost by developing heat resistance.

Our studies demonstrated that VRE may become inactivated in compost if conditions are optimal and that some enterococci may develop heat resistance during the thermophilic phase of composting. This is important because it suggests that improperly composted manures may serve as a means for the spread of VRE on food products intended for human consumption.

Introduction

Enterococci are a Gram-positive, facultativly anaerobic and are commensal organisms that occupy the intestinal tract of humans and other animals (Fischetti et al., 2000). Enterococci are known for their rapid acquisition of antibiotic resistance, especially to vancomycin, by either obtaining a resistance transposon from resistant enterococci or originating from an organism with a resistance gene inserted in its chromosome (Yoo, 2006). Vancomycin resistant enterococci (VRE) have been shown to survive concentrations of vancomycin over 256 µg/ml (Eisner, 2005), and display induced resistance when treatment is administered with antibiotics that the organism was previously susceptible to (Ike, 2002). Indeed, resistance to high levels of antibiotic can cause serious problems when attempting to treat an infection, therefore it is important to limit the introduction of VRE into the environment.

Another characteristic of VRE is their ability to survive at high temperatures. A study by Benachour et al. (2005) showed that enterococci were able to survive for 120 minutes at 62°C in a liquid matrix. The thermal tolerance of enterococci has been shown to result from heat shock induced sigma proteins that prevent protein mis-folding at high temperatures, allowing the organism to survive (Benachour et al., 2005). Therefore, VRE subjected to sub-lethal temperatures, such as those experienced during the mesophilic phase at the beginning of composting, could experience the extended survival.

The heat resistance of microorganisms is often quantified by determining their D and z-values (Batish, 1988; Sörqvist, 2003). The D-value is defined as the time required at a given temperature to reduce the bacterial population by 1 log CFU/g and the z-value

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is the temperature increase required to reduce the D-value by 1 log. Enterococci isolates from pasteurized milk showed D-values at 70ºC of 6.5 and 3.5 min for *E. faecium* and *E. faecalis*, respectively (Batish, 1988). Sörqvist (2003) determined the z-values for *E. faecalis* and *E. faecium* to be 9.5 and 9.6ºC, respectively, in a liquid matrix. Both D and z-values are commonly used to determine the amount of time and heat necessary to effectively inactive a bacterial population.

Enterococci and VRE have been detected in agricultural settings (Andrews et al., 2004; Lim, 2006). Andrews et al. (2004) found *E. faecalis* present in swine waste obtained from a farm at an initial concentration of 4.8×10^5 CFU/g. Lim (2006) reported that VRE were detected in 16.7 and 1.9% of chicken and pig waste samples, respectively, from a farm where avoparcin was used 4 years previously. Avoparcin, a growth inducing feed additive, has been shown to increase the occurrence of VRE on farms where it was used (Kruse et al., 1999).

Composting is used to convert organic waste into a soil amendment, and also to eliminate potentially dangerous pathogens from animal waste or other agricultural wastes. Composting is primarily driven by microbial activity where the microorganisms present in compost break down organic material and cause the subsequent rise in temperature (Ghaley et al., 2005). It is commonly accepted that elevated temperatures in composting is the major mechanism resulting in the elimination of pathogenic bacteria. If performed under optimal conditions, composting is an effective method for inactivating potentially pathogenic organisms, such as enterococci (Pourcher et al., 2005; Tonner-Klank et al., 2005). Enterococci have been observed to decline by 4 logs over the course of a 7-month composting trial using a sewage sludge and straw mixture (Pourcher et al.,

2005). In another study the enterococci population in a swine based compost mixture decreased by 4 logs over a 3 month composting trial (Venglovsky et al., 2005). However, there have been no studies performed on the fate of VRE during composting. The objectives of this study were to 1) determine the fate of both enterococci and VRE at different locations of compost heaps, which consisted of two types of compost materials in the 4-month composting trials, 2) characterize VRE isolates, 3) determine the relative heat resistance of those VRE isolates by determining their D and z-values, and 4) determine if the same strains of VRE were able to survive the entire composting process.

Materials and Methods

Composting on farm and sampling: A total of three compost heaps were constructed for this study. The compost heaps from Trials 1 and 2 consisted of sawdust-dairy manure mixture, old hay, waste cattle feed, and fresh hay, which were mixed thoroughly with a front loader at a ratio of 28:16:8:1, respectively, on a volume basis. The resulting compost mixture had a carbon to nitrogen ratio of 24:1 and a moisture content of ca. 60%. The compost heap from Trial 3 consisted of dairy manure, sawdust and calf manure, waste feed, and vegetable residues containing beans, kale, red cabbage, and lettuce, and the heap was mixed thoroughly with a front loader at a ratio of 8:4:1:1, respectively, on a volume basis. The resulting compost mixture of Trial 3 had a carbon to nitrogen ratio of 22:1 and a moisture content of ca. 47%. All three heaps measured 2 m

in diameter at the base, between 1.1 and 1.2 m in height, and were conical in shape. All on-farm composting trials were conducted on the research farms of Clemson University.

Thirty grams of the compost samples were taken from 4 different locations throughout the compost heaps as shown in Figure 1. For Trials 1 and 2, samples were taken from the surface, top, center, and bottom locations. There were 3 sample locations in Trial 3: surface, center, and bottom. The bottom, center, and top samples were collected from 30cm, 50cm, and 70cm from the stage of composting, respectively, and the surface samples were taken directly from the surface of the heap. Samples were taken on days 3, 7, 14, 21, 30, 60, and 120 into composting.

Temperature, pH, oxygen content, C:N ratio, and moisture determinations: A Demensta Instruments OT21 (Demensta Instruments, Arlington Heights, IL) temperature and oxygen sensor was used to record the temperature and oxygen content at each location on the sampling day prior to the opening of the compost heap (Shepherd et al., 2007). Composting temperatures were measured daily for the first week and then taken weekly. After 30 days, the temperature was taken monthly. For pH and moisture content determination, approximately 1 g of each sample was weighed in an aluminum dish and then dried in an oven (Blue M Electric Company, Blue Island, IL) at 105°C for 24 h (Shepherd et al., 2007). About 100 g of initial compost mixture in duplicate were sent to the Agricultural Service Lab at Clemson University for C:N ratio testing (Shepherd et al., 2007).

Enumerating enterococci from compost: Samples were aseptically collected in duplicate from each location of the compost heap. The samples were analyzed

microbiologically immediately upon arrival at the lab. Ten grams of each sample were mixed with 90 ml of sterile phosphate-buffered solution (PBS) and homogenized in a Stomacher 400 Circulator (Brinkman Instruments, Inc., Westbury, NY) at medium speed for 1 min. Serial dilutions were plated on both Bile Esculin Agar (BEA) (Becton, Dickenson and Company, Sparks, MD) and Bile Esculin agar containing 6 μg/ml of vancomycin (BEA-V) (Sigma-Aldrich Co., St. Louis, MO). The plates were incubated at 37ºC for 24 to 48 h. The colonies that had a dark center with a brown halo on BEA and BEA-V were presumed to be enterococci and VRE, respectively. The detection limit was 10 CFU/g.

Isolation and characterization of enterococci isolates: Based on colony morphology, five different VRE colonies were selected from the surface and center samples on each sampling day for each composting trial. Five enterococci isolates were also picked on each sampling day from Trial 1. The isolates for samples collected in all locations were re-streaked several times on BEA and TSAS plates, and then the purified isolates were preserved in Tryptic Soy Broth (TSB) (Becton, Dickenson) containing 20% glycerol, and stored in a -80ºC freezer. All isolates were then characterized by gram staining, ability to grow in a 6.5% NaCl solution at 45ºC, and catalase reaction testing as described by Brown (2008). Only those isolates that were gram positive, catalase negative, and able to grow in a 6.5% sodium chloride solution were presumed to be enterococci and were tested further.

Minimal Inhibitory Concentration: The VRE isolates kept in the -80°C were streaked onto Mueller Hinton Agar (MHA) (Becton, Dickenson and Company, Sparks, MD) and incubated at 37ºC for 24 h. A single colony was transferred to 5.0 ml of

Mueller Hinton Broth (MHB) (Becton, Dickenson and Company). The tubes were incubated on a shaker at 37ºC for 24 h. After incubation the bacterial cultures were pelleted by centrifugation (3,100 x g, 5 min, at room temperature) in a Fisher Scientific Centric™ Centrifuge (Thermo Fisher Scientific Inc., Waltham, MA) and resuspended in 0.85% saline solution to match the McFarland #2 standard (ca. 1 x 10^8 CFU/ml). The suspended inoculum was diluted 1:100 in sterile MHB to a final concentration of 1 x 10^6 CFU/ml. Samples were then added into a Costar® 96 well plate (Corning Incorporated, Corning, NY) yielding a final bacterial concentration of 5 x 10^5 CFU/ml. The samples were plated using a Replica Plater (Sigma, St. Louis, MO) in triplicate onto MHA containing different concentrations of vancomycin (8, 16, 32, 64, 128, and 256 µg/ml)(Sigma, St.Louis, MO). The samples were also plated in triplicate onto MHA containing 64 µg/ml teicoplanin (Supelco, Bellefonte, PA). The plates were incubated for 24 to 48 h at 37ºC and the growth was recorded.

PCR identification of genus, species, and vancomycin resistance gene of vancomycin resistant enterococci isolates: A total of 88 VRE isolates were obtained from compost heaps. Those VRE isolates were stored in a -80°C freezer and were then streaked onto Tryptic Soy Agar (TSA) (Becton Dickinson) and incubated for 24 h at 37ºC.

To prepare the bacterial DNA, a loopful of each isolate was added to 1 ml of sterile, distilled water and boiled for 5 min. The PCR primers, reaction mixtures, and conditions for *Enterococcus* genus, species, and *vanA* identification are listed in Tables 2.5, 2.6, and 2.7. The reaction mixtures were thermocycled in a Bio-Rad®iCycler (Bio-Rad Hercules, CA). The PCR products were electrophoresed on a 1.5% agarose gel.

After electrophoresis, the gel was stained in ethidium bromide for 15 min, destained for 15 min in distilled water, and photographed by GelDoc (Bio-Rad Laboratories). An *E. faecalis (*ATCC#29212) standard strain was used in genus and species identification as a positive control. Additionally, an *E. faecium* (ATCC#51229) standard strain containing the *vanA* gene was used as a positive control for species and *vanA* gene detection. All standard strains were obtained from the USDA-ARS (Charlene Jackson in Athens, Ga).

Heat tolerance screening: Two VRE isolates on each sampling day 0, 3, 7, 14, 30, 60, and 120 from Trial 1 compost heaps were grown on TSA for 24 h at 37°C. A single colony was transferred into a test tube containing 5 ml of Tryptic Soy Broth (TSB) (Becton Dickinson), vortexed, and incubated for 24 h at 37°C on a shaker. The tubes were then centrifuged at 3,100 x g for 5 min and the pellet was resuspended with 0.85% saline solution to obtain an OD at 640 nm of 0.5 (Biotek, Winooski, VT). The samples were then diluted with TSB to a final concentration of ca. 5 x 10^6 CFU/ml. Samples were tested in triplicate at 65°C at the following time intervals: 5, 10, 15, 20, 40, and 60 min. Heat screening was performed in a HaakeV26 water bath (Thermo Haake GmbH, Karlsruhe, Germany) and heated with a Haake DL 30 immersion coil apparatus accurate to ± 1ºC. J-Type Thermocouples (DCC Corporation, Pennsauken, NJ) were inserted in the water bath and inside a tube containing TSB to ensure the bacterial culture reached the desired temperature for the entire time interval. Thermocouples were connected to a HotMux Data Logger (DCC Corporation) and analyzed using Visual HotMux 2.5.1 software (DCC Corporation). After heating for the predetermined time interval, triplicate sample tubes were removed and placed into an ice bath immediately. The sample tubes were incubated at 37°C for 24 h and turbid growth was recorded.

D-value determination: Two VRE isolates on each sampling day 0, 7, 30, and 60 from composting Trial 1 were selected for D-value determination. The isolates were prepared using the same methods described for heat tolerance screening. Samples were tested in duplicate at 60°C, 65°C, and 70°C, and duplicate sample tubes were removed after heating for the predetermined time intervals and immediately placed into an ice bath. The samples were then serially diluted, plated onto TSA, and incubated for 24 h at 37°C. The D-values were calculated by plotting the thermal death curve of the bacterial cultures to obtain the time required to reduce the bacterial population by 1 log CFU/ml. The z-value was calculated from graphing the log D-values versus temperature to obtain the temperature shift required to reduce the D-value by 1 log. ATCC standard strains *E. faecalis* (ATCC#29212) and *E. faecium* (ATCC#51229) were also tested as controls.

Strain identification of selected VRE isolates by Box-PCR: The DNA of VRE isolates screened for heat resistance was prepared as described above. The Box-PCR assay was performed at a final volume of 20 μl consisting of 0.2 mM dNTPs, 2.0 μl of 10x buffer, 2.0 mM $MgCl_2$, 0.2 U AmpliTaq, 1.0 μl DMSO, 20μg BSA (Promega), 0.6 μl sterile, distilled water, and 2.9 pmol of BoxA1R primer (CTACGGCAAGGCGACGCTGACG) (Invitrogen). The PCR protocol was as follows: initial denaturing at 95ºC for 7 min followed by 35 cycles of 94ºC for 1 min, 53ºC for 1 min, and 65ºC for 8 min with a final extension at 65ºC for 16 min. The PCR product was electrophoresed in a 1.5% agarose gel for 3.5 h at 50 volts, and then stained, washed, and photographed as described above.

Statistical Analysis: Plate count data were converted to $\log_{10}$ CFU/g for statistical analysis. An analysis of variance (ANOVA) for a completely randomized design with repeated measures across dates was conducted to determine if general differences existed between treatment means using the general linear model (GLM) procedure. Tukey HSD (honestly significant difference) was employed to compare each sampling date for every heap location. For composting temperature data, an analysis of variance (ANOVA) for a completely randomized design with repeated measures across dates was conducted to determine if differences existed between different locations. All statistical analysis was performed using the Statistical Analysis System 9.1 (SAS: SAS Institute. Cary, NC).

Results

Three on-farm composting trials were conducted on the research farms of Clemson University. Trials 1, 2, and 3 began in October of 2005, April of 2006, and August of 2006, respectively, with each trial lasting 4 months. Shepherd et al. (2007) concurrently sampled from the same compost heaps as used in this study and were responsible for taking all temperature, pH, and moisture content data from all compost heaps, which served as our temperature, pH, and moisture content profiles.

Temperature profiles and oxygen content: A rapid rise in internal temperature of the compost heaps occurred soon after the initial mixing of the heap. Figures 2.2, 2.3,

and 2.4 reveal the temperature stratification of the compost heaps from all three trials. The compost temperatures from the highest to lowest at those locations were as follows: top > center > bottom > surface. While temperature stratification occurred throughout the heaps during active composting, temperatures were elevated above 50°C during thermophilic composting at every location in the heaps for at least 21 days in Trial 1, 30 days in Trial 2, and 14 days in Trial 3. Similarly, temperatures reach ca. 55°C for 25, 38, and 17 days for Trial 1, 2 and 3, respectively. The higher temperatures inside heaps observed in Trial 2 were probably affected by the high ambient temperatures experienced during the summer, while the other two were performed during the fall or spring months. For all three trials, the maximum temperature was recorded at the top location.

Oxygen contents of the heaps were low among all Trials, however, microaerophilic conditions existed within the heaps for all Trials with oxygen contents ranging from 0 – 6% (data not shown). In all trials the oxygen content was low, initially; however, as composting progressed, the moisture content of the heaps decreased while the oxygen content increased.

pH and moisture content determinations: The results from Trials 1, 2, and 3 were similar in that all compost mixtures were neutral to slightly alkaline at day 0 with pH values in the range of 6.57-8.53 (data not shown). Trials 1 and 2 recorded mild decreases in pH following the third sampling day while the pH of all heap locations in Trial 3 increased. The pHs of internal samples from Trials 1 and 2 rebounded back to alkaline, reaching a maximum of 10.01 at the bottom from day 21 in Trial 1 and 9.94 in the center from day 60 in Trial 2. Trial 3 reached a maximum pH of 9.49 at the center and bottom on day 21. For Trials 1, 2, and 3 the internal locations had significantly ($p <$

0.05) higher pH values than the surface following days 21, 60, and 30, respectively, for each trial. For the surface samples in each trial, the pH decreased gradually towards the end of the composting trials and was close to neutral.

Throughout most of the sampling days in Trials 1, 2, and 3, the moisture content of the internal samples changed only slightly (data not shown). Trials 1 and 2 were similar and had 3 sampling days where the internal samples were significantly ($p < 0.05$) different whereas Trial 3 had 4 days. However, the surface samples at most all sampling days contained significantly ($p < 0.05$) less moisture than the internal samples.

Survival of enterococci and VRE during composting: For the initial compost mixture, VRE accounted for 2% of the enterococci population in Trial 1. Within 3 days of composting, both enterococci and VRE were inactivated rapidly with population reductions of 2.0-2.2 and 1.2-1.8 logs throughout the heap, respectively. At the end of 120 days of composting the populations of VRE were reduced by 2.17, 4.25, 3.70 and 3.28 logs at the surface, top, center, and bottom of the heaps. A similar trend was observed for enterococci. By the end of 120 days of composting in Trial 1, the VRE population accounted for 50%, 54%, 19% and 8% of the total enterococci enumerated in samples on the surface, top, center, and bottom, respectively, from Trial 1 (Table 2.1).

VRE accounted for 19% of the initial compost mixture in Trial 2. Within 3 days of composting, both enterococci and VRE populations were inactivated rapidly with reductions of 0.77-1.00 and 0.04-2.32 logs at all locations, respectively. At the end of 120 days of composting the populations of VRE were reduced by ca. 5.29-5.41 logs at the below the detection limit at the top, center, and bottom of the heaps. A similar trend was observed for enterococci. By the end of 120 days of composting VRE was undetectable

in all internal heap locations, but remained at 1.35 logCFU/g on the surface. However,

on the last day VRE was detected in internal heap samples it accounted for 1.5%, 0.04%,

and 0.03% of the total enterococci enumerated in samples from the top, center, and

bottom, respectively.

In Trial 3, VRE accounted for 26% of the initial compost mixture. Within 3 days

of composting, both enterococci and VRE populations were inactivated with reductions

of 0.56-1.00 and 0.63-1.86 logs, respectively, throughout the heap. At the end of 120

days of composting the populations of VRE were below the detection limit at the center

and bottom locations and remained 1 log/CFU/g at the surface of the heap. A similar

trend was observed for enterococci. By the end of 120 days of composting in Trial 3, the

VRE population accounted for 44%, 50%, and 15% of the total enterococci enumerated

in samples on the surface, center, and bottom, respectively, from Trial 3.

Average log CFU/g reduction rates were calculated for the first 7 days of

composting for each location because of rapid internal heating of the heap and the linear

reduction in bacterial populations recorded during the first 7 days (Table 2.4). The log

reduction rates at heap locations for enterococci (BEA) and VRE (BEA-V) for all three

trials were in the following order: top > center > bottom > surface.   The bacterial

inactivation rates were highest in Trial 2 for both enterococci and VRE populations.

Statistical analysis comparing VRE and enterococci population inactivation

during composting at each location in the heap revealed that VRE and enterococci

populations inside the heap were reduced significantly ($p < 0.05$) after 3 days of active

composting throughout all three trials. However, there was an insignificant reduction in

VRE (Table 2.1 and 2.3) on the surface location in Trials 1 and 3. In Trial 2 the surface

location had a slower reduction in enterococci from day 14 until day 60 with similar results observed in the other two trials. In Trial 2 the VRE population in the top of the heaps was reduced significantly ($p < 0.05$) every sampling day until the population was undetectable and similar trends were observed in Trial 3 (Table 2.2 and 2.3).

Further statistical analysis comparing different heap locations revealed that the top location had significantly ($p < 0.05$) less VRE and enterococci than other locations following 7 days of composting throughout all three trials (Table 2.1, 2.2, and 2.3). All four locations in heaps from Trial 1 had significantly ($p < 0.05$) different levels of VRE after 14 days of composting in the following order: surface > bottom > center > top. Similar results were observed with enterococci.

Phenotypic characterization: A total of 105 presumptive VRE and 35 presumptive enterococci were isolated from all 3 composting trials. Characterization by gram staining, NaCl testing, and catalase reaction confirmed that 84% and 91% as VRE and enterococci, respectively.

Determination of Minimal Inhibition Concentration (MIC): MIC testing revealed that 85 out of 88 VRE isolates were resistant to > 256 µg/ml of vancomycin. Out of the remaining 3 isolates, two had a MIC for vancomycin of > 128 µg/ml and the remaining isolate was resistant to > 64 µg/ml. Three *E. faecalis* and two *E. faecium* isolates from Trial 1 had MICs for vancomycin of 32 µg/ml. Teicoplanin, a glycopeptide antibiotic, has been used to differentiate *vanA* and *vanB* phenotypes of enterococci. All 88 isolates were able to grow on TSA agar containing 64 µg/ml of teicoplanin.

Genus, species and *vanA* detection using PCR: Eighty-eight VRE isolates taken from all three composting trials were enterococci as confirmed with PCR by amplifying enterococcal genus-specific primers (Figure 2.5). PCR was also used for species identification using species-specific primers for *E. faecalis* and *E. faecium* identification. None of the 88 VRE isolates yielded a 215 bp (*E. faecalis*) or a 360 bp (*E. faecium*) amplicon (Figure 2.6). Species-specific PCR testing was also performed on 32 enterococci isolates from Trial 1, which were enumerated on media that didn't contain vancomycin. Of the 32 enterococci isolates from Trial 1 tested, 2 (6%) were confirmed to be *E. faecium* and 3 (9%) were *E. faecalis* (Figure 2.7). Those isolates MIC for teicoplanin was confirmed to be > 64 µg/ml. PCR was also used to detect the *vanA* gene for vancomycin resistance in those 88 VRE isolates isolated from Trials 1, 2, and 3, and 32 enterococci isolates from Trial 1. Out of the 88 VRE isolates screened, 83 (94%) produced the appropriate 732 bp amplicon, indicating the presence of the *vanA* gene (Figure 2.8). Out of the 32 enterococci isolates tested, 4 (13%) contained the *vanA* gene for vancomycin resistance; 1 from day 30, 2 from day 60 and 1 from day 120. However, none of the *vanA* VRE were either *E. faecalis* or *E. faecium*. In all PCR reactions, a negative control was used to rule out cross-reactivity of the primer sets.

Heat tolerance screening: VRE isolates taken at various sampling days from both the center (50 cm above the stage of the compost heap) and the surface of the heap were selected for heat resistance testing. Two trials of heat screening were performed in triplicate for each isolate at 65°C and the results revealed that isolates from days 30 and 60 were the most heat tolerant, with one isolate from day 30 surviving for 30 min (Figure

2.9). Figure 2.9 also reveals that the isolates from day 3 were the most heat susceptible, with one isolate surviving only 5 min.

D and z-value determination: D-values (time required to decrease viable count by 1 $\log_{10}$ CFU/ml) were determined at three temperatures for each isolate (Table 2.9). D-values of selected VRE isolates were in the range of 9.7-17.73 min at 60°C, 4.73-12.57 min at 65°C, and 1.59-4.44 min at 70°C. The center isolate from day 0 had significantly (p< 0.05) lower D-values at 65°C and 70°C than all other isolates analyzed from compost, whereas center isolates from day 30 had significantly (p<0.05) higher D-values than all other analyzed samples at all three temperatures. It's also important to note that the D-values of the center samples from days 30 and 60 were significantly (p<0.05) higher than the D-value of the surface isolate at the respective temperature except for the day 60 isolates at 60°C. The z-values were higher for all 4 of the center isolates when compared to the surface sample from the respective sampling day (Table 2.9), and z-values increased as composting proceeded. Both ATCC strains had the lowest D and z-values compared with the values of all isolates obtained from compost.

Box-PCR: Box-PCR was used to determine if the same species are able to persist throughout the entire composting trial, which would suggest that the organisms developed heat resistance. Box-PCR analysis of VRE isolates (n=12) from heat tolerance screening revealed that there were 8 unique genogroups (Figure 2.10), which were established based on the banding patterns of those isolates. Sixty-seven percent (7/12) had unique genogroups; however 42% (5/12) were in the same genogroup. Isolates of the same Box-PCR pattern originated from the center and surface of days 7, 14 and 60 of

composting, suggesting the persistence of this VRE isolate during composting. The D-values for the isolates from day 60 were significantly ($p<0.05$) higher at all tested temperatures than the D-values of the isolates obtained after 7 days in compost.

Discussion

Animal waste contains some bacterial species that are associated with food borne diseases, and therefore, limiting the introduction of these pathogens into the environment is of key importance. It is commonly accepted that elevated temperatures in composting is the major mechanism resulting in the elimination of pathogenic bacteria. If performed under optimal conditions, composting is an effective method for inactivating potentially pathogenic organisms, such as *E. coli* O157:H7 and enterococci (Shepherd, 2007; Tonner-Klank et al., 2005). The elimination of potentially pathogenic organisms through composting can ultimately reduce the amount of food borne illnesses. In our study, three, 4-month composting trials were surveyed for changes in enterococci and VRE populations. Due to the conical shape and heterogeneous makeup of compost, the temperatures in compost heaps may vary throughout (Shepherd et al., 2007), and therefore, would affect the survival of bacterial populations. To address this variability, samples were taken from 4 different locations in Trials 1 and 2, and 3 different locations in Trial 3 for enterococci and VRE enumeration.

The USDA recommends organic growers maintain their compost at a temperature in the range of 55-70°C for 3 days for a static, aerated pile system (NOSB, 2002). A carbon to nitrogen (C:N) ratio of 20:1-40:1 is acceptable for composting, but a 25:1-30:1 is ideal (Sherman, 2005). The C:N ratio of all three trials was within the acceptable range, and the temperature in each heap was above 55°C for 14 to 21 days. The temperature data from all three trials revealed that there was temperature stratification throughout the heaps. Inside the heaps the warmest location was near the top of the heap, 70 cm from the base, while the coolest location was at the bottom, slightly above the concrete pad. This temperature stratification occurred because the conical shape of the heaps allowed for the heat, produced from microorganism activity, to rise towards the top.

In this study, temperature, oxygen content, pH value, and moisture content were determined at each sampling location at each sampling interval (Shepherd et al., 2007). During the thermophilic phase of composting, microbial activity reduced the pH (data not shown) within 7 days, where after the pH began slightly to rise possibly because the organic acids present were consumed as ammonia was produced (Beck-Friis et al., 2003). The slight reduction in pH caused by organic acid production is evident in Trials 1 and 3 on day 3 where the pH of the surface location was significantly ($p < 0.05$) higher than the pH of internal samples probably due to various levels of microbial activity. The moisture content of the internal samples changed slightly during active composting in any of the 3 trials, suggesting that moisture content had a minimal effect on enterococci or VRE inactivation (data not shown). However, the initial moisture content of each compost mixture may have affected the heating in the early stages of composting, which would

have also affected enterococci and VRE inactivation. The oxygen content rose (data not shown) as composting progressed while there was a subsequent slight decrease in moisture content. These changes were brought on because the heat generated by microbial activity helped to dry out the heap, effectively increasing the amount of pore space in the heap and, in turn, increasing the oxygen content. Trials 1 and 2 included in this study had similar initial compost mixtures, moisture content (56.5%), and C:N ratios (22:1). Compared with Trials 1 and 2, Trial 3 had a higher C:N ratio (24:1), and a lower moisture content (47%). The large volume of vegetable scraps and sawdust, which contain large amounts of carbon, could have caused the higher C:N ratio recorded for Trial 3. Although the moisture content values for all 3 trials fall within acceptable values for composting, enterococci growth has been shown to decline when the moisture content drops below 50% (Ward, 1981). The lower moisture content in combination with temperatures sustaining 60°C for 14 days in the center of Trial 3 may have contributed to inactivate VRE to undetectable levels by sampling day 120 in internal locations. It is important that initial compost measurements are within acceptable ranges to ensure that conditions are optimal for microbial activity, which, in turn, will increase the temperature necessary for pathogen inactivation.

Previous studies have reported that enterococci can become inactivated from an initial population of 4-5 logs in 3-8 days in laboratory-scale bioreactors, under controlled environmental conditions (Niwagamba et al., 2008; Vinnerås, 2006). Fewer studies have addressed the inactivation of enterococci in composted manure heaps under field conditions. Furthermore, it was not indicated if those studies were performed using optimal C:N ratios or moisture content of composting materials. A study by Pourcher et

al. (2005) observed the decline of enterococci population by 4 $\log_{10}$ over the 7-month composting trial in a compost mixture consisting of rural sewage sludge. Another study, performed by Tonner-Klank et al. (2005), reported a 4 log reduction in enterococci cell numbers through composting over 3 months using compost toilets. Our results revealed that there were variations in the durations of enterococci and VRE as affected by many factors, such as compost compesition, seasonality, and locations inside the heap. Studies have reported the persistence of other pathogens in compost to different extents such as *E. coli* O157:H7 and *Salmonella* spp. Olson (2005) reported that *E. coli* O157:H7 and salmonella are inactivated in composted manure after 7 and 7-14 days, respectively. While Olson (2005) did not reveal the temperatures of the heap or the specific compost mixture, that study does however, suggest that salmonella may be able to persist for longer periods of time compared to *E. coli* O157:H7. Shepherd et al. (2007) examined the survival of *E. coli* O157:H7 in the same compost heaps as used in our study. *E. coli* O157:H7 at an initial cell concentration of ca. 10^7 CFU/g became inactivated at all internal heap locations after 2 weeks of composting, but in surface samples it survived for up to 4 months. In our study, enterococci and VRE survived up to 120 days in all internal heap locations in compost when the initial cell numbers were ca.10^7 CFU/g and composting was performed during the fall. This suggests that both enterococci and VRE are better able to persist for a much longer time in compost compared with *E. coli* O157:H7 and other foodborne pathogens.

The inactivation of pathogens in compost and the temperature maintained in the heap have been shown to have an inverse correlation (Shepherd et al., 2007). The enterococci and VRE populations became undetectable at the top of the compost heaps

after 60 and 30 days, respectively, when initial cell numbers were ca. 10^7 and composting was performed during the summer (Trial 2). Trial 2 was unique in that there was a significant ($p < 0.05$) reduction in the amount of both VRE and enterococci cell numbers after 3 days of composting at all heap locations. To demonstrate the effect of each location on enterococci and VRE inactivation, the average log reduction rates were calculated for the first 7 days of thermophilic composting for each location (Table 2.4). The inactivation rates also correlated with the temperature stratification at internal heap locations. The log reduction rates at heap locations for enterococci (BEA) and VRE (BEA-V) for all three trials are in the following order: top > center > bottom > surface. The log reduction rates were higher for the trials that experienced the most rapid inactivation of VRE, with Trial 2 recording the highest log reduction rates and inactivating VRE to below detection limits at all internal heap locations by day 60. Statistical analysis from Trials 1 and 3 revealed that surface locations were the only locations to not significantly ($p > 0.05$) reduce the amount of VRE after 3 days, suggesting that the lower temperatures experienced on the surface may have contributed to extended bacterial survival. The extended survival of VRE on the surface on the compost surface is problematic because they can spread VRE to the surrounding environment such as water, soil, and agricultural crops. In contrast, the top location in all three trials had significantly ($p < 0.05$) less enterococci and VRE than all other locations after 7 days of composting. This correlates with the temperature data and suggests that both enterococci and VRE inactivation is dependent on temperature. Variations in enterococci inactivation from different studies can be due to many different variables including the raw materials in the compost mixture, C:N of the mixture, moisture content,

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size and shape of the heaps, surrounding environment's temperature (seasonality), and the frequency of turning of the heaps (Pourcher, 2005; Tonner-Klank et al., 2005; and Shepherd et al., 2007).

In Trial 1 the initial VRE population constituted only 2% of the entire enterococci population, however on day 120 the VRE population accounted for 50%, 54%, 18%, and 8% of the enterococci populations at the surface, top, center, and bottom locations, respectively (Table 2.4). Similarly, VRE accounted for the largest percentage of total enterococci enumerated in the hottest heap location in all 3 trials. This suggests that some VRE may have developed heat resistance compared to non-VRE. Benachour et al. (2005) reported that enterococci develop elevated heat resistance by pre-exposure to sub-lethal heating and showed that enterococci were able to survive for 120 minutes at 62°C in a liquid matrix. Benachour et al. (2005) reported that the survival was due to a *sigV* gene's production of heat shock induced proteins that prevent protein mis-folding at high temperatures. However, the rate of temperature increases, the duration of elevated temperatures, and the initial microbial population of the compost mixture may affect the development of the heat-shock response. The top of the compost heap (70 cm from the stage of composting) from Trial 2 sustained temperatures of ca 60°C for 15 days (Figure 2) and enterococci and VRE still remained in the top location with cell numbers of 3.08 and 4.90 logs, respectively. This suggests that enterococci and VRE present in compost may have survived by the production of heat shock proteins.

All 88 suspected VRE isolates were previously screened using various phenotypic tests for enterococci, but genetic confirmation using PCR can be a rapid and accurate method to identify the genus of those isolates as enterococci (Haugland et al.,

2005; Shanks et al., 2008; Iapichino et al., 2008). PCR using primers specific for a fragment of the 16s RNA gene specific for enterococci confirmed that all 88 suspected VRE isolates were enterococci. These data suggests that the phenotypic tests were in agreement with the PCR results.

Studies have shown that *E. faecalis* and *E. faecium* are the two most dominant species, making up roughly 90% and 10% of the enterococci population, respectively (Witte et al., 1999). Due to the wide distribution of *E. faecium* and *E. faecalis* in animals and the environment, it is important to determine their presence in compost containing cattle waste. Several genes have been identified that have species-specific variable regions including the genes for: elongation factor EF-Tu, manganese-dependent super oxide dismutase (*sodA*), heat shock protein 60, and D-Ala:D-Ala ligase (Ke et al., 1999; Poyart et al., 2000; Goh et al., 2000; Ozawa et al., 2000). Multiplex PCR was performed to identify *E. faecium* and *E. faecalis* using species-specific primers for the *sodA* gene due to the high variability of the gene between species. The results revealed that none of the 88 VRE isolates tested were either *E. faecalis* or *E. faecium*, however, 3 (9%) of the enterococci isolates tested were confirmed as *E. faecalis* and 2 (6%) as *E. faecium*. These results reveal that although *E. faecium* and *E. faecalis* are the two most predominant species found in cattle guts and may therefore be predominant in cattle feces, they may not adapt well in a compost ecosystem.

Enterococci are well known for having high levels of antibiotic resistance; most notably with the glycopeptide antibiotic vancomycin. Vancomycin resistance is conveyed to enterococci via different genes: *vanA*, *vanB*, *vanC*, *vanD*, *vanE* and *vanG* (Garnier et al., 2004). The *vanA* gene is responsible for varying to high levels of

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resistance to vancomycin and resistance to teicoplanin in excess of 256 µg/ml. Whereas, the *vanB* gene conveys varying to low levels of resistance to vancomycin but is susceptible to teicoplanin at concentrations as low as 4 µg/ml (Moellering, 1998). Therefore, testing the MIC for vancomycin isn't enough to differentiate between the *vanA* and *vanB* phenotypes. *VanC*, *vanD*, *vanE,* and *vanG* occur infrequently, have low (from > 2 µg/ml to > 64 µg/ml) levels of resistance to vancomycin, and are susceptible to teicoplanin (Moellering, 1998; Depardieu et al., 2003). In this study, susceptibility testing with teicoplanin was performed to identify the vancomycin resistance phenotypes. The MIC data suggests that all 88 VRE isolates may contain the *vanA* vancomycin resistance gene due to their ability to grow in the presence of 64 µg/ml of teicoplanin, whereas no isolates contained the *vanB* gene. Although not all the VRE isolates had a high (> 256 µg/ml) MIC for vancomycin, previous studies have reported some enterococci containing the *vanA* gene to have a relatively low MIC for vancomycin. Aleyasin et al. (2007) found selected *vanA* isolates having MIC values as low as > 32 µg/ml.

The *vanA* and *vanB* genes are the two most commonly occurring vancomycin resistance gene found in enterococci, accounting for almost 60 and 40% of all isolated VRE, respectively (Clark et al., 1993). *VanA* can be found in many different species however, *vanB* has only been found in *E. faecalis* and *E. faecium* (Moellering, 1998). Due to the teicoplanin resistance (> 64 µg/ml), all 88 of our VRE isolates were tested for the *vanA* gene using the PCR method. The results revealed that 83 out of 88 (94%) of VRE isolates contain the *vanA* gene for vancomycin resistance. This suggests that the

antibiotic testing was an effective means for differentiating those VRE isolates containing the *vanA* gene.

In addition to resistance to multiple antibiotics, enterococci are well known for high levels of heat resistance (Houben, 2003). Studies have shown that *E. faecalis* contain sigma proteins, coded by the *sigV* gene, that are activated at elevated temperatures and prevent protein mis-folding (Benachour et al., 2005). A mutant *sigV E. faecalis* had increased susceptibility to heat shock when compared to the wild type (Benachour et al., 2005). Therefore, enterococci that have been exposed to elevated, sub-lethal temperatures like those experienced during composting, may have increased heat resistance. To quantify an organism's heat resistance D and z-values are often determined. D and z-values are especially important to determine for organisms present in compost to ensure that the compost heap sustains sufficient temperatures to inactivate potentially hazardous pathogens. The heat tolerance screening results revealed that the isolates present at the beginning of Trial 1 are relatively heat susceptible whereas the isolates from day 30 and 60 have increased levels of heat resistance (Figure 2.9). Both D and z-values of selected isolates also confirmed that isolates from day 30 and 60 are significantly ($p < 0.05$) more heat resistant that isolates from day 0. These data may suggest that VRE may develop heat resistance during the thermophilic phase of composting or the elevated temperatures encountered during composting may select for a subpopulation of VRE with high thermal resistance. However, since only a limited number of VRE isolates were tested in this study, a systematic evaluation of VRE isolates during composting should be conducted to verify our preliminary results reported here. Our data revealed that the center isolates from day 30 had significantly ($p<0.05$) higher

D-values than the surface isolate from day 30 at all three of the temperatures tested and that the z-values for all 4 center isolates were higher than those of the surface samples from the respective sampling day. Our data suggests that the elevated temperatures experienced in the center of the compost heap caused VRE to develop elevated levels of heat resistance compared with those VRE isolates present on the surface of the heap. Therefore, the temperatures inside in the compost heaps may be high enough to kill most microorganisms, but allow for a few heat-adapted cells to exhibit thermal resistance. Our results also suggest that VRE isolates present at the early stages of composting have similar thermal resistance compared to ATCC strains of *E. faecium* and *E. faecalis* (Table 2.9). These data are especially important due to compost's use as a fertilizer on organic crops and the potential for heat resistant VRE to persist in compost and subsequently contaminate crops. The prevention of these microorganisms from contaminating crops can ultimately reduce the amount of food borne illnesses.

It is important to determine whether the strains of VRE present at the end of composting were present at the beginning. If the strains are the same it would suggest that the specific strain developed heat resistance while in compost, which allowed for its extended survival. However, if different strains are present at the beginning and end it would infer that there may be a sub-population of VRE present that have elevated heat resistance intrinsically and become more predominant as the less heat tolerant strains become inactivated as thermophilic composting takes place. Several studies have applied pulsed field gel electrophoresis (PFGE) for identifying different strains of enterococci (Chiew and Hall, 1998; Saeedi et al., 2003; Turabelidze et al., 2000). Box-PCR has been shown to provide results comparable with those from PFGE and has proven to be a

reliable and quick method for differentiating genogroups of enterococci (Hassen et al., 2004; Jackson et al., 2005). Our Box-PCR results revealed 8 unique banding patterns, or genogroups, and that 42% (5/12) of the VRE isolates shared the same banding pattern (Figure 2.10). Genogroup 3 strains were present in the center on days 7, 14, and 60, as well as on the surface on days 7 and 14. These data provide some evidence that genotype 3 VRE were able to persist in compost up to 60 days and may adapt to the elevated temperatures by developing heat resistance.

Conclusions

Our results indicate that the temperature stratification at different locations of the heaps affected the rates of enterococci and VRE inactivation; however, up to 7.4 and 6.7 log CFU/g of enterococci and VRE inside compost heaps could be inactivated within 120 days and 30 days, respectively, when composting was performed during the summer months. Importantly, our results revealed that both enterococci and VRE can survive up to 4 months on the surface of compost heaps and this could serve as a source of pathogen contamination of the surrounding environment and a recontamination of the compost. The microbiological analysis of Trials 1 and 3 revealed that VRE may have elevated heat resistance compared to non-VRE due to the VRE population making up over half of the total enterococci population at the top and center locations, respectively; the hottest heap location of each of the respective Trials. Additionally, other factors including moisture

content and the C:N ratio may affect the inactivation of VRE and enterococci. It is important to keep these values within the optimal range to allow for effective composting. The MIC data revealed that 97% of our VRE isolates from compost were resistant to > 256 µg/ml of vancomycin and 100% were resistant to > 64 µg/ml of teicoplanin. These data indicates that all 88 VRE isolates are phenotypically resistant to vancomycin. Our results also indicate that *E. faecium* and *E. faecalis* are present in compost, however they had relatively low levels of vancomycin resistance (> 32 µg/ml). PCR methods confirmed that 94% of 88 VRE isolates from all Trials contain the *vanA* gene for vancomycin resistance. Our results revealed that VRE isolates taken from compost are more heat resistant than those that have not been subjected to sub-lethal heating and that some VRE isolates may gain heat resistance during the thermophilic phase of composting, which was confirmed by Box-PCR analysis of VRE isolates collected at different stages of composting.

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<center>**Figure Legend**</center>

Figure 2.1: Diagram displaying the 4 sampling locations of compost heaps for Trials 1, 2, and 3.

Figure 2.2: The temperature profile of compost Trial 1 during composting. Temperatures were measured at the surface ($\Diamond$), the top (), the center (Δ), and the bottom ($\times$).Arrows represent days where precipitation occurred. Graph used with permission from Shepherd et al. (2007).

Figure 2.3: The temperature profile of compost Trial 2 during composting. Temperatures were measured at the surface ($\Diamond$), the top (), the center (Δ), and the bottom ($\times$). Arrows represent days where precipitation occurred. Graph used with permission from Shepherd et al. (2007).

Figure 2.4: The temperature profile of compost Trial 3 during composting. Temperatures were measured at the surface ($\Diamond$), the top (), the center (Δ), and the bottom ($\times$). Arrows represent days where precipitation occurred. Graph used with permission from Shepherd et al. (2007).

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Figure 2.5: Enterococci genus identification of VRE isolates by PCR. Lanes 1-17, 17 VRE isolates; Lane 18, negative control; Lane 19, Positive control *E. faecalis* (ATCC #29212); Lane 20, 50kb ladder of molecular weight standard.

Figure 2.6: Detection of *E. faecalis* and *E. faecium* species of VRE isolates by PCR. Lanes 1-16, 16 VRE isolates; Lane 17, negative control; Lane 18, positive control *E. faecalis* (ATCC #29212); Lane 19, positive control *E. faecium* (ATCC #52122); Lane 20, 50kb ladder of molecular weight standard.

Figure 2.7: Detection of *E. faecalis* and *E. faecium* species of non-VRE isolates isolated from trial 1 by PCR. Lanes 1-7, 7 non-VRE isolates; Lane 8, negative control; Lane 9, positive control *E. faecium* (ATCC #52122); Lane 10, positive control *E. faecalis* (ATCC #29212); Lane 11, 50kb ladder of molecular weight standard.

Figure 2.8: Detection of the *vanA* gene in VRE isolates from trial 1 by PCR. Lanes 1-5, 5 VRE isolates; Lane 6, positive control *E. faecium* containing *vanA* (ATCC #51559);Lane 7, negative control; Lane 8, 100kb ladder of molecular weight standard.

Figure 2.9: Heat tolerance screening at 65°C for center (▲)and surface (■) isolates collected at different stages of composting from Trial 1.

Figure 2.10: Box-PCR analysis of VRE isolates collected at different stages of composting. Lane 1: Isolate #2 from the surface on Day 0. Lane 2: Isolate #5 from the center on Day 0. Lane 3: Isolate #11 from the surface on Day 7. Lane 4: Isolate #13 from the center on Day 7. Lane 5: Isolate #15 from the surface on Day 14. Lane 6: Isolate #18 from the center on Day 14. Lane 7: Isolate #22 from the surface on Day 30. Lane 8: Isolate #23 from the center on Day 30. Lane 9: Isolate #24 from the surface on Day 60. Lane 10: Isolate #27 from the center on Day 60. Lane 11: Isolate #28 from the surface on Day 120. Lane 12: Isolate #32 from the center on Day 120. Lane 13: negative control. Lane 14: positive control *E. coli* O157:H7. Lane 15: Eco R1 DNA Ladder. Number listed below each lane designates the genogroup of each isolate.

Table 2.1: Fate of enterococci and VRE at different locations of heaps during composting (Trial 1).

		Avg log CFU/g at days of composting[a]							
Media	Location	0	3	7	14	21	30	60	120
BEA									
	Surface	[a]7.10±0.08A	[a]5.37±0.03B	[a]5.19±0.04B	[a]5.03±0.08C	[a]4.82±0.04C	[a]4.49±0.04D	[a]4.11±0.11E	[a]3.41±0.11F
	Top	[a]7.10±0.08A	[b]4.94±0.08B	[d]4.42±0.08BC	[c]3.92±0.26CD	[c]3.61±.034DE	[c]3.33±0.26E	[c]2.78±0.33F	[c]1.38±0.71G
	Center	[a]7.10±0.08A	[b]4.98±0.31B	[c]4.61±0.14BC	[b]4.32±0.32C	[b]4.24±0.24C	[b]3.78±0.22C	[b]3.26±0.33E	[b]2.37±0.42F
	Bottom	[a]7.10±0.08A	[ab]5.10±0.39B	[b]5.14±0.09B	[a]4.81±0.11BC	[a]4.69±0.17C	[a]4.39±0.09D	[a]3.81±0.11E	[ab]3.03±0.26F
BEA-V									
	Surface	[a]5.36±0.04A	[a]5.16±0.04AB	[a]5.08±0.04BC	[a]4.76±0.07CD	[a]4.51±0.05D	[a]4.17±0.07E	[a]3.94±0.08F	[a]3.19±0.11G
	Top	[a]5.36±0.04A	[b]3.53±0.57B	[c]3.36±0.06B	[d]2.71±0.19C	[d]2.62±0.12C	[d]2.26±0.14CD	[d]1.99±0.11D	[d]1.11±0.16E
	Center	[a]5.36±0.04A	[b]4.10±0.23B	[b]3.76±0.09C	[c]3.04±0.06D	[c]2.96±0.09D	[c]2.66±0.08E	[c]2.21±0.12F	[c]1.66±0.11G
	Bottom	[a]5.36±0.04A	[b]3.87±0.82B	[b]3.87±0.13B	[b]3.81±0.08B	[b]3.64±0.09BC	[b]3.28±0.13C	[b]2.56±0.09D	[b]2.08±0.06E

[a]Values (BEA or BEA-V) with different capitalized letters within each row are statistically different (p <0.05) among sampling days.

[b]Values (BEA or BEA-V) with different superscript letters within each column are statistically different (p < 0.05) among different locations on each sampling day.

Table 2.2: Fate of enterococci and VRE at different locations of heaps during composting (Trial 2).

Media	Location	Avg log CFU/g at days of composting[a]							
		0	3	7	14	21	30	60	120
BEA									
	Surface	^{a}7.43±0.06A	^{a}6.66±0.09BC	^{a}6.98±0.07AB	^{a}6.34±0.56CD	^{a}6.33±0.04BCD	^{a}6.33±0.06CD	^{a}6.14±0.08D	^{a}3.22±0.16E
	Top	^{a}7.43±0.06A	^{c}6.43±0.03B	^{c}5.35±0.13C	^{c}4.90±0.03CD	^{d}4.29±0.12D	^{c}2.48±0.53E	ND	ND
	Center	^{a}7.43±0.06A	^{b}6.51±0.03B	^{b}5.86±0.07C	^{b}5.20±0.08D	^{c}4.71±0.07DE	^{b}4.39±0.07E	^{c}1.49±0.94F	ND
	Bottom	^{a}7.43±0.06A	^{b}6.56±0.02B	^{b}6.07±0.04C	^{b}5.45±0.05D	^{b}4.97±0.04E	^{b}4.43±0.19F	^{b}2.77±0.11G	^{b}1.35±0.05H
BEA-V									
	Surface	^{a}6.70±0.03A	^{a}6.66±0.05B	^{a}6.67±0.05B	^{a}5.60±0.02C	^{a}5.49±0.04D	^{a}5.40±0.05D	^{a}2.89±0.06E	^{a}1.35±0.05F
	Top	^{a}6.70±0.03A	^{c}4.38±.020B	^{b}3.40±.019C	^{c}3.08±0.06D	^{d}2.47±0.15E	ND	ND	ND
	Center	^{a}6.70±0.03A	^{b}5.04±0.31B	^{b}3.72±0.22C	^{b}3.38±0.07C	^{c}2.87±0.08D	^{b}1.00±0.53E	ND	ND
	Bottom	^{a}6.70±0.03A	^{b}5.09±0.37B	^{b}3.92±0.25C	^{b}3.69±0.19CD	^{b}3.08±0.08D	^{b}1.00±0.53E	ND	ND

[a]Values (BEA or BEA-V) with different capitalized letters within each row are statistically different (p <0.05) among sampling days.

[b]Values (BEA or BEA-V) with different superscript letters within each column are statistically different (p < 0.05) among different locations on each sampling day.

[c]ND: Values were not detected with our detection limit of 10 CFU/g.

Table 2.3: Fate of enterococci and VRE at different locations of heaps during composting (Trial 3).

Media	Location	Avg log CFU/g at days of composting[a]							
		0	3	7	14	21	30	60	120
BEA									
	Surface	^{a}6.46±0.06A	^{a}5.90±0.06B	^{a}5.46±0.02C	^{a}4.98±0.07D	^{a}3.78±0.10E	^{a}3.32±0.08F	^{a}2.79±0.05G	^{a}1.35±0.05H
	Center	^{a}6.46±0.06A	^{b}5.55±0.07B	^{c}4.65±0.02C	^{c}3.03±0.07D	^{c}2.65±0.06D	^{c}2.21±0.11D	^{c}1.30±0.05E	^{a}1.00±0.82E
	Bottom	^{a}6.46±0.06A	^{b}5.46±0.03B	^{b}5.12±0.03C	^{b}4.13±0.05D	^{b}3.21±0.02E	^{b}2.87±0.07F	^{b}2.03±0.08G	^{a}1.00±0.00H
BEA-V									
	Surface	^{a}5.87±0.08A	^{a}5.24±0.02AB	^{a}4.98±0.01BC	^{a}4.32±0.01CD	^{a}3.99±0.01DE	^{a}3.45±0.02EF	^{a}2.99±0.05F	^{a}1.00±0.82G
	Center	^{a}5.87±0.08A	^{b}4.13±0.01B	^{c}3.49±0.03BC	^{c}3.01±0.05C	^{c}2.88±0.06C	^{c}2.12±0.03D	^{b}1.00±0.82E	ND
	Bottom	^{a}5.87±0.08A	^{c}4.01±0.06B	^{b}3.70±0.03BC	^{b}3.49±0.07C	^{b}2.95±0.04D	^{b}2.65±0.02D	^{b}1.21±0.38E	ND

[a]Values (BEA or BEA-V) with different capitalized letters within each row are statistically different (p < 0.05) among sampling days.

[b]Values (BEA or BEA-V) with different superscript letters within each column are statistically different (p < 0.05) among different locations on each sampling day.

[c]ND: Values were not detected with our detection limit of 10 CFU/g.

Table 2.4: Summary of inactivation rates of enterococci and VRE during composting.

Trial	Location in heap	Avg log reduction/day[a]		VRE percentage of enterococci[b]	
		BEA	BEA-V	Day 0	Last day detected (day)
#1	Surface	0.27	0.04	2%	50% (120)
	Top	0.38	0.29	2%	54% (120)
	Center	0.36	0.23	2%	19% (120)
	Bottom	0.28	0.21	2%	8% (120)
#2	Surface	0.06	0.03	19%	1% (120)
	Top	0.30	0.47	19%	1.5% (21)
	Center	0.22	0.43	19%	0.04% (30)
	Bottom	0.20	0.40	19%	0.03% (30)
#3	Surface	0.14	0.13	26%	44% (120)
	Center	0.26	0.34	26%	50% (60)
	Bottom	0.19	0.31	26%	15% (60)

[a]Average log reductions per day were calculated based on log reductions during the first 7 days of composting. Values were calculated by subtracting the log values of enterococci (BEA) and VRE (BEA-V) at day 7 from their initial log values at day 0 and dividing by 7, respectively.

[b] Percentage of VRE of entire enterococci population. The percentage was calculated by dividing the VRE CFU/g by the enterococci's CFU/g for either the first or last sampling days that both populations were detected.

Table 2.5: List of PCR primers and products for enterococci genus, species, and *vanA* detection.

Target	Primer	Sequence (5'->3')	Product size (bp)	Ref
Enterococcus spp.	ECF	AGAAATTCCAAACGAACTTG	92	Shank et al. 2008
	ECR	CAGTGCTCTACCTCCATCA		
E. faecalis species	FL1	ACTTATGTGACTAACTTAACC	215	Sedgley et al. 2005
	FL2	TAATGGTGAATCTTGGTTTGG		
E. faecium species	FM1	GAAAAAACAATAGAAGAATTAT	360	Jackson et al. 2005
	FM2	TGCTTTTTTGAATTCTTCTTTA		
VanA gene	VanA1	GGGAAAACGACAATTGC	732	Kilic et al. 2004
	VanA2	GTACAATGCGGCCGTTA		

Table 2.6: List of PCR reaction mixtures for enterococci genus, species, and *vanA* detection.

	PCR Reaction Mixture						
Target	10x Buffer	dNTPs	H$_2$0	MgCl$_2$	Amplitaq	Primer[a]	DNA
Genus	2.0 µl	0.2 mM	9.8 µl	1.5 mM	0.2 U	4 pmol	2.0 µl
Species	2.0 µl	0.2 mM	7.3 µl	2.5 mM	0.2 U	10/5 pmol[b]	2.5 µl
vanA	2.0 µl	0.5 mM	4.0 µl	3.1 mM	0.4 U	10 pmol	2.0 µl

[a]Amount of primer listed was added for each forward and reverse primer for the respective target listed in Table 2.5.

[b]10 pmol of *E. faecalis* primers and 5 pmol of *E. faecium* primers were added to the multiplex reaction mixture.

Table 2.7: List of PCR condition for enterococci genus, species, and *vanA* detection.

Target	Denaturation	Number of cycles	Heating	Annealing	Extension	Final Extension
						PCR conditions
Genus	95°C 1 min	40	95°C 15 s	60°C 1 min	-[a]	72°C 1 min
Species	95°C 4 min	30	95°C 30 s	55°C 1 min	72°C 1 min	72°C 7 min
vanA	95°C 10 min	30	94°C 30 s	58°C 30 s	72°C 30 s	72°C 10 min

[a] Genus detection required no additional extension time because of the small (92bp)

amplicon.

Table 2.8: Detection of enterococci genus, species, and the *vanA* gene.

Trial	Isolate group (n)	PCR detection			
		Enterococci	*E. faecium*	*E. faecalis*	*vanA* [c]
1	VRE (32)	32 (100%)	0	0	29 (91%)
	enterococci (32)[a]	35 (100%)	2 (6%)[b]	3 (9%)[b]	4 (13%)
2	VRE (27)	27 (100%)	0	0	25 (93%)
3	VRE (29)	29 (100%)	0	0	29 (100%)

[a] Enterococci were enumerated on BEA containing no vancomycin.

[b] *E. faecium* and *E. faecalis* isolates had an MIC of 32 µg/ml for vancomycin.

[c] All VRE isolates were confirmed to have an MIC for teicoplanin of > 64µg/ml.

Table 2.9: The D and z-values for VRE isolates from Trial 1 along with two ATCC standard strains for *E. faecalis* and *E. faecium*.

Isolate	Compost age (day)	Location	D-value (min) at temperature (°C)			Z-value (°C)
			60	65	70	
#2	0	Surface	11.45±0.24CD	6.26±0.09D	1.66±0.05DE	11.92
#5	0	Center	9.7±0.47DE	4.73±0.29E	1.59±0.25DE	12.73
#11	7	Surface	12.04±0.11C	6.34±0.36D	2.68±0.17BC	13.76
#13	7	Center	11.87±0.46C	7.8±0.26C	2.08±0.29CD	14.33
#22	30	Surface	13.3±.32BC	8.58±0.24 C	3.19±0.20B	16.13
#23	30	Center	17.73±1.45A	12.57±0.45A	4.44±0.32A	16.63
#24	60	Surface	14.19±0.79B	8.36±0.79C	3.13±0.65B	15.23
#27	60	Center	15.09±0.84B	10.97±0.41B	4.16±0.53A	17.87
E. faecalis ATCC#29212	-	-	8.28±0.70E	4.44±1.04E	1.12±0.25E	11.51
E. faecium ATCC#51229	-	-	9.84±1.32DE	4.06±0.40E	1.73±0.30DE	13.25

[a] Values with different capitalized letters in each column are statistically different ($p < 0.05$) at the temperature.

[b] Values are averaged from 4 replicates for each isolate.
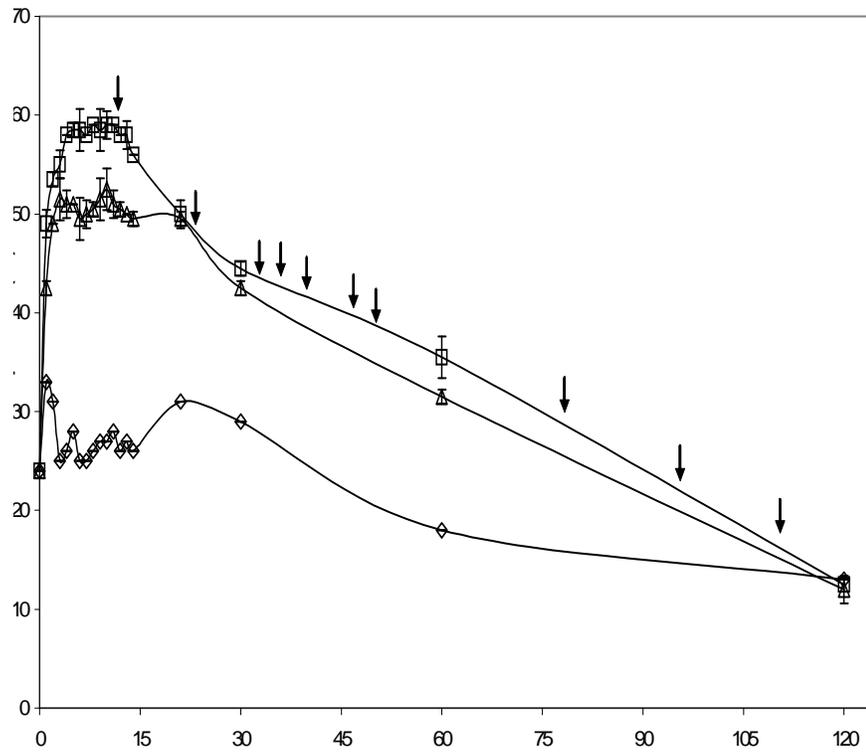
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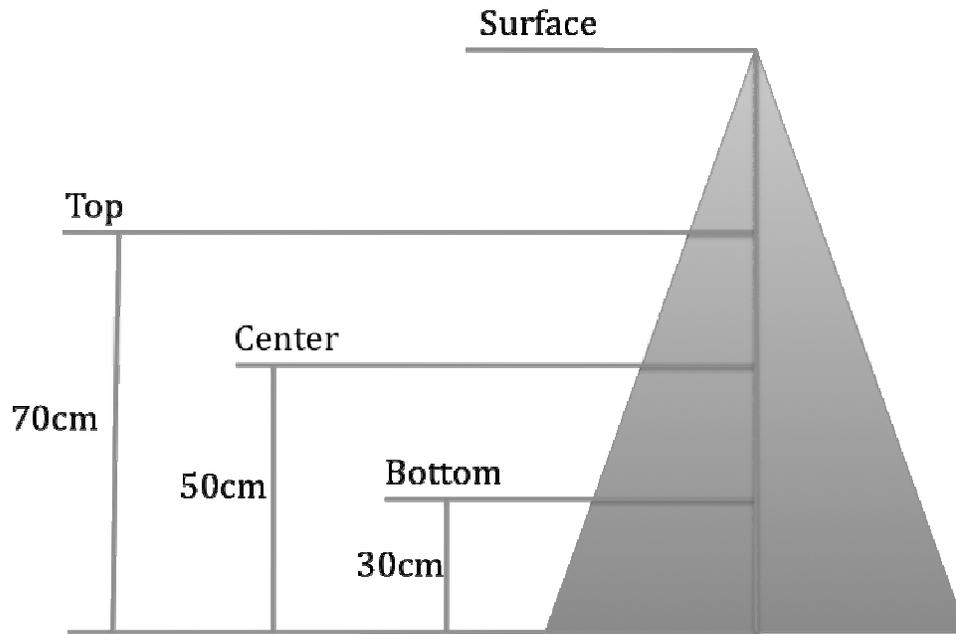
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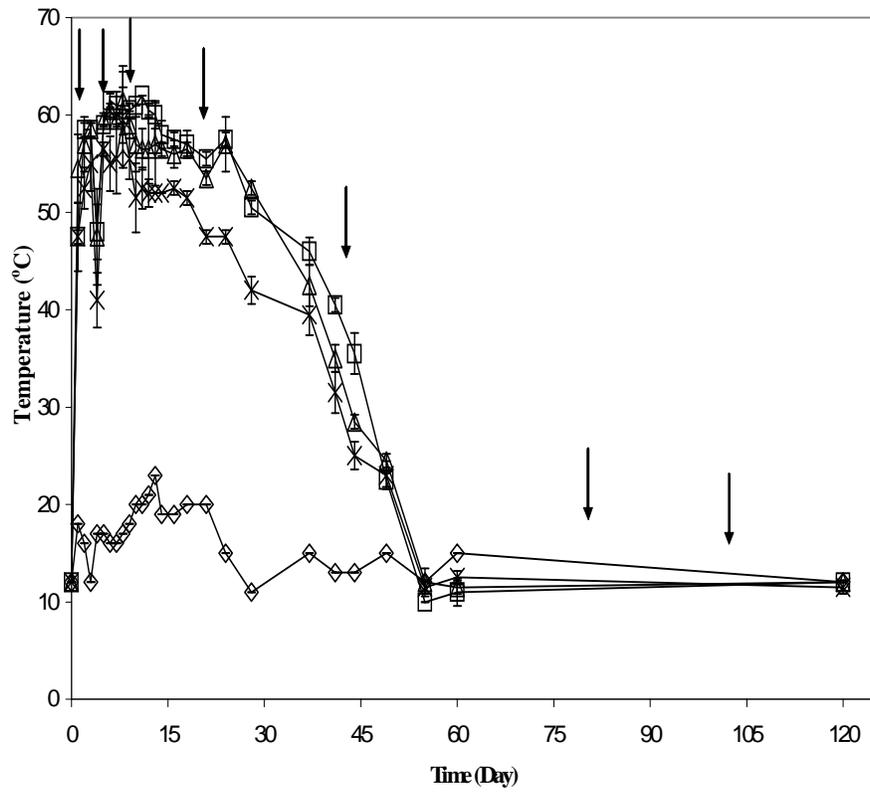
Used with permission from Shephard et al. (2007)
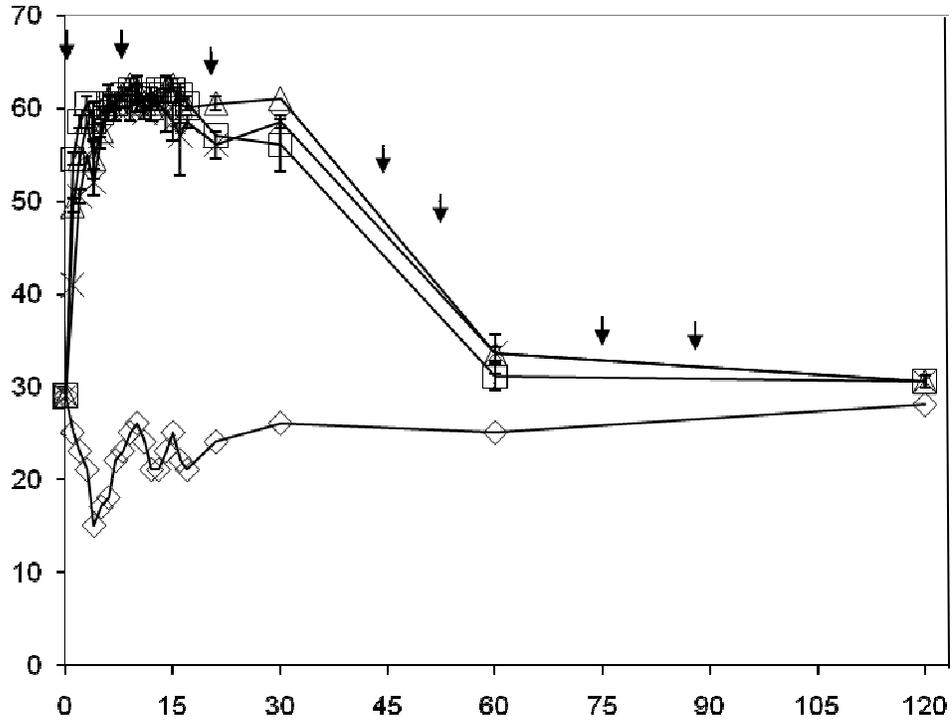
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Used with permission from Shephard et al. (2007)
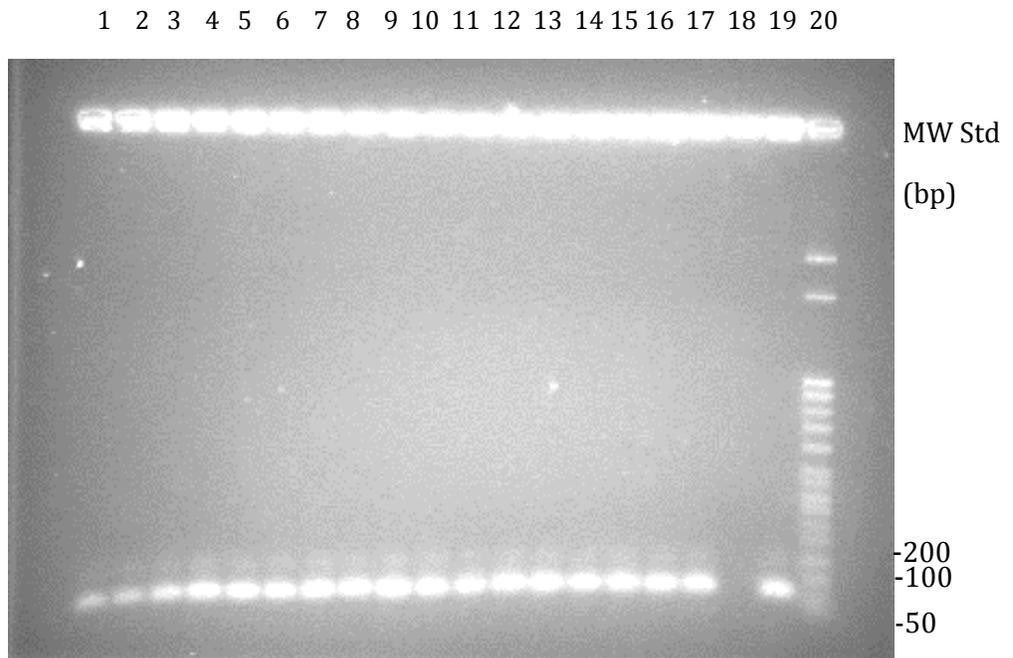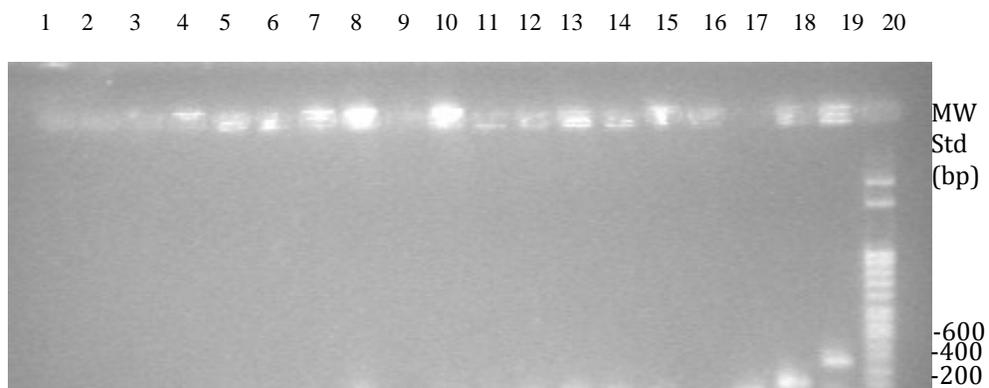
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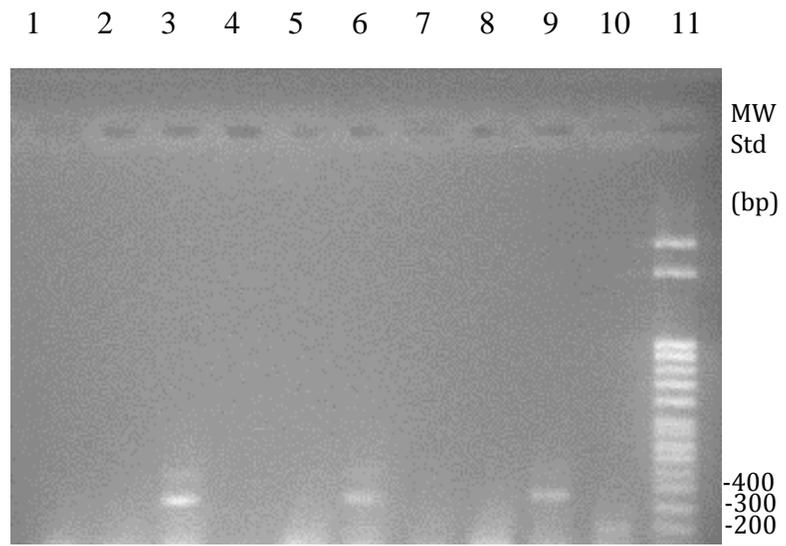
Figure 2.5:

Figure 2.6:
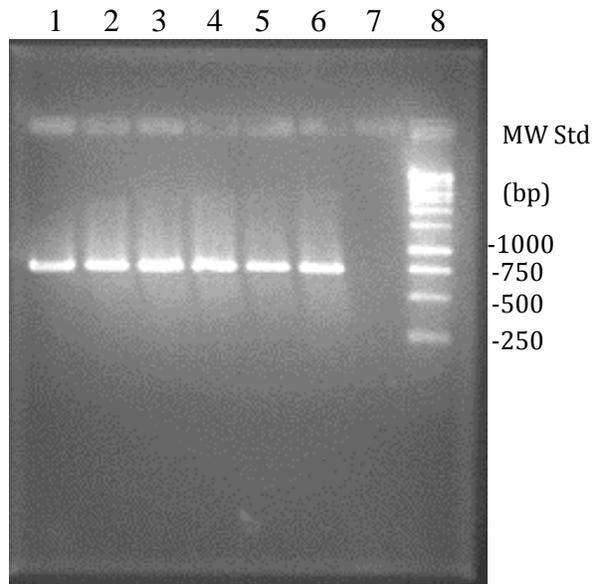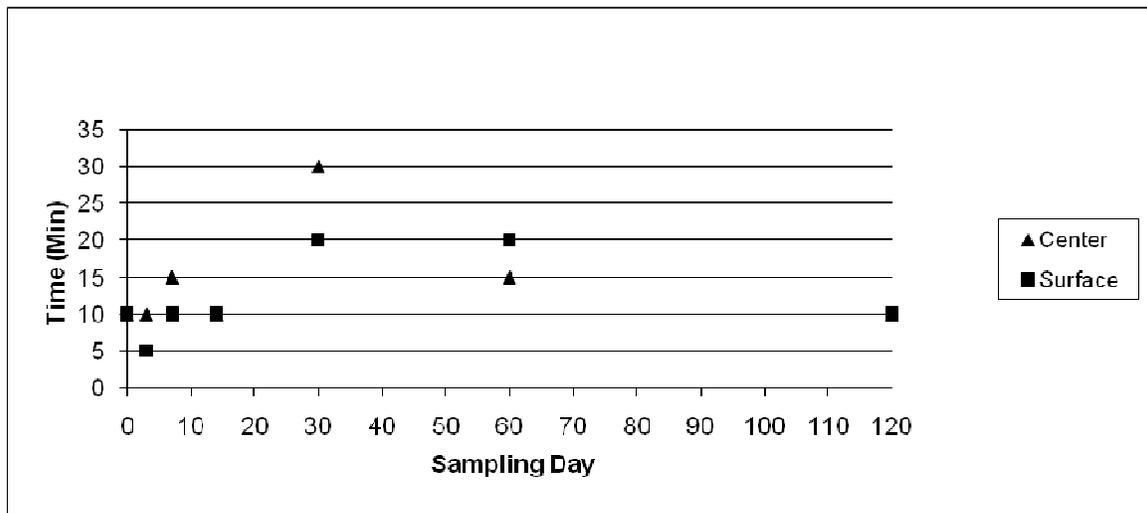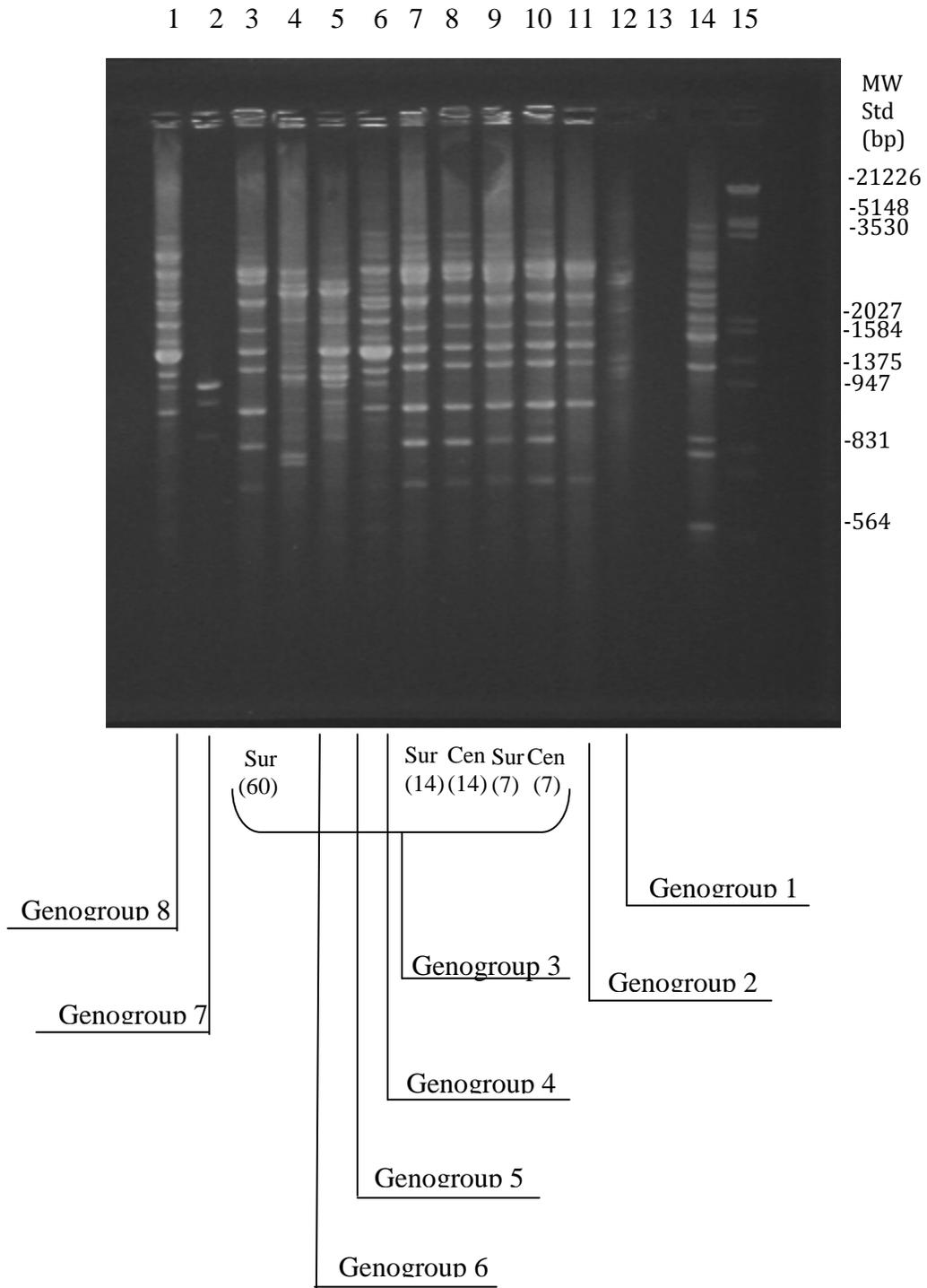
Figure 2.7:

Figure 2.8:

Figure 2.9:

Figure 2.10:

CHAPTER THREE

CONCLUSION

In this study, a microbiological analysis of enterococci and vancomycin resistant enterococci (VRE) in compost and the characterization of those VRE isolates from compost were conducted. Extended survival of both enterococci and VRE was observed in the compost's surface for at least 4 months. However, both enterococci and VRE were inactivated rapidly inside the heap. Our results revealed that the population of 7.4 log CFU/g of enterococci and 6.7 log CFU/g of VRE may become inactivated at internal heap locations within 60 and 30 days, respectively, after the onset of composting when performed during the summer months. However, some microorganisms were able to persist in all heap locations for at least 120 days when composting was performed in the fall. Results from the heat resistance experiments indicated that while some enterococci become inactivated with elevated temperatures during composting, some cells inside the compost heaps may develop heat resistance probably due to induction of a heat-shock response. Our results also revealed that *E. faecalis* and *E. faecium* are less prevalent in a compost ecosystem, whereas other enterococcal species containing the *vanA* gene for vancomycin resistance are able to survive for 120 days in compost.   In conclusion, our results demonstrated that composting under appropriate conditions can result in the inactivation of VRE inside the heaps and that some enterococci populations may be able to adapt to elevated temperatures in compost.