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MICROBIOLOGICAL SAFETY OF ORGANIC FERTILIZERS USED FOR PRODUCE PRODUCTION

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MICROBIOLOGICAL SAFETY OF ORGANIC FERTILIZERS USED FOR PRODUCE PRODUCTION

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ABSTRACT

Over the last few decades there has been an increase in the use of organic fertilizers due to the rise in popularity of organic food production. However, if the fertilizers are not adequately sanitized there is the possibility of contamination of agricultural crops by foodborne pathogens from the spreading of organic fertilizers onto the crops. This is especially important for animal waste-based fertilizers since animals frequently carry foodborne pathogens. Furthermore, antibiotic use in food animal production may induce antimicrobial resistance in gut bacteria which can be spread to crops and the environment through fertilizer use. The objectives of this study were to analyze various organic fertilizers for microbial quality and the presence of antimicrobial resistance as well as evaluate the growth potential of foodborne pathogens in selected fertilizers.

One hundred and three organic fertilizer samples were surveyed for moisture content, water activity, pH and microbial quality, including enumeration of total bacteria count, *Enterobacteriaceae*, total coliforms, and *Escherichia coli* as well as the presence of foodborne pathogens *Salmonella*, *Escherichia coli* O157:H7 and *Listeria monocytogenes*. Moisture content ranged from 0.98% to 86.37% with the water activities between 0.298 and 0.999 and the pH between 3 and 10 with an average of 7.77 ± 1.28.Total bacteria, *Enterobacteriaceae*, coliforms, and *E. coli* were in the range of 3 to 9, <1 to 7, <1 to 6, and <1 to 6 log CFU/g, respectively. No *Salmonella*, *E. coli* O157:H7, or *L. monocytogenes* were found.

The growth potential of *Salmonella* spp. and *E. coli* O157:H7 was evaluated in selected organic fertilizers with high (≥5 log CFU/g) and low (<5 log CFU/g) levels of background bacteria. In fertilizers with high levels of background bacteria, *Salmonella* and *E. coli* O157:H7 increased

ca. 1 log CFU/g in plant-based compost and fish emulsion-based compost, respectively. In fertilizers with low levels of background bacteria, pathogen growth was observed in bone, blood, and feather meal and the mixed source fertilizer by ca. 3 and 4 log CFU/g for *Salmonella* and *E. coli* O157:H7, respectively.

E. coli isolates (n=72) from organic fertilizers were further characterized for phylogenetic group, the presence of shiga-toxins, resistance to antibiotics and presence of integrons. All of the isolates were negative for shiga-toxins (stx1 and stx2). The majority of the isolates belonged to phylogenetic group B1 followed by group A. Eleven isolates had antimicrobial resistance with five resistant to $\geq$2 antibiotics and two resistant to $\geq$10 antibiotics. Five of the eleven isolates had class 1 integrons present.

In conclusion, our study revealed a wide range of microbiological quality among organic fertilizers, and identified some factors affecting the growth of foodborne pathogens in these fertilizers.

DEDICATION

I dedicate this thesis to my Mom, Dad, Kelly and Michael for always believing in me and helping me stay sane throughout this process.

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CHAPTER ONE

LITERATURE REVIEW

Introduction

Millions of tons of animal feces are produced each year requiring more efficient methods of waste disposal. Animal manure has traditionally been applied to land as an agricultural amendment. However, it has become apparent that this process leads to the transmission of pathogens to crops that are then consumed by humans. One method for the reduction of pathogens is recycling of wastes by composting, yet this process can still produce products that are unsafe if not done properly. Compost quality standardization is a relatively new concept that was unknown worldwide as recently as 1985 (Brinton 2000). With the increasing popularity of organic farming in the last few decades, the use of organic fertilizers of animal waste origins has also increased drastically. In 2006 alone, manure was spread as a fertilizer on about 15.8 million acres of U.S. cropland (MacDonald et al. 2009). However, some recent outbreaks of human enteric pathogens have been traced back to fresh produce contaminated in the field by animal manure or improperly produced compost (Sivapalasigam et al. 2004; US FDA 2007; CDC 2006).

Composting is a thermophilic process and the high temperatures generated can kill microorganisms, including bacteria, viruses, and parasites under the right conditions. There are three distinct phases of the composting process including a mesophilic phase (40-50^{o}C), a thermophilic phase (50-65^{o}C), and a cooling/maturation phase (≤30^{o}C) (Hassen et al. 2002). While temperature is important, pathogen reduction during composting can be affected by a variety of factors. Some of these factors include competition with indigenous microorganisms,

desiccation, nutrient depletion, pH, and storage time. The U.S. Environmental Protection Agency (EPA) has set up guidelines for the minimum temperature reached by the compost and the length of time the compost is held at that temperature. Compost must reach a temperature of at least 55^{o}C for 3 days for static piles and 15 days for windrows with a minimum of 5 turnings. This standard was part of the 40 CFR Part 503 regulations for pathogens in biosolid compost which also included standards for bacterial levels of finished compost (EPA 1999). For Class A biosolids, fecal coliform counts must be less than 1,000 MPN/g of compost and *Salmonella* levels must be less than 3 MPN/4g of compost. Class B biosolids are allowed to have higher levels of indicator microorganisms, but there is a restriction on where the biosolids can be applied. Composts that meet these requirements are considered safe for use on agriculture. However, if these time and temperature criteria are not met, pathogens may still be present in the composts. Also, exposure of pathogens to sublethal temperatures may allow for extended survival of pathogens through heat adaptation. The heat-shock response is induced by the activation of the *rpoS* gene which regulates bacterial stress responses (Loewen and Hengge-Aronis 1994). Studies reveal that heat adapted pathogens survive longer than control strain in laboratory and field studies (Singh et al. 2010; Shepherd et al. 2010). Pathogens that survive the composting process may regrow to hazardous levels (Zaleski et al. 2005; Hess et al. 2004; Russ and Yanko 1981). Another way in which pathogens can become a problem in finished compost is the reintroduction of microorganisms by an outside source. These microorganisms are not subject to the high temperatures that kill off human pathogens, and the compost heap may have conditions that allow pathogen growth such as high moisture content, rich nutrients, mesophilic temperature range, and neutral pH (Kim et al. 2009).

Another major concern is the rise of antimicrobial resistance in pathogens that may be present on produce consumed by humans. Antibiotics are used to a great extent in the production of food animals as therapeutic treatment as well as growth promotion. However, the use of antimicrobials in animal production systems may select for resistance in enteric microorganisms (Smith et al. 2007; Jiang et al. 2006; Walk et al. 2007). Animal manures, commonly used as soil amendments or organic fertilizers in the form of compost or slurry, can carry antimicrobial resistant bacteria (Heringa et al. 2010; Sengeløve et al. 2003). High levels of antimicrobial resistant bacteria present in fertilizers can lead to contamination of agricultural crops by pathogenic bacteria resistant to antibiotics (Natvig et al. 2002; Solomon et al. 2002; Ingham et al. 2004; Islam et al. 2004; Islam et al. 2005).

Mutations that confer antimicrobial resistance can be transferred from one bacterium to another through horizontal transfer of mobile genetic elements such as plasmids, transposons, and integrons. Integrons are genetic elements containing components of a site-specific recombination system that recognizes and captures mobile gene cassettes, and they generally include an integrase gene (*int*) and an adjacent recombination site (*attI*) (Fluit and Schmitz 2004). The transfer of mobile genetic elements can occur in the intestine of animals (Blake et al. 2003) or in the environment (Götz and Smalla 1997; Guan et al. 2007).

Compost quality is the main issue with regard to composting and biological treatment in general. It determines the outlets where the product can be used, capability of the treatment plant, and the long-term acceptability of the composting process as a valuable waste treatment option (Lasaridi 1998). The numerous characteristics involved in the composting process make it hard to define what makes a “quality” compost. Quality assessment surveys are one way to determine if finished organic fertilizers are meeting the standards required of them. However,

there are few studies that investigate compost quality. Therefore, more studies need to be done in order to clearly define what makes stable, quality finished composts.

Organic Fertilizers Commonly used in Agriculture Production

Organic fertilizers are generally by-products of animals, vegetables and minerals. The three main nutrients provided by organic fertilizers are nitrogen (N), phosphorus (P), and potassium (K) which are released slowly over time. In order to make the nutrients available to plants, the organic matter must first be broken down and converted to a water soluble form that plants can absorb. In addition, organic fertilizers help to improve soil quality by breaking up heavy clay and improving air circulation and drainage (Toronto Master Gardeners).

The most common types of organic fertilizers are animal manures (Watson et al. 2002). Since manure may contain high levels of human and plant pathogens it must be incorporated into the soil and cannot be left on the soil surface. Also, uncomposted manure requires a minimum interval between application and harvest of crops (USDA NOP). If the crops have parts that come in contact with the soil, a minimum of 120 days is required, whereas a 90 day interval is required for all other crops intended for human consumption. In order to eliminate pathogens before application, manure can be composted. A compost mixture must have a carbon:nitrogen ratio between 25:1 and 40:1 as well as reach a temperature of at least 55°C for 3 days for static piles to be considered safe to use on crops used for human consumption (Baker 2005).

Composts used as organic fertilizers may also be made up of waste material from commercial sources such as parks and gardens (greenwaste), pack house wastes, and food industry wastes (Watson et al. 2002). These types of compost can be a good source of all three essential nutrients (N, P, and K) (Toronto Master Gardeners), but they are often contaminated

with “impurities” such as plastic from trash bags or residues from genetically modified organisms (GMO’s), so products should be used carefully (Sullivan 2001; Watson et al. 2002).

In addition to animal manures, animal by-products are commonly used as organic fertilizers. Blood meal is dried slaughterhouse waste and contains about 12% nitrogen. However, it can burn plants with ammonia, lose much of its nitrogen through volatilization, or encourage fungal growth, so it is not the best source of nitrogen. Feather meal is a by-product of the poultry slaughter industry. The total nitrogen in feather meal is fairly high (7 to 10%), but feather meals release their nitrogen very slowly. Bone meal contains 27% total phosphate nearly all of which is available for use by plants, but is expensive to use. Fish emulsion is made from whole fish and fish parts that are digested to form slurry by use of either phosphoric acid or special enzymes. The emulsion may be fortified with chemical fertilizer, so products with a nitrogen content over 5% should be used carefully in organic farming (Sullivan 2001).

Another group of organic fertilizers comes from plant by-products. Alfalfa meal made from the alfalfa plant contains 3% nitrogen and is said to contain unknown growth factors which help make its mineral content more effective as a plant nutrient. Cottonseed meal is rich in nitrogen (7%), however, because of the amount of insecticides used on cotton in the U.S. most organic certification programs restrict or prohibit the use of cottonseed meal. Soybean meal also contains about 7% nitrogen but is expensive to use as a fertilizer (Sullivan 2001). Kelp by-products can be used as a source of trace minerals and is often used in the form of seaweed tea where dried kelp is added to water and steeped like a tea. Kelp by-products contain relatively concentrated amounts of plant auxins, growth regulators, and stimulants that promote rooting and delay senescence and decay. Ashes from wood and other plant residues can also be used as fertilizer. These are a good source of calcium and potash and can be blended with a compost to

balance their nutrient levels. However, ash is usually alkaline and can have an adverse effect on soil pH (Baker 2005).

Mined minerals are a further nutrient source in organic farming and the most commonly used include rock phosphate, gypsum, limestone, potassium sulfate, and magnesium sulfate. Rock phosphates are usually derived from ancient marine deposits and are added directly to livestock manure. The manure acids dissolve much of the total phosphate while the phosphate stabilizes the nitrogen in the manure (Sullivan 2001). Gypsum and limestone are applied for their calcium content to help balance the soil pH. Some mined minerals are restricted because of their high solubility, high salt index (Baker 2005).

There are many different types of organic fertilizers available each with their own nutrient levels. However, quality control of organic fertilizers is mainly centered on manures and manure-based composts. More work needs to be done on the microbiological quality of organic fertilizers that are not based on animal wastes.

Pathogen Survival in Manure and Manure-Amended Soil

Manure has been used as a fertilizer for many years in order to provide nutrients to crops and improve overall soil quality. In 2006, manure was spread onto 15.8 million acres of cropland in the United States accounting for 5% of the total U.S. cropland (MacDonald et al. 2009). A significant proportion of this manure is generated by animal feeding operations (Gerba and Smith 2005). Manure is usually stacked for later use (weeks or months) or applied fresh. Sometimes manure is mixed with bedding before stacking to generate high temperatures as in composting. Other times, manure is washed out with water into ponds, and the slurry is later spread onto fields (Himathongkham et al. 2000).

Healthy agricultural animals can act as reservoirs for human pathogens such as *Salmonella* spp. and *Escherichia coli* O157:H7. Fresh dairy manure may contain from 2 to 7 log CFU/g of *Salmonella* and 2 to 5 log CFU/g of *E. coli* O157:H7 (Himathongkham et al. 1999). These pathogens may persist in the animal manure for weeks or months, but survival time depends on various abiotic factors such as temperature, aeration, location in manure pile, and composition. Himathongkham et al. (2000) showed that an increase in temperature resulted in an increase in elimination rate of pathogens. Kudva et al. (1998) supported this by reporting that *E. coli* O157:H7 survived for the shortest period of time at higher temperatures (37, 45, and 70°C). Aeration of ovine manure decreased the survival of *E. coli* O157:H7 when compared with nonaerated manure piles (Kudva et al. 1998). *E. coli* O157:H7 and *Salmonella* spp. survived longer in the top layers of stacked manure piles in contrast to the middle-bottom layers (Himathongkham et al. 1999 and 2000). The origin of the manure seemed to have some effect on the survival of pathogens with a more rapid die-off of both *E. coli* O157:H7 and *Salmonella* Typhimurium in poultry manure versus cow manure (Himathongkham et al. 1999; Himathongkham et al. 2000), and a longer survival period for *E. coli* O157:H7 in ovine manure as compared with bovine manure (Kudva et al. 1998). Biotic factors such as background microflora play a role in pathogen survival as well. Semenov et al. (2007) found that the background microbial community suppressed the growth of *E. coli* O157:H7 as well as *Salmonella* Typhimurium in manure, with *E. coli* O157:H7 more sensitive to competition by the native microflora than *Salmonella*.

The pathogens that survive in manure and manure slurry may be transferred to the soil when effluent is added as a fertilizer. Addition of fresh biosolids to soil caused *E. coli* levels to rise to significantly greater values due to unknown mechanisms involving the existing microflora

of natural soils. However, when sterile biosolids were added to soil there was also an increase in pathogen numbers indicating that the addition of organic nutrients alone may result in multiplication of pathogens (Unc et al. 2006). On the other hand, studies have shown that higher concentrations of manure in the soil contribute to an increased number of background microflora in the soil (Jiang et al. 2002), and high levels of indigenous microorganisms help with the inactivation of *E. coli* O157:H7 (Jiang et al. 2002; Vidovic et al. 2007). Nutrient-limited soils and low temperatures also cause a decline in *E. coli* O157:H7 numbers (Vidovic et al. 2007). When combined these factors may work synergistically and result in a higher mortality rate for the pathogen. Rainfall shortly after the application of manure and slurry to soil will cause pathogens to percolate deeper into the soil. However, in soil with plant roots present the pathogens are unable to move below the rooting depth (Semenov et al .2009). The pathogen may then be able to survive or grow for a period of time in the soil.

The survival of pathogens in soil leads to concerns that manure-amended soils harboring pathogenic bacteria may contaminate crops such as vegetables and fruits. Therefore, animal manure needs to undergo proper treatment to inactivate human pathogens in order to be safe for application on agricultural lands. One such process is thermophilic composting.

The Composting Process

Composting is the decomposition of organic matter such as plant materials and animal manure by microorganisms in order to create a humus-like material that can be used as a soil amendment. During aerobic composting microorganisms produce heat that can inactivate pathogens present in the compost material. There are three common types of composting systems including passively or actively aerated static piles, windrow systems in which compost is

stacked into long, narrow rows and turned on a regular schedule, and in-vessel systems in which the compost is enclosed in a constructed structure containing some form of forced aeration (Erickson et al.).

Studies have shown that a large variety of mesophilic, thermotolerant, and thermophilic aerobic microorganisms are present in composts. These microorganisms include bacteria, actinomycetes, yeasts, and other fungi. During the composting process the diversity of the microorganisms changes due to temperature fluctuations. There are three main phases of composting and each phase includes different types of microorganisms. The first stage is the mesophilic phase during which temperature increases to reach around 40 to 50^{o}C. During this phase mesophilic microorganism populations increase and mainly include fungi and bacteria. Mesophilic bacteria can reach as much as 10^{8} to 10^{10} bacteria/g waste dry weight (WDW) (Hassen et al. 2002; Herrmann and Shann 1996). This stage is accompanied by a drop in pH because of a build up of volatile organic acids, such as acetic and lactic acid (Erickson et al.). The second phase of composting is marked by high temperatures and is known as the thermophilic phase. Temperatures during the thermophilic phase reach 50 to 70^{o}C, and actinomycetes and thermophilic bacteria including *Bacillus*, *Thermonospora*, and *Micropolyspora* dominate while mesophilic bacteria are inactivated (Herrmann and Shann 1996; Strom 1985; Carpenter-Boggs et al. 1998). Microbial activity will decline if temperatures are near 70^{o}C or if oxygen levels inside compost piles get too low. However, turning of the compost pile or applying forced aeration will allow the microorganisms to become active again. Also, water is lost at the surfaces of the compost pile during this phase causing the materials to dry out (Erickson et al.). Eventually, the microbial activity will slow down causing temperatures to drop to ambient temperature in the cooling or maturation phase. During this phase there is a resurgence of growth of mesophilic

bacteria (Hassen et al. 2002). Actinomycetes and fungi are also common during this stage (Herrmann and Shann 1996). The curing phase is marked by continuation of decomposition at a slower rate and stabilization of the carbon:nitrogen (C:N) ratio (Erickson et al.; Hassen et al. 2002).

Pathogen Survival during Composting

Numerous studies have demonstrated that, under the right conditions, pathogens can be inactivated during the composting process. Time and temperature are the major factors in determining inactivation of pathogens during composting. Déportes et al. (1998) found that total coliforms, fecal coliforms, *E. coli*, and *Salmonella* were below the detection limit by day 27 for municipal solid waste composts whose maximum temperature reached 70-80^{o}C. During thermophilic anaerobic digestion of manure from dairy cattle, Aitken et al. (2007) determined the inactivation rate of *E. coli* O157:H7 at 55°C to be 3 logs within 30 minutes and 4 logs within 100 minutes. Shepherd et al. (2007) showed that *E. coli* O157:H7 inside of dairy manure-based compost was eliminated within 21 days under field conditions. Hess et al. (2004) found that in straw and cattle manure-based compost composted in small-scale laboratory reactors *E. coli* and *E. coli* O157:H7 levels were undetectable after 1 day if composts reached 55 and 65°C. Lung et al. (2001) showed that Rifampin resistant (Rif^{R}) *E. coli* O157:H7 was undetectable in 3 days when the compost reached a maximum temperature of 45°C, whereas Rif^{R} *Salmonella* was more sensitive to composting at 45°C and reached undetectable levels in only 2 days. Thermal death times for *Salmonella* Newport in composted sewage sludge were determined by Wiley and Westerberg (1969), and the results showed that at 60°C, 40 minutes were needed to reduce $1.9x10^{9}$ CFU/g of *S.* Newport to undetected levels. During the composting of biowaste and

garden waste, *Salmonella* Senftenberg strain W775 survived less than 10 hours and 2 days in two separate piles where the internal temperature of the compost was ≥60^{o}C. (Ceustermans et al. 2006).

To make compost, manures rich in nitrogen are mixed with one or more carbon amendments. Carbon and nitrogen provide required nutrients and energy for microorganisms and may affect their survival during the composting process. Erickson et al. (2009) looked at the effect of C:N ratio on survival of *Salmonella* spp. in cow manure compost using a bioreactor. The last day that *Salmonella* was detected were days 3, 5, and 7 for the 20:1, 30:1, and 40:1 formulations, respectively. A C:N ration of 20:1 was found to be optimal because *Salmonella* spp. were inactivated at temperatures 15-fold less than required for 40:1 formulations and pH was slightly more acidic which in turn contributed to pathogen inactivation.

Some studies reported that pathogens are able to survive for longer periods of time in compost. One reason for this may be the location of the sample in the compost pile. Pathogens on the surface of the compost heap survive for longer periods than pathogens in the sub-surface (Shepherd et al. 2007; Erickson et al. 2009). Temperature also has an impact on survival of pathogens. Pathogens can survive longer during mesophilic composting (<45°C) than those in composts whose temperature reach thermophilic limits (Lung et al. 2001; Hess et al. 2004; Grewal et al. 2007). Also, lower moisture content and lower levels of indigenous microflora were shown to increase the survival time of *Salmonella* Senftenberg W755 in biowaste compost (Ceustermans et al. 2006).

Regrowth and Recolonization of Pathogens in Finished Compost

Once the thermophilic composting process is finished pathogens may regrow or recolonize in the compost. Regrowth in compost is an increase in viable numbers of microorganisms following a decline in population during active composting. In contrast, recolonization is the reintroduction of bacteria to compost followed by growth (Zaleski et al. 2005). Zaleski et al. (2005) suggest that a threshold number of pathogenic cells need to be present in the compost in order for regrowth to occur. This is especially important when indigenous microorganisms are present because of biotic competition. Most of the research done on the growth potential of bacteria in finished compost focuses on recolonization by seeded bacteria, but a few papers show that regrowth can occur.

One condition where regrowth of pathogens in finished compost may occur is in the presence of excess moisture, such as exposure to rainfall. Zaleski et al. (2005) reported the regrowth of heterotrophic plate count bacteria by ca. 1 log CFU/g in biosolids in a drying bed after rainfall events, as well as a slight increase of *Salmonella* population in the drying beds. Temperature is another factor that affects the regrowth of pathogens in compost. Hess et al. (2004) showed that *E .coli* O157:H7 regrew during composting of bovine manure inside a bioreactor when the composting temperature was in the range of 40 to 50^{o}C. There was a 1 and 2 log CFU/g increase of *E. coli* O157:H7 when the composting temperature was maintained at >50^{o}C for 2 days and 40 to 50^{o}C for 1 day, respectively. Studies also demonstrate that regrowth of pathogens occurred in the presence of a low population of background microflora. Russ and Yanko (1981) found that the population of *Salmonella* in sewage sludge (20% moisture) with low levels of background bacteria increased more than 4 orders of magnitude when kept in the

mesophilic temperature range of 20 to 40°C, whereas *Salmonella* in sludge with moisture contents of 16% and 9% incubated at 44 or 4°C showed no growth.

Studies have demonstrated that if pathogens are reintroduced into compost after the thermophilic phase they can quickly grow to hazardous levels. The growth potential may differ depending on environmental conditions such as moisture content, temperature, indigenous microflora, pH, and season. Moisture content is one of the main factors that affect the recolonization of pathogens in organic fertilizers. Bacteria need a high water activity environment in order to survive and grow and this usually means higher water content is also needed. Yeager and Ward (1981) illustrated that background bacteria in sludge grew rapidly with moisture contents above 25%, but no growth occurred in sludge with a moisture content less than 15%. *Salmonella* persisted up to 12 weeks in moist irradiated composts (45%), whereas it declined rapidly in air-dried compost within 4 weeks (Hussong et al. 1985). Pietronave et al. (2004) showed that *Salmonella* and *E. coli* grew in compost with a moisture content between 40% and 80% but not at 10%. Payne et al. (2007) found that poultry litter with a water activity of 0.96 or higher will allow growth of *Salmonella*. However, the effects of high moisture content on pathogen growth may differ depending on other characteristics of the compost, such as background microbial populations and storage temperature.

The recolonization of pathogens can be affected by the number and diversity of the background microflora in a compost pile. The indigenous microorganisms in compost come from human and animal wastes as well as other compost ingredients. Thermophilic microorganisms (*Bacillus*, *Thermonospora*, and *Micropolyspora*) generate heat during the thermophilic phase which is the key step for pathogen inactivation (Strom 1985). Another way that background microbes help get rid of pathogenic bacteria is competition for nutrients and water and/or

produce antimicrobial compounds. Millner et al. (1987) reported that prior occupation of the substrate by microbes may suppress pathogen growth when compared to simultaneous inoculation. The most common way to compare the impact of high and low indigenous bacterial levels on pathogen growth is to sterilize the compost either by irradiation or autoclaving. *E. coli*, *Salmonella*, *E. coli* O157:H7, and *Listeria monocytogenes* all had a greater growth potential in sterilized compost as compared to nonsterilized compost (Sidhu et al. 1999; Sidhu et al. 2001; Pietronave et al. 2004; Hussong et al. 1985; Kim and Jiang 2010; Yeager and Ward 1981). Hussong et al. (1985) showed that it was not just the presence of microflora that suppressed *Salmonella* growth, rather the high activity of the indigenous microorganisms. The suppressive effect of the background bacteria also depends on the diversity of microorganisms in the population of microflora (Pietronave et al. 2004; Millner et al. 1987). It is possible that readily available nutrients, high moisture content, and optimal temperature result in a higher activity of the indigenous microflora which may out-compete pathogens present in the compost (Sidhu et al. 2001).

Storage temperature is another important characteristic of compost that affects recolonization. During the composting process high temperatures eliminate pathogens, but if pathogens are introduced after the thermophilic phase they may encounter temperatures conducive to their growth and survival. Pietronave et al. (2004) reveal that increased temperature can have a positive effect of the growth of *Salmonella* and *E. coli* and that the influence of temperature has an inverse relationship to moisture content. Kim and Jiang (2010) found that *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* grew to higher levels in autoclaved dairy compost during warm seasons, such as the spring and summer months. In some cases, temperature may have little effect on the growth potential of pathogens depending

on the type of compost. Elving et al. (2009) found that *Salmonella* grew in household waste composts at three different temperatures (14, 24, and 37°C), whereas in beef cattle manure-based compost and fresh dairy cattle manure little to no growth was seen at any temperature.

Compost maturity and pH also have an effect on the growth of pathogens in finished compost. Maximum growth of *Salmonella* and *E. coli* O157:H7 declined as the maturity of the compost increased (Sidhu et al. 2001; Kim et al. 2009). Payne et al. (2006) determined the effect of pH on the growth of *Salmonella* in poultry litter. They found that in environments with a neutral to slightly alkaline pH (7 and 9) *Salmonella* showed growth of ca. 2 logs. The most drastic reduction in pathogen population occurred in low-pH environments. Overall, composting has numerous different factors that affect the growth of pathogenic bacteria each one impacting the others.

Current Regulations on Composting

If the right conditions are not met during composting, pathogens may not be eliminated. To ensure the microbiological safety of composted products, the United States Environmental Protection Agency (USEPA) set up the 40 CFR Part 503 guidelines for pathogen reduction in biosolids. For Class A standards fecal coliform levels must be less than 1,000 MPN/g of dry compost and *Salmonella* numbers must be less than 3 MPN/ 4 g of dry compost. Class A biosolids are safe to be applied to soil where it comes into contact with humans, such as lawns or agricultural products including produce. Class B biosolids can have higher levels of the above microorganisms but there are restrictions on where they can be used. For example, Class B biosolids can be applied to forest lands, reclamation sites, and other areas where there is a low potential for public exposure. To ensure that these levels are met the USEPA set up specific time

and temperature regulations. During composting temperatures above 55°C and below 65°C must be met for 3 days in aerated static piles or in-vessel systems, or 15 days for windrow systems with at least five turnings (USEPA 1999). The USEPA also recommends that a 0.3 m (1 foot) layer of insulating material such as finished compost be placed over the surface of static piles to ensure that every particle of the compost be subjected to the time-temperature criteria. Turning would negate this practice because it is assumed that by turning, all material at the surface of the pile will eventually end up at the middle of the pile (USEPA 2003). In order to determine if the composting meets the USEPA requirements testing should be done at the end of the composting process.

Quality Assessment of Composts

Compost quality refers to the physical, chemical, and biological characteristics of a batch and is an issue that affects the way composts can be used and marketed. However, quality is hard to define because it means different things to different people based on their background and the regulations in place where they live. Quality is determined by a number of different parameters that make up the compost including moisture content, organic matter, carbon:nitrogen ratio, heavy metals, pH, microbial diversity, and maturity or stability (Lasaridi et al. 2006). A few studies have surveyed various types of composts in order to gather information on composting practices and determine the occurrence of pathogenic bacteria in finished products.

Hussong et al. (1985) determined the level of *Salmonella* in sewage sludge composts collected throughout the United States. Water activity of the composts averaged 0.9953 with an average moisture content of 51%, and the pH averaged 6.7. Fecal coliforms were detected in

two samples with populations of $1.3x10^7$ MPN/g and $1.1x10^5$ MPN/g. Three of the composted sludges contained *Salmonella* with one sample having an MPN value $1.7x10^4$ MPN/g and two samples positive by enrichment only. The sample with countable numbers of *Salmonella* was stored at -20° for one year then retested. The compost was still positive for the pathogen but at a density 1 log less than previously found ($2.4x10^3$ MPN/g).

Yanko (1988) analyzed 260 composted sewage sludges from across the United States to determine the numbers of pathogenic organisms present. The highest concentrations of microorganisms were found in samples from static pile systems, whereas the lowest concentrations were found in pelletized sludge from a heat-drying process. When various materials were added after composting in order to produce commercial soil amendments, significantly higher bacterial and fungal concentrations were seen, suggesting nutrient-related growth. *Salmonella* was the most frequently detected potentially pathogenic bacteria, whereas toxigenic *E. coli* levels were very low. It was determined that the occurrence of pathogenic bacteria in these sludges represented a potential hazard risk.

Skanavis and Yanko (1994) collected samples of composted sewage sludge (n=45); raw amendment materials including aged redwood (n=15), firbark (n=15), redwood chips (n=10), rice hulls (n=15), and sawdust (n=38); and four bagged commercial soil conditioners including recycled compost (n=45), compost made with rice hulls (n=45), compost made with wood chips (n=45), and modified sludge compost (n=45) weekly for one year. Coliform levels were lower in composted sludge as compared with the four commercial compost-based products. *Salmonella* was not found in any of the composted sludge or bulking agent samples. The *Salmonella* positive rates were 27, 22, 22, and 36% for the recycled compost, rice hull-based compost, wood chip-

based compost, and modified sludge compost, respectively. The *Salmonella* isolates were serotyped, and the results show that *Salmonella* Ohio was the most common (45% of isolates).

A study in Massachusetts (Soares et al. 1995) surveyed 16 operating compost facilities in the state to assess the status of the process for biosolids management. Twelve of the 16 facilities used aerated static piles to compost their treated biosolids; three facilities used an in-vessel system with agitated bins, aeration and compost turning equipment; and one used a Taulman continuous feed vertical silo system. Five of the facilities exceeded EPA Class A standards for *E. coli* with Facility D even exceeding Class B standards. It was concluded that failure to maintain an appropriate moisture level will cause a failure to promote activity of indigenous microflora and degrade available carbon which can lead to unstable compost products.

Christensen et al. (2002) used two different methods, direct process evaluation and spot test analysis, to evaluate physical characteristics of the compost as well as microbiological parameters from four different facilities in Europe. Facility 1 in Norway used open-air windrows to compost household, yard, and forestry waste. Facility 2 in Denmark used open-air windrows to compost sewage sludge mixed with yard waste and straw. At Facility 3 in Sweden household and yard waste were composted in force-aerated boxes. Facility 4 in Finland composted sewage sludge, wood chips, and peat in fully enclosed force-aerated tunnels. The pH ranged from ca. 5 to 8 and the C:N ratio ranged from ca. 10:1 to 25:1. For the microbiological parameters, *E. coli* concentrations were above the detection limit in only 4 of the 47 samples. There was a correlation between fecal coliforms and *E. coli*. No samples were positive for *Salmonella* in the finished compost except at Facility 2 where two of the five samples were positive. The two

methods tested in this article had conflicting results which suggests that careful testing of finished compost is needed for accurate results.

Lasaridi et al. (2006) assessed the quality of 28 composts in the Greek market and examined their compliance with Greek and EU standards. Moisture content of the composts ranged from 6% for an MSW compost to 70% for a peat-based product. All samples tested had almost neutral pH except for one peat-based product with the pH ranging from 6.3 to 8.9. Total mesophilic bacteria reached levels above 8 log CFU/g in most composts with the exception of six samples where the counts ranged from 6 to 8 log CFU/g. Spore-forming bacteria were found in all samples tested exceeding 6 log CFU/g, with eight samples exceeding 8 log CFU/g. Yeasts were detected in eight samples with numbers ranging from 2 to 6 log CFU/g. Fecal coliforms were found to be higher than $5x10^2$ CFU/g, whereas *Salmonella* spp. were not detected in any of the composts tested. Due to high variability in the compost quality the authors suggest a need to establish quality assurance procedures in Greece in order for composting to be a more reliable waste treatment method. There is also a need for the development of EU quality standards in order to standardize compost markets.

Briancesco et al. (2008) determined the quality of compost derived from raw wastes from 20 plants in Italy based on the reduction of microorganisms. *Salmonella* in raw wastes had an average population of 10^2 MPN/g. In the finished product very low levels of the pathogen remained except in two facilities where no reduction was observed. *Clostridium perfringens* spores had an average of 2 log CFU/g in finished composts with a reduction rate of two orders of magnitude in all samples. There were a higher number of samples positive for *Giardia* cysts as compared to *Cryptosporidium* oocysts in the raw material entering the compost plants, but neither of the parasites was detected in the final products. However, helminth ova were still

present in one sample after the composting process. They concluded that compost containing sewage sludge seems to have a better hygienic quality as compared to compost containing green waste and municipal solid waste.

Brinton et al. (2008) investigated the occurrence of fecal indicators and pathogenic bacteria in market-ready recycled organic matter (ROM) composts in Washington, Oregon, and California. For Washington State, 23% of samples exceeded the EPA 503 limit for fecal coliforms of 1,000 MPN/g. One sample was positive for *Salmonella*, but at 1.8 MPN/g it was below the EPA 503 limit of 3 MPN/ 4g. For Oregon, 44% of samples exceeded the EPA regulations, whereas 36% had no detectable levels for fecal coliforms. For California, only 20% of the samples exceeded the EPA limit for fecal coliforms. *E. coli* O157:H7 was detected at three plants in the large facility group. These plants were retested 3 months later, and three of the five facilities were still positive for the pathogen. No samples from Oregon and California were positive for *Salmonella*. Apparently, additional research is needed to discover critical factors that affect pathogen levels in finished compost.

Most recently, Saha et al. (2010) analyzed municipal solid waste compost quality in different cities in India. Moisture content varied from 3.6% to 45.4% with a median value of 17.2%. The pH ranged from 6.64 for 'Neem cake' fortified compost to 9.63 with a median value of 7.50. The C:N ratio ranged from 7.2 to 36.5 with a median of 16.4. *Salmonella* could not be detected in any of the compost samples, but total coliforms were detected in 17% of samples belonging to six cities. India has the potential to produce as much as 4.3 million tons of compost each year, but inappropriate solid waste management and production of poor quality composts are preventing utilization of such large amounts of fertilizer.

Antimicrobial Resistance

There is a serious concern worldwide about the increased prevalence of antimicrobial resistance in microbial communities. The use of antibiotics in food animal production is the main risk factor for increased resistance in pathogenic bacteria. Therapeutic treatments are intended for diseased animals. However, for some animals, such as poultry and fish, mass medication is the only feasible means of treatment, while in other animals it is just more efficient to treat entire groups by medicating feed or water. Producers may also use antimicrobials in relatively low concentrations to promote growth. This type of "nontherapeutic" use often occurs early in production and is typically discontinued once the animals mature (McEwen and Ferdorka-Cray 2002).

The use of antimicrobials in animal production systems selects for resistance in enteric microorganisms. Smith et al. (2007) found that Minimum Inhibitory Concentrations (MICs) for sarafloxacin were higher for *E. coli* isolates from commercial flocks that were given sarafloxacin than for isolates from a farm with no recent use of fluoroquinolones. Similarly, Jiang et al. (2006) showed that calves treated with ceftiofur shed significantly higher numbers of ceftriaxone-resistant bacteria than control animals. When comparing conventional and organic dairy farms, there was a high prevalence of resistant *E. coli* from phylogenetic group A on conventional farms as opposed to phylogenetic group A isolates on organic farms with limited antimicrobial usage (Walk et al. 2007).

Resistant bacteria can be introduced into the farm environment through animal feces. Repeated introduction of resistance genes from fecal microorganisms can lead to a build-up of antimicrobial resistance in bacterial communities. Smith et al. (2007) found that 75% of the *E. coli* isolates from commercial broiler chicken flocks were resistant to three or more

antimicrobials. Carson et al. (2008) determined that only 2% of *E. coli* isolates from beef cattle farms were resistant to more than five antimicrobials. Of the 105 ceftriaxone-resistant commensal bacterial isolates found on a dairy and a poultry farm by Yang et al. (2006), 97.1% were resistant to three or more antimicrobial agents, 68.6% were resistant to 5 or more agents, and 34.3% were resistant to all nine agents tested.

The resistant bacteria can be spread throughout the farm environment. Yang et al. (2006) determined that drinking water and feed on a dairy farm and a poultry farm, old manure slurry on the dairy farm, and bedding from the poultry farm had high levels of commensal bacteria with reduced susceptibility to ceftriaxone. Not only can this affect the immediate farm environment, but antimicrobial residues have been found in environmental waters near swine and poultry farms (Campagnolo et al. 2002).

Animal manures are commonly used as organic fertilizers in the form of compost or slurry, and antimicrobial resistant bacteria present in the manure can be transferred to the fertilizers. Heringa et al. (2010) tested 277 compost samples for the presence of antimicrobial resistant *E. coli* and found that 17 out of 118 isolates were resistant to two or more antibiotics. This could lead to increased levels of antimicrobial resistant bacteria in the soil where the compost is used as a fertilizer. In contrast, Sengeløve et al. (2003) saw a decrease in the number of tetracycline-resistant bacteria in soil after the spread of pig manure slurry.

Numerous studies have determined that microbes in manure or organic fertilizers can be transferred to fresh produce in the field. *E. coli* has been shown to contaminate carrots, lettuce, and radishes after fertilization with bovine manure (Natvig et al. 2002; Ingham et al. 2004). *E. coli* O157:H7 was detected on lettuce, parsley, carrots, and onions grown in soil amended with dairy manure compost (Islam et al. 2004; Islam et al. 2005). *Salmonella*

Typhimurium was detected on radishes and arugula grown in soil mixed with inoculated bovine manure (Natvig et al. 2002). Solomon et al. (2002) found that *E. coli* O157:H7 can be taken up by the root system of and localized within lettuce leaf tissue. Apparently, high levels of antimicrobial resistant bacteria in fertilizers can lead to contamination of agricultural crops by these microorganisms. Ruimy et al. (2010) surveyed conventional and organic fruits and vegetables for antimicrobial resistant bacteria and determined that raw produce is frequently associated with resistant gram-negative bacteria and similar levels were found regardless of conventional or organic production.

Resistance Integrons

Antimicrobial resistance usually comes from a mutation of certain genes in a susceptible bacterial strain that allows the mutant to make a specific protein that inactivates an antimicrobial agent or otherwise evades the agent's damaging effects. Mutations can develop on the bacterial chromosome, but more commonly, resistance genes are imported from outside via genetic vectors such as plasmids, transposons, and integrons. These mobile genetic elements can be transferred horizontally to other strains or species (O'Brien 2002). Blake et al. (2003) showed that resistant genes can be transferred from commensal *E. coli* to other commensal *E. coli* as well as pathogens, such as *E. coli* O157:H7 and *Salmonella* within the intestine of swine. Horizontal transfer can occur in the environment as well with efficiency depending on factors such as substrate and temperature. Manure added to the soil enhanced the mobilization of resistance genes (Götz and Samlla 1997). Additionally, conjugative transfer of plasmids occurred between *E. coli* cells in chicken manure at 23°C but not in compost at temperatures above 50°C (Guan et al. 2007).

Integrons are genetic elements containing components of a site-specific recombination system that recognizes and captures mobile gene cassettes, and they generally include an integrase gene (*int*) and an adjacent recombination site (*attI*). There are three known classes of integrons. Most belong to class 1. The core structure of class 1 integrons is made up of *intI* integrase and the *attI* recombination site (Fluit and Schmitz 2004). Class 2 integrons are embedded in the Tn7 family of transposons and include an integrase followed by three gene cassettes (*dfrA1*, *sat2*, and *aadA1*) (Hansson et al. 2002). Little is known about class 3 integrons, but their structure is comparable to that of class 2 integrons (Collis et al. 2002). Gene cassettes normally contain a single gene and a recombination site known as the 59 base element or *attC.* A large proportion of integrons contain the *aadA* gene encoding resistance to streptomycin-spectinomycin as well as the *sul-1* gene which confers sulfonamide resistance (Fluit and Schmitz 2004).

Integrons have been identified from numerous antimicrobial resistant isolates. Cocchi et al. (2007) found that 44 out of 120 *E. coli* carried class 1 integrons while only 4 strains carried class 2 integrons. The class 1 integrons carried 11 distinct gene cassettes with the most common cassette encoding the *aadA* gene. The class 2 integrons all had the same cassette array: *dfrA1*, *sat2*, and *aadA1*. Zhao et al. (2001) found nine shiga toxin-producing *E. coli* isolates carrying class 1 integrons with a majority of the isolates containing the *aadA* gene. Yang et al. (2010) detected class 1 integrons from a beef ranch, dairy farm, and city locations. Again the *aadA* gene cassette was most common. Heringa et al. (2010) found nine integron containing isolates out of the 136 tested containing gene cassettes from the *aadA* and *dfr*A1 families.

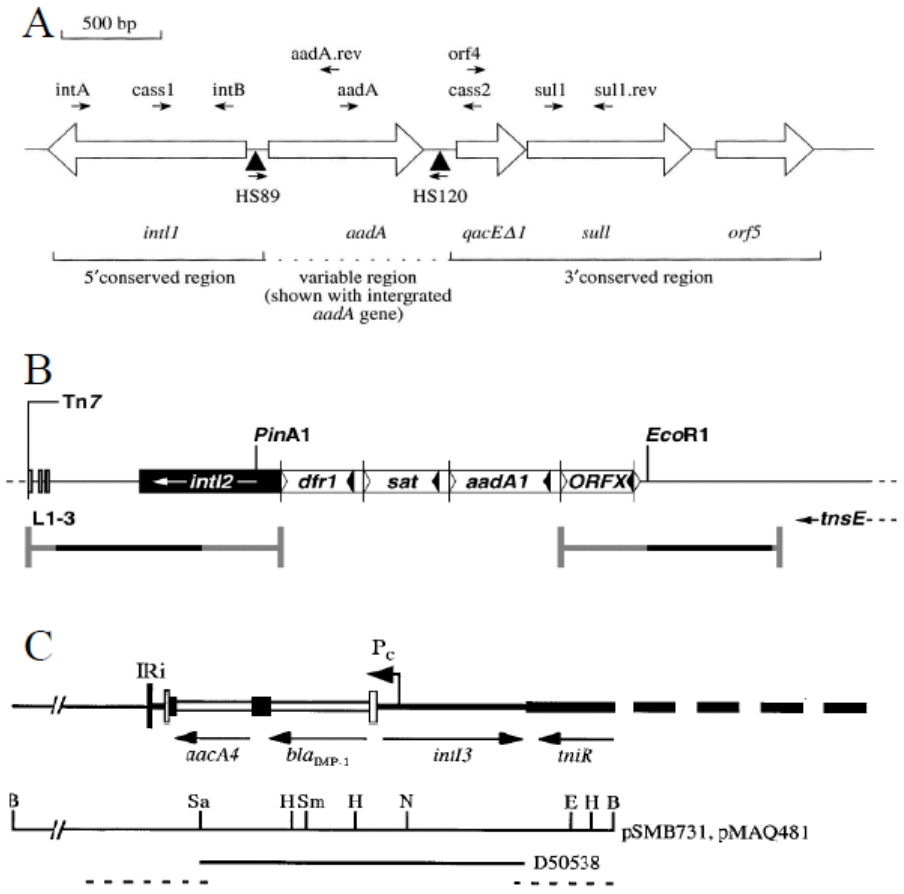

Figure 1.1. Structure of resistance integrons. A-class 1 (Rosser and Young 1999), B-class 2 (Hansson et al. 2002), and C-class 3 (Collis et al. 2002).

Conclusion

During recent years a growing number of food-borne disease outbreaks have been associated with the consumption of fresh produce. One of the major sources of contamination is thought to be animal manure applied to fields as an organic fertilizer. Composting is one biological method to eliminate pathogens from manure and biosolids before land application. However, if thermophilic composting does not occur, pathogens may survive and regrow to hazardous levels. Pathogens can also be reintroduced into the compost pile by animals or

contaminated farm equipment. If this happens, pathogens may be subject to favorable conditions and grow to high numbers. The USEPA has set standards for sewage sludge that encompass composting as well. However, composting is a complex process driven by microbial activities which are subject to changes by a variety of factors. Additionally, the concern of antibiotic resistant microorganisms makes it more crucial that pathogens are eliminated during composting. More thorough studies need to be done to completely understand the relationship between pathogens and the composting environment.

Objectives of this Study

1. Microbiological survey of various types of organic fertilizers
2. Growth potential of *E. coli* O157:H7 and *Salmonella* spp. on select organic fertilizers with different levels of background microflora
3. Antimicrobial resistance and presence of integrons in *E. coli* isolated from organic fertilizers

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CHAPTER TWO

MICROBIAL ANALYSIS OF ORGANIC FERTILIZERS AND EVALUATION OF THE GROWTH POTENTIAL OF FOODBORNE PATHOGENS IN VARIOUS FERTILIZERS

Abstract

Composting is a sanitizing process to reduce the population of pathogens in animal wastes. However, inadequate composting may not be able to get rid of all of the pathogenic bacteria which may be subsequently transferred to food products such as fresh produce. A microbiological survey was conducted on 103 organic fertilizers collected from across the United States. The moisture content ranged from ca. 1% to 86.4% with the water activities between 0.298 and 0.999. The average pH was 7.77 ± 1.28. The total background bacteria ranged from ca. 3 to 9 log CFU/g. *Enterobacteriaceae* populations were in the range of less than 1 log to as much as 7 log CFU/g while coliform levels varied from less than 1 log to ca. 6 log CFU/g. Thirty samples (29%) were positive for *Escherichia coli* with levels reaching ca. 6 log CFU/g. However, there were no confirmed positives for *E. coli* O157:H7, *Salmonella* spp. or *Listeria monocytogenes*. The growth potential of *E. coli* O157:H7 and *Salmonella* spp. was determined in organic fertilizers with high (≥5 log CFU/g) and low (<5 log CFU/g) levels of background bacteria. In the presence of high levels of background bacteria, *Salmonella* and *E. coli* O157:H7 grew ca. 1 log CFU/g within the first day of incubation in plant-based compost and fish emulsion-based compost, respectively. With low levels of background bacteria, *Salmonella* grew ca. 2.6, 3.0, 3.0, and 3.2 log CFU/g in the blood, bone, and feather meals and the mixed-source fertilizer, respectively, whereas *E. coli* O157:H7 grew ca. 4.6, 4.0, 4.0, and 4.8 log CFU/g, respectively. Our results revealed a wide range of microbiological quality among different types of organic fertilizers, and

identified the environmental factors affecting the growth of *E. coli* O157:H7 and *Salmonella* spp. in some organic fertilizers.

Introduction

Over the last couple of decades organic farming and food production have increased in popularity. In 1990, the USDA set up the Organic Food Production Act in order to establish national standards for the production and handling of 'organic' foods (Gold 2007). In the U.S., certified organic crop acreage more than doubled between 1997 and 2005 and now encompasses all 50 states. However, this only accounts for 0.5% of all U.S. cropland and 0.5% of all U.S. pasture land. In 2005, 5% of vegetable acreage and 2.5% of fruit and nut acreage were certified organic while only 0.2% of corn and soybean crops were grown using organic practices (Greene et al. 2009). One major practice used in organic farming is the application of organic fertilizers. The most common organic fertilizers applied to soil are composted animal manures usually supplemented with rock powder, plant by-products, and additional animal by-products like blood, bone, and feather meals (Kuepper 2003). In 2006, manure was spread as a fertilizer on about 15.8 million acres of U.S. cropland, accounting for 5% of the total (organic and conventional) 315.8 million acres of cropland in the U.S. (MacDonald et al. 2009).

The number of outbreaks caused by food-borne pathogens has also increased due to the increasing consumption of ready-to-eat foods. From 1973 to 1997, outbreaks associated with fruit, vegetable, salad, and fruit juice have increased from 0.7% in the 1970s to 6% in the 1990s. Both *Salmonella* and *E. coli* O157:H7, important foodborne pathogens, are frequently implicated in fresh produce-related outbreaks, such as lettuce, melon, tomato, carrot, spinach, and green onions (Sivapalasingam et al. 2004). A multi-state outbreak of *Salmonella*

Typhimurium in states east of the Mississippi river was linked to tomatoes consumed at restaurants (CDC 2006a). One outbreak of *E. coli* O157:H7 from shredded iceberg lettuce at Taco John restaurants in Minnesota and Iowa was traced to dairy farms near a lettuce growing region in California's Central Valley (USFDA 2007). Another outbreak of *E. coli* O157:H7 was due to contaminated spinach from three counties in California (CDC 2006b). Contamination of produce on the farm can be linked to contaminated irrigation water and wash water, improperly composted manure, and direct contact with feces of livestock (Sivapalasingam et al. 2004). With the increased interest in organic farming, produce contamination due to organic fertilizers is expected to increase.

There are few reports on the microbiological quality of organic fertilizers. Brinton et al. (2009) surveyed 94 market-ready recycled organic composts, and found that 23, 44, and 20% of samples exceeded the EPA 503 limit of 1,000 MPN/g for fecal coliforms in Washington, Oregon, and California, respectively. One sample from Washington was positive for *Salmonella*, while samples from 3 large scale composting facilities in California were positive for *Escherichia coli* O157:H7. Lasaridi et al. (2006) examined 28 compost samples from the Greek market to determine their quality profile. Most composts had background populations greater than 10^8 CFU/g, whereas fecal coliforms exceeded 10^3 CFU/g in all of the samples. No *Salmonella* spp. was found in any of the samples tested.

Thermophilic composting is commonly used as a sanitizing method to reduce the occurrence of pathogens in animal waste products. However, there is still the possibility that the composting process may not fully eliminate pathogens or that pathogens may be reintroduced into the finished product due to cross-contamination on the farm. Several studies evaluated the growth and survival of pathogens in finished compost as affected by environmental factors.

Salmonella and *E. coli* grew and survived longer in compost with a high moisture content (>20%) (Hussong et al. 1985; Pietronave et al. 2004). *E. coli*, *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* all had greater growth potential in sterilized compost as compared to nonsterilized compost (Sidhu et al. 2001; Pietronave et al. 2004; Hussong et al. 1995; Kim and Jiang 2010; Yeager and Ward 1981). Pietronave et al. (2004) revealed that increased temperature can have a positive effect on the growth of *Salmonella* and *E. coli*. Payne et al. (2006) found that *Salmonella* grew in poultry litter ca. 2 log CFU/g in neutral to slightly alkaline environments (pH 7 and 9).

The objectives of this study were to analyze the physical and biological characteristics of organic fertilizer samples with different compositions from multiple states and evaluate the growth potential of *E. coli* O157:H7 and *Salmonella* spp. in selected types of organic fertilizers under various conditions.

Materials and Methods

Sample Collection: Samples of different types of organic fertilizers were collected aseptically from multiple states in the United States including Arizona, California, Georgia, Kentucky, Maryland, New York, North Carolina, South Carolina, and Tennessee from 2007 to 2010. The composition and age of the compost as well as the location collected were recorded. The samples were transported at room temperature and stored in a refrigerator (5°C) upon arrival.

Water Activity, Moisture Content, and pH measurements: The water activity of each sample was measured using a dew-point water activity meter (Aqualab series 3TE, Decagon Devices, Pullman, WA). Moisture content was determined by drying ca. 2-3 g of sample overnight in an oven (Thelco Model 27, Precision Scientific Co., Chennai, India) at 105°C. For measurement of

pH, one gram of sample was mixed with 50 ml of distilled water for 5 minutes and pH was measured using a pH meter (Orion Star meter, Thermo Fisher Scientific Inc., Fort Collins, CO).

Microbiological Analysis: Twenty five grams of each organic fertilizer were added to 225 ml of universal preenrichment broth (UPB; Neogen, Lansing, MI) in a Whirl-Pak® sampling bag and shaken vigorously for 1 minute. For enumeration of total background bacteria serial dilutions were made from each UPB suspension, spiral-plated on tryptic soy agar (TSA; Becton & Dickinson and Co., Sparks, MD), and incubated overnight at 37ºC. For enumeration of *Enterobacteriaceae*, 1 ml of the UPB suspension was mixed with 15 mL of violet red bile agar with glucose (VBRG) (Becton & Dickinson) by pour-plating then overlaid with 5 mL of VBRG and incubated overnight at 37ºC. Coliforms and *E. coli* were enumerated by plating 1 ml of the UPB suspension onto *E. coli*/coliform Petrifilm® (3M, St. Paul, MN), which was incubated overnight at 37ºC. The remaining UPB suspensions were incubated overnight at 37ºC with shaking for pathogen detection.

Pathogen Detection: One milliliter of each overnight culture of UPB was transferred to 9 ml of Rappaport-Vassiliadis (RV) broth, modified-tryptic soy broth (mTSB; Becton Dickinson) with novobiocin (20µg/ml) (Oxoid Ltd., Basingstoke, Hants, UK), and Fraser broth (Becton Dickinson) for selective enrichment of *Salmonella* spp., *E. coli* O157:H7, and *L. monocytogenes*, respectively, and incubated overnight at 37ºC with shaking. Samples were then streaked onto sorbitol MacConkey agar (Becton Dickinson), Oxford agar (Becton Dickinson), and xylose-lysine-tergitol 4 agar (XLT4; Becton Dickinson) as well as hektoen enteric agar (HE; Neogen, Lansing, MI) for detection of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* spp., respectively.

Real-time PCR Confirmation of Presumptive Positives of Pathogens: Presumptive positive colonies of the target pathogen were purified by streaking onto TSA several times. Suspected

colonies were aseptically transferred to an Ependorf tube containing 1 ml of sterile distilled water followed by boiling for 5 to 10 minutes for DNA extraction. Extracted DNA was used to perform real-time PCR using primers O157BF (5'-AAATATAAAGGTAAATATGT GGGAACATTTGG-3') and O157BR (5'-TGGCCTTTAAAATGTAAACAACGGTCAT-3') (Fortin et al. 2001) for detection of *E. coli* O157:H7, HLYF (5'-TCCGCAAAAGATGAAGTTC-3') and HLYR (5'-ACTCCTGGTGTTTCTCGATT-3') for detection of *L. monocytogenes,* and SF (5'-CCTTTCTCCATCGTCCT GAA-3') and SR (5'-TGGTGTTATCTGCCTG ACC-3') (Wang et al. 2004) for detection of *Salmonella* spp. The PCR reaction was carried out in a Bio-Rad iCycler system (BioRad, Inc., Hercules, CA). The reaction mixture (20µL) contained 1.5µM each of forward and reverse primer, 4µL of DNA template, and 10µL of iQ SYBR Green Supermix (BioRad, Inc., Hercules, CA). The PCR protocol for *E. coli* O157:H7 detection consisted of an initial denaturation at 95ºC for 10 min; 40 cycles of denaturation at 94ºC for 45 s, annealing at 52ºC for 45 s, and elongation at 72ºC for 45 s (Fortin et al. 2001). The PCR protocol for *L. monocytogenes* and *Salmonella* detection consisted of an initial denaturation at 95°C for 10 min; 30 cycles of denaturation at 95°C for 8 s, annealing at 55°C for 5 s, and elongation at 72°C for 10 s (Wang et al. 2004).

Bacterial Cultures: A three strain mixture of both *E. coli* O157:H7 and *Salmonella* spp. was used as the inocula. Strains F06M-0923-21 (Spinach outbreak, from California Department of Health), F07M-020-1 (Taco John outbreak, from California Department of Health), and B6914 (*stx* 1^- and *stx* 2^-, from Dr. Fratamico, USDA-ARS-ERRC) were selected for *E. coli* O157:H7, as well as, *Salmonella* serovars Enteritidis H2292 (from Dr. Mike Doyle, University of Georgia), Heidelberg 21380 (from Dr. Shaohua Zhao, CVM-FDA), and avirulent Typhimurium 8243 (from Dr. Roy Curtis, Washington University). These strains were induced to be resistant to 100 µg rifampin per milliliter (Fisher Scientific, Fair Lawn, NJ, USA) through the gradient plate method (Rice and

Bonomo 2005) and stored at -80ºC in tryptic soy broth (TSB; Becton Dickinson) with 20% glycerol (Fisher Scientific).

Growth Potential of Pathogens in Composts: The growth of *E. coli* O157:H7 and *Salmonella* was evaluated in selected types of fertilizers with different levels of background microorganisms. The organic fertilizers with a high level of background bacteria (≥5 log CFU/g) tested for potential growth of *E. coli* O157:H7 included fish emulsion-based compost (n=1), cow manure-based compost (n=2), and spent mushroom compost (n=2). The growth potential of *Salmonella* was evaluated separately in the following fertilizers: spent mushroom compost, super-heated chicken litter, plant-based compost, horse manure- based compost, and hen manure-based compost. Moisture content was adjusted to 40% for the composted fish emulsion and to 50% for the chicken litter in order to achieve a water activity above 0.99. All other organic fertilizers were used at their original moisture content. Organic fertilizers (n=5) with a low level of background bacteria (<5 log CFU/g) were tested including bone meal, blood meal, feather meal, cow manure based-compost, and a mixed source organic fertilizer. The moisture content of all these organic fertilizers was adjusted to achieve the highest possible water activity (0.95-0.99).

An inoculum cocktail of three rifampin-resistant strains of *E. coli* O157:H7 or *Salmonella* was sprayed onto compost to a final concentration of ca. 5 log CFU/g using a sterile spray nozzle. The compost was then mixed by hand while wearing sterile gloves until the inoculum was evenly distributed. The samples were stored in sampling bags at 22 or 30°C, and taken at 16 h, 1, 2, 3, 5, and 7 days to determine pathogen populations. Each growth study was conducted for two trials.

Statistical Analysis: Bacterial counts were converted to log CFU/g dry weight and subjected to analysis of variance (ANOVA) using SAS (ver. 9.1; SAS Institute, Inc., Cary, NC, USA). *P*-value

below 0.05 was considered significantly different. Tukey's honestly significantly different test was used to determine if the growth of the pathogens differed at each sampling time.

Results

A total of 103 organic fertilizer samples were collected from multiple states across the United States (Table 1). The greatest percentage of samples (16.5%) were mixed source while the greatest numbers from a single predominate source include cow manure-based composts (n=15), spent mushroom composts (n=11), horse manure-based composts (n=10), and poultry-based samples (n=11) (5 super-heated chicken litter and 6 poultry-manure based composts). The moisture content ranged from ca. 1% to 86.4%, with water activities between 0.298 and 0.999. The vermicomposts had the highest average moisture content at ca. 75%, while the bone, blood, and feather meals had the lowest average at 8.1%. The spent mushroom composts, vermicomposts, alpaca manure, and mixed animal waste composts all had an average water activity above 0.99. The pH of most samples was neutral with the average pH at 7.77. However, the pH of the liquid fish emulsion samples was around 3~4, as compared with pH of ca. 10 for the dairy cow manure-based composts collected from Arizona.

The total background bacterial counts ranged from ca. 3 to 9 log CFU/g. The highest average total bacterial counts of 7.98 and 7.95 log CFU/g were from the horse manure-based compost and the rabbit manure-based compost, respectively. Both the liquid fish emulsion and the blood, bone, and feather meal groups had lower average total bacterial counts at around 4 log CFU/g. Individual samples with low total bacterial levels included Arizona dairy cow manure compost (n=1), super-heated poultry litter (n=1), and mixed source fertilizer (n=1). *Enterobacteriaceae* populations were in the range of less than 1 log to as much as 7 log CFU/g.

All vermicompost, rabbit manure-based compost, greenwaste, and alpaca manure samples were positive for *Enterobacteriaceae*. Coliform levels varied from less than 1 log to ca. 6 log CFU/g with an average of 3.37 log CFU/g. Thirty samples (29%) were found to be positive for *E. coli* with the biggest proportion of those coming from the horse manure-based composts. The average number of *E. coli* was 2.74 log CFU/g with the highest reaching 6.34 log CFU/g. The super-heated chicken litter, liquid fish emulsion, composted fish emulsion, and plant-based compost were all negative for *Enterobacteriaceae*, coliforms and *E. coli*. About 75% of the bone, blood, and feather meals were positive for coliforms, but all samples were negative for *E. coli*. Overall, no samples were confirmed positive for *E. coli* O157:H7, *Salmonella* spp. or *L. monocytogenes* after species-specific PCR analysis.

E. coli O157:H7 was tested for potential growth in organic fertilizers with high levels of background bacteria (≥5 log CFU/g) at both 22 and 30°C for 7 days (Fig. 1). The composts, including composted fish emulsion (n=1), composted cow manure (n=3), and spent mushroom compost (n=2), were inoculated with a three-strain mixture of *E. coli* O157:H7 at an initial level of ca. 5 log CFU/g. *E. coli* O157:H7 grew ca. 0.8 logs in 1 day at both 22 and 30°C in the composted fish emulsion sample only. The *E. coli* O157:H7 populations in the two spent mushroom composts declined faster than in the composted cow manure samples Since the growth potential for *E. coli* O157:H7 was not different at 22ºC than at 30ºC, the following growth studies were conducted at room temperature only.

The growth potential of *Salmonella* spp. was tested in 5 different types of organic fertilizers with high levels of background bacteria (≥5 log CFU/g) at room temperature (22°C) including super-heated chicken litter, hen manure-based compost, spent mushroom compost, horse manure-based compost, and plant-based compost (Table 2). At an initial inoculum level of

ca. 5 log CFU/g the plant-based compost supported growth of *Salmonella* spp. by ca. 1 log within 1 day then stayed at a steady state until day 7 (Fig. 2). There were fluctuations in *Salmonella* population in super-heated chicken litter throughout the 7 days of incubation. For example, the *Salmonella* spp. level was 5.4 logs at day 0, decreased slightly to 5 logs by day 1, then went back up to 5.4 logs by day 3 before falling to 3.6 logs on day 7. Within 7 days of incubation, there was a slight decline (< 1 log) in *Salmonella* population in both the hen manure compost and the horse manure compost, but a moderate decline of 2 logs in the mushroom compost.

The potential growth of *E. coli* O157:H7 and *Salmonella* spp. was also tested in organic fertilizers with low levels of background bacteria (<5 log CFU/g) at 22°C (Fig. 3). The same organic fertilizers were used for both pathogens including bone meal, blood meal, feather meal, high pH cow manure based compost, a mixed source fertilizer, whereas a neutral pH cow manure compost with a high background bacteria level (ca. 7 log CFU/g) was used as a control (Table 2). All samples were adjusted to moisture contents between 30 and 50% prior to pathogen inoculation to yield water activity of at least 0.95. At an initial inoculum level of ca. 5 log CFU/g, *E. coli* O157:H7 grew significantly ($P < 0.05$) in all of the samples except for the two cow manure-based composts. Within 7 days of incubation, *E. coli* O157:H7 populations increased ca. 4.6, 4.0, 4.8, and 4.0 log CFU/g in the bone meal, blood meal, mixed source fertilizer, and feather meal, respectively. There was a slight decline in *E. coli* O157:H7 population in the cow manure-based compost with a neutral pH compared with a rapid die off of the pathogen in the cow manure compost with a pH of 10.

The growth of *Salmonella* spp. in low background (<5 log CFU/g) organic fertilizers followed a similar pattern as *E. coli* O157:H7, but the increase in population was less. *Salmonella* populations increased ca. 2.6, 3.0, 3.2, and 3.0 log CFU/g in the bone meal, blood meal, mixed

source fertilizer, and feather meal, respectively. The neutral pH cow manure sample had a small decrease in pathogen population of about 0.7 logs by day 7, while the cow manure compost with pH 10 had no detectable *Salmonella* spp. soon after inoculation.

Discussion

The thermophilic phase of the compost process can eliminate pathogenic bacteria in animal wastes under optimal conditions. Most other organic fertilizers analyzed in this study also went through some types of heat treatments such as dry-heat for superheated chicken litter and boiling for fish emulsion. Therefore, finished organic fertilizer should be expected to have none or very low levels of potentially pathogenic bacteria such as *E. coli* as well as other indicator microorganisms. Our survey found 30 out of 103 organic fertilizer samples positive for *E. coli* (29%). The average count of *E. coli* was 2.74 log CFU/g with the highest population reaching 6.34 log CFU/g. All of the organic fertilizer samples analyzed in this study were found to be free of *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes*. Brinton et al. (2009) surveyed 94 compost samples and detected *E. coli* levels ranging from below detection limit to 7.30 log MPN/g with a third of the samples having less than 10 MPN/g and another third exceeding 1,000 MPN/g. One out of 94 samples was found to be positive for *Salmonella* but was below the EPA limit of 3 MPN/4g. *E. coli* O157:H7 was detected in samples from three large composting facilities and was still present at the facilities when retested 3 months later. The above results indicate that if optimal conditions are not met, the composting process may not be adequate to eliminate all pathogenic bacteria.

Indicator bacteria such as *E. coli* and coliforms are commonly used to judge the quality of finished composts. The California Leafy Green Marketing Agreement (LGMA) sets Good

Agricultural Practice (GAP) standards for producing fresh and fresh-cut lettuce and leafy greens. The LGMA guidelines for fecal coliforms in the finished compost are <1,000 MPN/g (California Leafy Green Products Handler Marketing Agreement 2010). In this study, although we did not enumerate fecal coliforms, coliform bacteria were found in 63% of the samples tested with a range from 1 to 6.70 log CFU/g. Coliforms were present in 100% of the vermicompost, rabbit manure-based compost, and the alpaca manure samples with an average population of 3~5 log CFU/g. All fertilizer groups with coliforms present were also positive for *E. coli* except for the blood, bone and feather meal group and the greenwaste group. There was a significant positive correlation between fecal coliforms and *E. coli* in market ready composts as reported by Brinton et al. (2009). Elving et al. (2009) also found a positive correlation between total coliforms and *S.* Typhimurium. This illustrates that coliforms and fecal coliforms are useful as an indication of compost hygiene.

There are many ways that pathogens may get introduced into organic fertilizers after the thermophilic phase of composting or heating treatment is over. For example, insects and wild animals, water runoff, and equipment used in fertilizer handling can contaminate the organic fertilizers. Since organic fertilizers are high in nutrients, pathogens may have the opportunity to multiply when reintroduced. High numbers of pathogens in organic fertilizer may then be transferred to food products such as fresh produce. Therefore, in this study we evaluated the growth potential of *Salmonella* spp. and *E. coli* O157:H7 in selected types of fertilizers under various conditions.

The growth potential of *E. coli* O157:H7 and *Salmonella* was evaluated in organic fertilizers with high levels of background bacteria (≥5 log CFU/g) and low levels of background bacteria (<5 log CFU/g). For fertilizers with high levels of background bacteria, *E. coli* O157:H7

and *Salmonella* grew in the fish emulsion-based compost and plant-based compost, respectively, by ca. 1 log CFU/g within 1 day. In fertilizers with low background bacteria, both pathogens multiplied within 1 day in bone and blood meals and the mixed source fertilizer, and 5 days in feather meal with the growth potential in the range of ca. 3 to 5 log CFU/g. Numerous studies have analyzed the effect of indigenous microflora on growth potential. *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* all have a greater growth potential in sterilized compost as compared to nonsterilized compost (Kim and Jiang 2010; Yeager and Ward 1981; Hussong et al. 1985; Sidhu et al. 2001; Pietronave et al. 2004). Composts with a lower level of background microflora may support the growth of pathogens because there is less competition for water and nutrients. However, the suppressive effect of indigenous microflora also depends on the diversity of the microorganisms present (Pietronave et al. 2004; Millner et al. 1987). Although the plant-based compost in this study had high total bacteria counts, it still allowed growth of *Salmonella*. The presence of certain types of indigenous microflora may explain the growth of *Salmonella* in the plant-based compost as well as the growth of *E. coli* O157:H7 in the fish emulsion-based compost. Further study is needed on analyzing microbial composition in compost.

Moisture content has been investigated as one of the main factors that affects the growth potential of pathogens in finished compost (Kim et al. 2009; Yeager and Ward 1981; Hussong et al. 1985). Studies have demonstrated that the growth potential of bacteria in compost is positively correlated with moisture content (Yeager and Ward 1981; Hussong et al. 1985). Yeager and Ward (1981) determined that background bacteria in sludge grew rapidly with moisture contents above 25%, but no growth was seen in sludge with less than 15% moisture content. Pietronave et al. (2004) showed that *E. coli* grew in compost with a moisture

content of between 40 and 80% but not at 10%. Among the fertilizers tested in this study, *E. coli* was present in all groups with an average moisture content of at least 40% except for the plant-based compost and the liquid fish emulsion. These results indicate that organic fertilizers with high moisture contents are more favorable to the growth conditions of pathogens.

Another factor that affects the survival of pathogens in finished organic fertilizers is pH. *E. coli* was found in fertilizer samples with a pH that ranged from 5.39 to 9.57. Most of the samples were relatively neutral in pH, but the samples that were more alkaline (ca. 10) or more acidic (ca. 4) in pH had no *Enterobacteriaceae*, coliforms, or *E. coli* present. In our challenge study, both *E. coli* O157:H7 and *Salmonella* inoculated into dairy compost (pH 10) died off rapidly during the 7 days of incubation (Fig. 3). Payne et al. (2006) determined that environments with a neutral to slightly alkaline pH (7 to 9) allowed the growth of *Salmonella* as opposed to low pH (4) environments. Apparently, organic fertilizers with neutral pH are more favorable to growth and survival of pathogens than fertilizers with alkaline or acidic pH.

Conclusions

In conclusion, the microbiological quality of organic fertilizers can vary greatly. Each sample is unique based on their specific moisture content, water activity, pH, nutrient make-up, background microflora, and other factors. Overall, *E. coli* was present in nearly 30% of all organic fertilizer samples, whereas no sample was positive for *E. coli* O157:H7, *Salmonella* spp., or *Listeria monocytogenes*. Results from this survey of organic fertilizers provide some baseline data on the microbiological quality of these products. Based on our study, growth of *E. coli* O157:H7 and *Salmonella* spp. may be more likely in organic fertilizers with low indigenous microflora than with high indigenous microflora. However, all animal waste-based composts

tested in this survey failed to support the growth of these two pathogens. Therefore, growth potential cannot be decided by one factor but needs to take into consideration many components that make up the fertilizers.

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Figure Legends

Figure 2.1. Growth potential of *E. coli* O157:H7 in compost with high levels of background bacteria at 30°C (A) and 22°C (B). ■ #72-composted fish emulsion, ◊ #4-composted cow manure, ▲ #5-composted cow manure, **X** #BK-composted cow manure, **+** #25-mushroom compost, ● #28-mushroom compost. Dashed line indicates detection limit (1.6 log CFU/g).

Figure 2.2. Growth of *Salmonella* at 22°C in composts with high levels of background bacteria (≥5 log CFU/g). ◊ #25-mushroom compost, ■ #39-super-heated chicken litter, ▲#79-plant-based compost, **X** #20-horse manure-based compost, **+** #42-hen manure-based compost.

Figure 2.3. Growth potential of *E. coli* O157:H7 (A) and *Salmonella* spp. (B) in organic fertilizers with low levels of background bacteria (<5 log CFU/g) at 22°C. ◊ #56-feather meal, ■ #58-bone meal, ▲ #96-mixed source fertilizer, **X** #59-blood meal, **+** #2-high pH composted cow manure, ● #BK-neutral pH composted cow manure. Dashed line indicates detection level (1.6 log CFU/g).

Table 2.1. Analysis of Organic Fertilizers.

Source	n	Moisture Content (%) Avg. ± SD	Water Activity (a_w) Avg. ± SD	pH Avg. ± SD	TBC (Median/ Avg. log CFU/g)	*Enterobacteriaceae* (%[1]/median/ Avg. log CFU/g)	Coliforms (%/median/ Avg. log CFU/g)	*E. coli* (%/median/ Avg. log CFU/g)
Cow Manure Compost	15	39.38 ± 16.41	0.931 ± 0.18	8.28 ± 1.13	6.71/6.67	60/4.31/4.51	47/3.53/3.41	13/3.62/3.62
Horse Manure Compost	10	49.40 ± 15.27	0.987 ± 0.02	8.65 ± 0.76	7.83/7.98	94/5.15/4.76	94/3.1/3.84	67/2.53/3.04
Spent Mushroom Compost	11	62.95 ± 11.06	0.991 ± 0.01	7.86 ± 0.97	7.83/7.86	94/4.98/4.55	94/4.33/3.72	53/1.15/1.76
Super-heated Chicken Litter	5	14.18 ± 7.95	0.660 ± 0.06	7.83 ± 0.14	5.33/5.21	0/NA[2]/0	0/NA/0	0/NA/0
Poultry Manure	6	43.57 ± 22.64	0.890 ± 0.20	8.00 ± 0.71	6.66/6.66	83/4.16/4.64	67/3.90/4.16	50/3.04/2.97
Vermi-compost	5	74.97 ± 5.17	0.998 ± 0.00	7.04 ± 0.76	7.35/7.26	100/5.4/5.28	100/3.06/3.35	80/1.34/1.46
Liquid Fish Emulsion	4	56.65 ± 9.21	0.926 ± 0.02	3.72 ± 0.77	4.02/4.04	0/NA/0	0/NA/0	0/NA/0
Blood, Bone, Feather Meal	4	8.11 ± 4.18	0.465 ± 0.18	6.76 ± 0.90	3.94/3.89	25/4.43/4.43	25/3.57/3.57	0/NA/0
Rabbit Manure Compost	3	64.46 ± 22.69	0.966 ± 0.05	8.58 ± 0.18	8.03/7.95	100/6.04/6.08	100/4.70/4.65	67/4.27/4.27
Greenwaste Compost	9	31.00 ± 25.61	0.982 ± 0.04	7.34 ± 0.71	6.11/6.29	100/2.43/2.47	67/1.51/1.77	0/NA/0
Composted Fish Emulsion	2	26.92 ± 10.75	0.956 ± 0.02	8.78 ± 0.81	6.27/6.27	0/NA/0	0/NA/0	0/NA/0
Alpaca Manure Compost	2	54.43 ± 11.48	0.999 ± 0.00	7.19 ± 0.78	7.51/7.51	100/6.17/6.17	100/5.45/5.45	100/2.33/2.33
Plant Compost	1	44.42 ± 0.00	0.987 ± 0.00	8.52 ± 0.00	NA/9.14	0/NA/0	0/NA/0	0/NA/0
Mixed Animal Compost	6	68.34 ± 8.50	0.998 ± 0.00	8.52 ± 0.43	7.43/7.33	83/5.18/5.09	83/2.36/3.12	50/4.08/3.44
Mixed Source Fertilizers	20	40.66 ± 22.73	0.875 ± 0.23	7.73 ± 1.09	6.53/6.79	75/3.40/3.67	60/2.67/2.73	20/3.15/3.36

[1]: Percent positive among samples tested in each category

[2]: Not enough data to calculate a media

Table 2.2. Characteristics of Organic Fertilizers Used for Growth Potential Studies.

Compost	pH ± SD	Moisture content (% ± SD)	Total bacteria count (log CFU/g ± SD)	Growth Potential[1]	
				Salmonella	*E. coli* O157:H7
#20-Horse Manure	8.71 ± 0.01	56.06 ± 0.31	7.90 ± 0.09	-[2]	N/A
#25-Mushroom	9.02 ± 0.01	62.31 ± 0.54	8.38 ± 0.03	-	-
#39-Super-heated Chicken Litter	7.86 ± 0.40	51.00 ± 0.00	5.00 ± 0.35	-	N/A
#42-Hen Manure	8.04 ± 0.07	51.48 ± 0.08	6.50 ± 0.07	-	N/A
#79-Plant-based	8.52 ± 0.03	44.42 ± 0.70	9.14 ± 0.11	+	N/A
#4-Cow Manure	8.04 ± 0.11	36.54 ± 0.74	7.76 ± 0.03	N/A	-
#5-Cow Manure	8.19 ± 0.18	41.40 ± 3.10	7.11 ± 0.41	N/A	-
#28-Mushroom	8.08 ± 0.04	63.88 ±0.09	7.97 ± 0.27	N/A	-
#72-Fish Emulsion	8.20 ± 0.01	38.37 ± 1.70	7.15 ± 0.03	N/A	+
#BK-Cow Manure	6.70 ± 0.10	39.45 ± 0.30	6.53 ± 0.05	-	-
#2-Cow Manure	10.15 ± 0.06	43.64 ± 0.04	4.53 ± 0.29	-	-
#56-Feather Meal	6.22 ± 0.02	46.95 ± 3.82	3.38 ± 1.02	+	+
#58-Bone Meal	7.14 ± 0.11	29.82 ± 2.01	2.36 ± 0.32	+	+
#59-Blood Meal	7.83 ± 0.01	50.53 ± 1.10	4.50 ± 0.45	+	+
#96-Mixed Source	6.43 ± 0.03	35.21 ± 1.10	4.84 ± 0.20	+	+

[1]: Growth potential was defined as the difference of plate counts from the inoculum level
[2]: +, growth, -, no growth, N/A, not tested

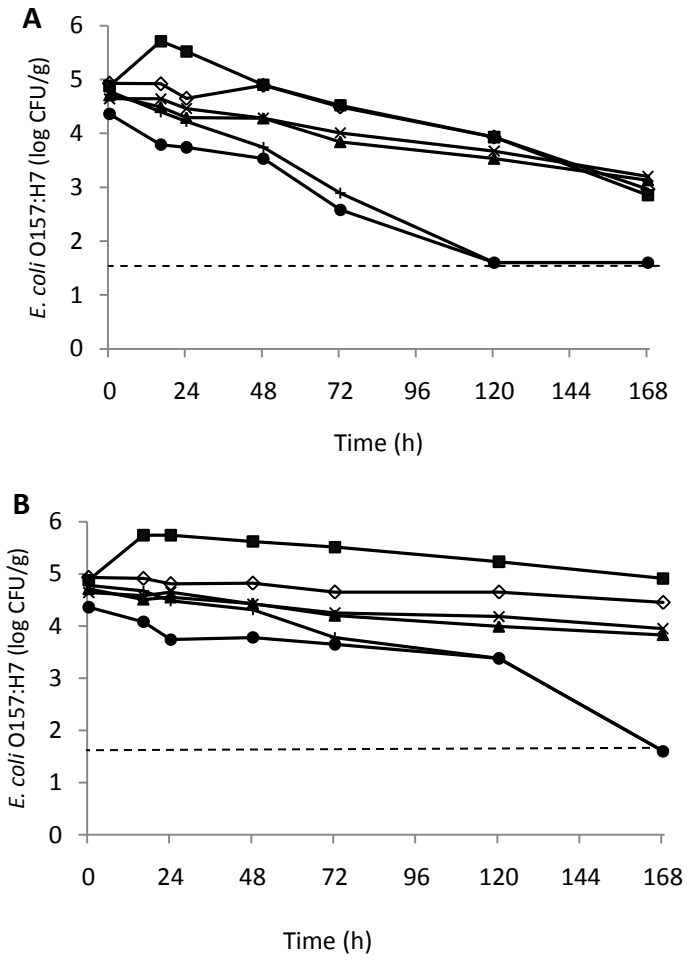

A
E. coli O157:H7 (log CFU/g)
0
1
2
3
4
5
6
0
24
48
72
96
120
144
168
Time (h)
B
E. coli O157:H7 (log CFU/g)
0
1
2
3
4
5
6
0
24
48
72
96
120
144
168
Time (h)

Figure 2.1

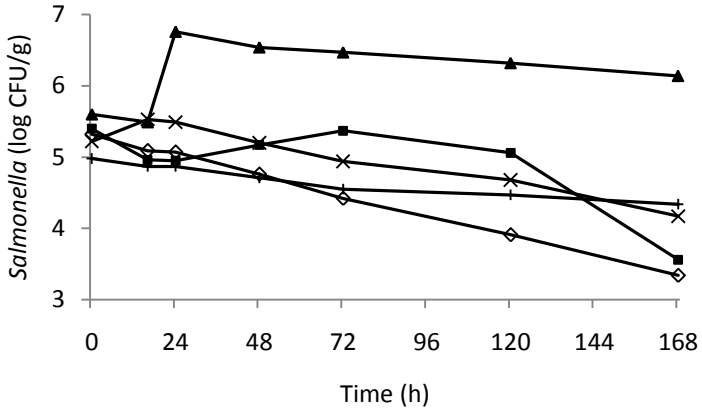
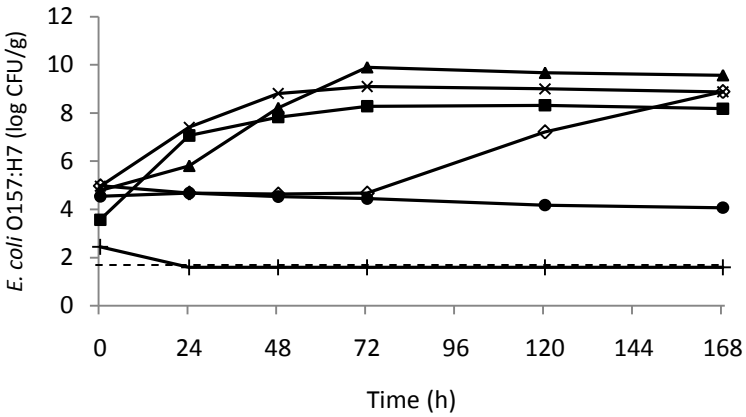


Figure 2.2


12
10
8
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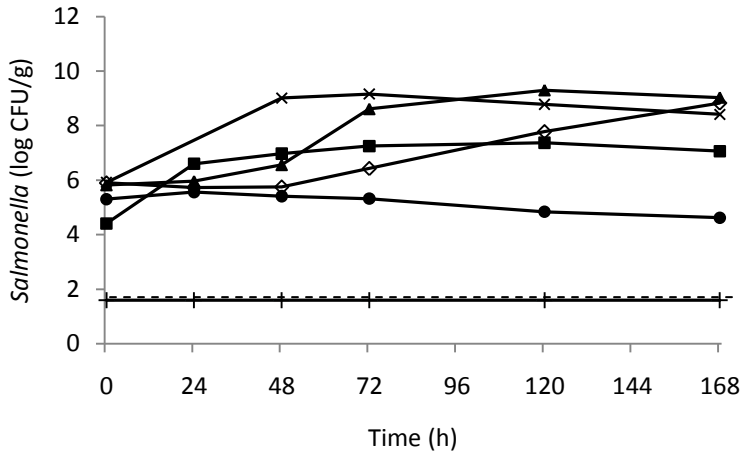
E. coli O157:H7 (log CFU/g)
0
24
48
72
96
120
144
168
Time (h)


12
10
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Salmonella (log CFU/g)
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168
Time (h)

Figure 2.3

CHAPTER THREE

PRESENCE OF ANTIBIOTIC RESISTANCE AND INTEGRONS IN *ESCHERICHIA COLI* ISOLATED FROM ORGANIC FERTILIZERS

Abstract

With the rise of organic food production comes an increase in the use of organic fertilizers on agricultural crops. These fertilizers may contain bacteria carrying antimicrobial resistance genes on mobile genetic elements such as integrons. Resistance genes can be transferred to pathogenic bacteria in the fertilizer and may cause contamination of crops where the fertilizer is spread. One hundred and three organic fertilizers were analyzed for the presence of *Escherichia coli*. Thirty samples (29%) were found to be positive for *E. coli* with the biggest proportion of those coming from the horse manure-based composts. PCR was used to determine the phylogenetic groups of the *E. coli* isolates, with the majority of the isolates from group B1 (48%) and group A (32%). Resistance to 16 antibiotics was examined for the 72 *E. coli* strains isolated from the organic fertilizers. Eleven isolates had resistance to at least one antibiotic. Among them 5 isolates were resistant to $\geq$2 antibiotics, whereas 2 isolates were resistant to $\geq$10 antibiotics. Five of the eleven isolates showing antibiotic resistance had an integron present. The results of this study demonstrate that antibiotic resistance genes are present in nonpathogenic *E. coli* from organic fertilizers.

Introduction

There is concern worldwide about the increased prevalence of antimicrobial resistance in microbial communities. The use of antibiotics in food animal production to prevent disease

and promote growth is considered the main risk factor for increased resistance in pathogenic bacteria (McEwen and Fedorka-Cray 2002; van den Bogaard and Stobberingh 2000). The use of antimicrobials in animal production systems selects for resistance in enteric microorganisms (Jiang et al. 2006; Smith et al. 2007), and these resistant bacteria can be introduced into the farm environment through animal feces (Yang et al. 2006; Smith et al. 2007; Carson et al. 2008) and spread to neighboring water sources and agricultural crops (Campagnolo et al. 2002).

Antimicrobial resistance usually comes from a mutation of certain genes in a susceptible bacterial strain that allows the mutant to make a specific protein that either inactivates an antimicrobial agent or evades the agent's damaging effects. Mutations can develop on the bacterial chromosome, but more commonly, resistance genes are imported from outside via genetic vectors such as plasmids, transposons, and integrons (O'Brien 2002). Integrons are genetic elements containing components of a site-specific recombination system that recognizes and captures mobile gene cassettes, often coding for antibiotic resistance, and they generally include an integrase gene (*int*) and an adjacent recombination site (*attI*) (Fluit and Schmitz 2004). These mobile genetic elements can be transferred horizontally to other strains or species in the gut of livestock as well as in manure and composted animal wastes (Blake et al. 2003; Götz and Smalla 1997; Guan et al. 2007). Most importantly, antibiotic resistance genes can be transferred from commensal bacteria to pathogenic strains (Blake et al. 2003).

Escherichia coli is an organism that naturally occurs in the gut of humans and animals. Although most strains are nonpathogenic commensal organisms, there are pathogenic strains that can cause illness in humans. The abundance of *E. coli* in the gut implicates them as likely vehicles for the spread of resistance genes and genetic vectors (O'Brien 2002). *E. coli* fall into four main phylogenetic groups (A, B1, B2, and D). Virulent extra-intestinal strains mainly belong

to groups B2 and D, while most commensal strains are restricted to group A (Clermont et al. 2000). Pathogenic *E. coli* associated with foodborne outbreaks usually belong to groups B1 and B2 (Bando et al. 2007; Tramuta et al. 2008).

With the increase of organic food production, animal waste-based fertilizers are being used more and more. Since animal wastes are the major sources of many foodborne pathogens (Pell 1997), they need to undergo treatment to eliminate pathogens, such as composting. However, there are variations in the effectiveness of sanitization methods used as well as the potential for regrowth and recolonization of bacterial populations after the sanitization process (Hess et al. 2004; Zaleski et al. 2005; Kim et al.2009). Heringa et al. (2010) reported antimicrobial resistant *E. coli* in compost. Furthermore, there is the potential for the contamination of the surface of produce as well as internalization of bacteria by crops grown in soil where inadequately composted compost is used as a fertilizer (Islam et al. 2004; Islam et al. 2005; Solomon et al. 2002). The objectives of this study were to isolate *E. coli* from various organic fertilizers and evaluate their antimicrobial resistance.

Materials and Methods

Organic Fertilizer Sampling: Organic fertilizer samples (n=103) were collected aseptically from multiple locations across the United States between September 2008 and June 2010. These samples included cow manure-based compost (n=15), horse manure-based compost (n=10), spent-mushroom compost (n=11), poultry manure-based compost (n=6), super-heated chicken litter (n=5), vermi-compost (n=5), liquid fish emulsion (n=4), blood, bone, and feather meals (n=4), rabbit manure (n=3), greenwaste compost (n=9), fish emulsion-based compost (n=2), alpaca manure (n=2), plant-based compost (n=1), mixed animal manure-based compost (n=7),

and mixed source fertilizers (n=20) from Arizona, California, Georgia, Kentucky, Maryland, New York, North Carolina, South Carolina, and Tennessee. The samples were transported at room temperature and stored in a refrigerator (5°C) upon arrival.

Isolation and Characterization of E. coli *Isolates*: Twenty five grams of each organic fertilizer were added to 225 ml of universal preenrichment broth (UPB; Neogen, Lansing, MI) in a plastic sampling bag and shaken vigorously for 1 minute. *E. coli* were enumerated by plating 1 ml of the UPB suspension onto *E. coli*/coliform Petrifilm® (3M, St. Paul, MN), which was incubated overnight at 37°C. After incubation, three to five typical *E. coli* colonies (blue with gas bubble formation) per sample were picked from all positive plates and streaked onto levine eosin methylene blue agar (Difco, Detroit, MI). Typical *E. coli* colonies (blue-black colonies with green metallic sheen) were purified by streaking onto tryptic soy agar (TSA; Becton & Dickinson and Co., Sparks, MD) several times.

Presumptive positive *E. coli* isolates were verified by real-time polymerase chain reaction (RT-PCR) of the glutamate decarboxylase gene (*gad*) (Table 1). Gad detection has been commonly used as a highly specific method of *E. coli* identification and was performed as previously described by Chen et al. (2006). The SYBR green-based method was performed using the Bio-Rad iCycler™ system (BioRad, Inc., Hercules, CA). The PCR reaction mixture (20μL) contained 10μL of SYBR Green Supermix (BioRad), 1.5μM each of forward and reverse primer, and 4μL of DNA template.

Quality control strains used for PCRs are listed in Table 1. *E. coli* O157:H7 F07M-020-1 and *Salmonella* Typhimurium CVM 786 were kindly provided by California Department of health and Dr. Shaohua Zhao at the FDA Center for Veterinary Medicine, respectively. All confirmed *E.*

coli isolates used for the following study were stored at -80°C in tryptic soy broth (TSB, Becton & Dickinson) with 20% glycerol (Fisher Scientific, Fair Lawn, NJ).

Antimicrobial Susceptibility Testing: All *E. coli* isolates were tested for susceptibility to a series of antimicrobial agents on Mueller Hinton agar (Difco) using the agar dilution method according to the procedures described by the Clinical and Laboratory Standards Institute (CLSI, 2006). *E. coli* isolates were grown overnight in Mueller Hinton broth (Difco) at 37°C. The OD_{600} was adjusted to between 0.4 and 0.5, and each culture was diluted 1:10 in 90 µL of saline in a 96-well microplate (Costar, Corning NY). A sterile replica plater (Sigma, St. Louis, MO) was used to inoculate Mueller-Hinton plates supplemented with five different concentrations of each antibiotic. Each experiment was performed in triplicate. The antimicrobial agents (n=15) tested were chosen based on the National Antimicrobial Resistance Monitoring System's (NARMS) 96-well plate format and included amikacin (16-256 µg/ml), ampicillin (8-128 µg/ml), ceftriaxone (16-256 µg/ml), chloramphenicol (8-128 µg/ml), ciprofloxacin (1-16 µg/ml), trimethoprim/sulfamethoxazole (1/19-16/304 µg/ml), cefoxitin (8-128 µg/ml), gentamicin (4-64 µg/ml), kanamycin (16-128 µg/ml), nalidixic acid (8-128 µg/ml), sulfisoxazole (128-2048 µg/ml), streptomycin (16-256 µg/ml), tetracycline (4-64 µg/ml), ceftiofur (2-32 µg/ml), and cefotaxime (1-16 µg/ml) (Sigma). The minimum inhibitory concentration (MIC) results were interpreted by the use of the CLSI breakpoints for *Enterobacteriaceae*. The resistance breakpoints for the tested antibiotics were 4 µg/ml for ciprofloxacin and cefotaxime; 8 µg/ml for ceftiofur; 16 µg/ml for gentamicin and tetracycline; 32 µg/ml for ampicillin, chloramphenicol, cefoxitin, and nalidixic acid; 64 µg/ml for amikacin, ceftriaxone, kanamycin, and streptomycin; 4/76 µg/ml for trimethylprim/sulfamethoxazole; and 512 µg/ml for sulfisoxazole. *E. coli* 25922 (ATCC, Manassas, VA) was used as the control strain for antimicrobial susceptibility testing.

Disc diffusion susceptibility testing was used for amoxicillin/clavulanic acid according to the procedures described by the CLSI. *E. coli* isolates were grown overnight in Mueller Hinton broth (Difco) at 37°C. The OD_{600} was adjusted to between 0.4 and 0.5. A sterile swab was used to streak Mueller-Hinton agar plates with each *E. coli* culture for a lawn of growth. After the plates were dry, sterile amoxicillin/clavulanic acid (20/10μg/ml) discs (Becton & Dickinson) were placed onto the plates using a pair of sterile forceps. The plates were incubated at 37°C overnight. The diameter of the zone of inhibition was measured and compared to the CLSI table of interpretive standards. A zone of inhibition with a diameter of ≤13mm was considered resistant. Each test was performed in triplicate.

Detection of Virulence Genes, Integrons, and Phylogenetic Groups by PCR: Presumptive *E. coli* isolates were aseptically transferred to an Ependorf tube containing 1 ml of sterile distilled water followed by boiling for 5 minutes for DNA extraction. The primers used for this study are listed in Table 1. All primers were synthesized by Invitrogen Co. (Carlsbad, CA). A multiplex real-time PCR for shiga toxin genes (*stx1* and *stx2*) was carried out as described previously using 0.4 μM of each primer (Lang et al. 1994). The PCR program contained a 5 min initial denaturation at 94°C, followed by 30 cycles each of 94°C for 1 min, 58.5°C for 1 min, and 72°C for 1.5 min. The real-time detection of class 1 integrons (*int1*) was performed using parameters previously described by Jiang et al. (2006).

A triplex PCR was carried out for phylogenetic analysis of the *E. coli* isolates as described by Clermont et al. (2000). The PCR program consisted of denaturation for 5 min. at 94°C; 30 cycles of 30 s. at 94°C, 30 s. at 55°C, and 30 s. at 72°C; and a final extension step of 7 min. at 72°C. The specificity of amplification of all real-time PCR products was confirmed by melting-curve analysis, and the amplicon size was checked by gel electrophoresis in a 1.5% agarose gel.

Results

A total of 103 organic fertilizer samples were collected from multiple states across the United States. Eighty-six putative *E. coli* isolates were collected and 72 were confirmed by Gad PCR. The isolates were screened for shiga toxins 1 and 2 by real-time PCR and none were positive for either virulence gene. PCR was used to determine the phylogenetic groups of the *E. coli* isolates with a 94% positive identification. The majority of the isolates were from group B1 (n=35) (48%) followed by group A (n=23) (32%). Isolates from groups B2 (n=3) and D (n=7) were 4% and 9%, respectively. Isolates from cow manure-based compost, spent mushroom compost, poultry manure-based compost, vermi-compost, and alpaca manure were mostly from group B1 with 60, 83, 40, 85, and 67%, respectively. On the other hand, isolates from horse manure-based compost, rabbit manure, mixed animal compost, and mixed source fertilizers were mainly from group A with 88, 40, 50, and 88%, respectively.

Resistance to 16 antibiotics was examined for all 72 *E. coli* isolates. Among eleven isolates resistant to at least 1 antibiotic, 5 isolates were resistant to $\geq$2 antibiotics and 2 isolates showed resistance to $\geq$10 antibiotics. The largest percentage of resistant isolates was from poultry manure-based compost (27%) followed by isolates from stacked rabbit manure (18%), whereas no isolates from the mixed animal compost or the mixed source fertilizer had resistance. The two isolates that showed resistance to more than 10 antibiotics were from the alpaca manure and the poultry manure-based compost samples. All *E. coli* isolates with antibiotic resistance had a different resistance phenotype except for isolates 87-3 (vermi-compost) and 95-1 (rabbit manure) which shared the same resistance to cefoxitin. The majority of isolates were resistant to cefoxitin (45%), ampicillin, tetracycline and streptomycin (36%). No isolates were resistant to amikacin, ciprofloxacin, or sulfisoxazole. Of the eleven isolates with

antibiotic resistance, 5 contained class 1 integrons (Fig. 1). Three of the integron carrying isolates were resistant to ≥2 antibiotics, but neither of the 2 isolates resistant to ≥10 antibiotics had an integron present. Four of the 11 antibiotic resistant isolates and 2 of the 5 isolates carrying multiple antibiotic resistance belonged to phylogenetic group B1. Two out of 5 isolates positive for integrons were also from group B1.

Discussion

This study determined the prevalence and resistance patterns of *E. coli* from various types of organic fertilizers from across the United States. Fecal coliforms such as *E. coli* are useful indicators of compost hygiene (Brinton et al. 2009; Elving et al. 2009), and antibiotic profiles for *E. coli* may give some insight into the development of resistance in other foodborne pathogens. Also, the abundance of *E. coli* implicates them as likely vehicles for the spread of resistance genes (O'Brien 2002).

Beta-lactam antibiotics prevent the bacterial cell wall from forming by interfering with penicillin-binding proteins (PBPs) during peptidoglycan synthesis. Variations in activity of different β-lactams come from differences in affinity of PBPs for the antibiotic. Extended-spectrum aminobenzylpenicillins, such as ampicillin and amoxicillin, have a high activity against gram-negative bacteria, but acquired resistance of β-lactamase in bacteria has reduced the effectiveness of these drugs. Clavulanic acid is a β-lactamase inhibitor that is commonly administered along with amoxicillin (Prescott et al. 2000). Ampicillin is commonly used in treatment of bacterial infections in horses (Wilson 2001), and ampicillin resistant isolates have been found in horse manure (Hoyle et al. 2006; White et al. 2002). Penicillin is used for growth and feed efficiency as well as treatment of infection in the poultry industry and may confer β-

lactam resistance (McEwen and Fedorka-Cray 2002).We had four isolates with resistance to ampicillin (from horse manure-based compost, vermin-compost, alpaca manure, and poultry manure-based compost) and three isolates resistant to amoxicillin/clavulanic acid (from alpaca manure, and two poultry manure-based compost samples).

Cephalosporins are one class of β-lactam antibiotics, and are generally classified as first-, second-, third- or fourth-generation, according to their *in vitro* spectrum of activity (narrow, expanded, broad, and extended, respectively). First-generation cephalosporins are active against gram-positive bacteria and have very limited activity against gram-negative bacteria. Second-generation cephalosporins are active against the same gram-positive bacteria as first-generation cephalosporins, but they have more activity against gram-negative bacteria although the range is limited. Third-generation cephalosporins, on the other hand, were designed to have enhanced activity against gram-negative bacteria, while retaining their activity against gram-positive bacteria (Hornish and Kotarski 2002). Many first-generation and one second-generation cephalosporins are used in dairy cattle, while cefoperazone and ceftiofur are the only third-generation cephalosporins approved for use in animals and are used in many different species (Hornish and Kotarski 2002). In our study, five isolates had resistance to cefoxitin, a second-generation cephalosporin, while ceftriaxone, cefotaxime and ceftiofur, third-generation cephalosporins, had less resistant isolates with 2, 2, and 0, respectively.

Aminoglycoside antibiotics bind to the 30S subunit of the ribosome and cause misreading of the genetic code on the messenger RNA (mRNA). These antibiotics are useful against aerobic gram-negative bacteria. The order of clinical introduction of some common aminoglycosides is as follows: streptomycin (1944), kanamycin (1957), gentamicin (1963), and amikacin (1972) (Prescott et al. 2000). A number of different aminoglycosdies are used in food

animal production as growth promoters and therapeutic drugs (McEwen and Fedorka-Cray 2002). Resistance to older aminoglycosides is widespread and becoming a problem with newer drugs (Prescott et al. 2000). Our results showed higher numbers of resistant isolates to older aminoglycosides than to newer antibiotics, with streptomycin, kanamycin, gentamicin, and amikacin having 4, 2, 1 and 0 resistant isolates, respectively.

Tetracyclines are classic broad-spectrum antibiotics because of their activity against gram-positive and gram-negative aerobic and anaerobic bacteria. Tetracyclines inhibit protein synthesis by binding to the 30S ribosomal subunit and interfering with the binding of aminoacyl-transfer RNA to the acceptor site on mRNA. They tend to be used as first-line antibiotics in food animals, but widespread resistance particularly by gram-negative bacteria limits their use (Prescott et al. 2000). Tetracycline is commonly used to treat infections in the poultry industry and chlortetracycline is used as a growth promoter (McEwen and Fedorka-Cray 2002). We found four isolates with resistance to tetracycline from vermi-compost, alpaca manure, rabbit manure, and poultry manure-based compost. A high prevalence of tetracycline resistant *E. coli* isolates were found in broiler chickens and turkey manure (Smith et al. 2007; van den Bogaard and Stobberingh 2000).

Class 1 integrons are considered vectors for the dissemination of antimicrobial resistance genes. Integron-containing isolates are more likely to have antimicrobial resistance than those lacking an integron (Fluit and Schmitz 2004). Five *E. coli* isolates with antibiotic resistance were found to carry a class 1 integron. Three of the five isolates also showed resistance to $\geq$2 antibiotics. The *aadA* gene cassette is commonly carried by integrons and encodes resistance to streptomycin-spectinomycin (Fluit and Schmitz 2004). Two of the isolates carrying integrons had resistance to streptomycin. Sulfonamide resistance is also commonly

carried on integrons as the *sul1* gene (Cocchi et al. 2007), but none of the isolates with an integron showed resistance to sulfamethoxazole or sulfisoxazole. Neither of the two isolates that were resistant to ≥10 antibiotics had integrons present. They may instead be carrying a different type of genetic element conferring antibiotic resistance.

E. coli are divided into 4 phenotypic groups: A, B1, B2, and D. The majority of the *E. coli* isolated in this study was from groups B1 (48%) and A (32%) made up mostly of commensal *E. coli* (Clermont et al. 2000). Similar results were reported by Heringa et al. (2010) and Walk et al. (2007). The antibiotic resistant isolates were mostly from group B1 (36%), whereas 2 out of 5 integron carrying isolates were also from group B1. This was the opposite of the results found by Heringa et al. (2010) where more isolates with antibiotic resistance were from groups B2 and D, and 6 of the 9 isolates carrying integrons were from groups B2 and D. However, Houser et al. (2008) found higher levels of *E. coli* isolated from dairy cattle with antibiotic resistance in groups A and B1, and Cocchi et al. (2007) found that integrons were more prevalent in *E. coli* isolates from group A. These results suggest that the presence of antibiotic resistance genes and integrons in *E. coli* from the four phylogenetic groups varies. However, due to the small sample size in this study, a comprehensive and large scale study is needed to determine the association of antibiotic resistance with phylogenetic group.

Conclusions

This study reports the presence of antibiotic resistance and integrons in nonpathogenic *E. coli* isolated from organic fertilizers from multiple sources. The presence of antibiotic resistance as well as integrons in many different types of organic fertilizers demonstrates the pervasive nature of resistance genes in the environment. Since many of the isolates that had

antimicrobial resistance were from animal manure-based fertilizers, resistant strains may be the direct result of antibiotic use in animal production systems. Since resistance genes can be transferred from commensal to pathogenic bacteria (Blake et al. 2003) and manure may enhance the mobilization of these genes (Götz and Smalla 1997), there is the possibility that foodborne pathogens in the compost or the environment can acquire antimicrobial resistance. If these organisms contaminate fresh produce after application of fertilizers to the soil, they may cause illness in humans with lower susceptibility to antibiotics. The production of good quality organic fertilizers is an important factor in limiting the spread of antibiotic resistance to pathogenic bacteria.

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Figure Legends

Figure 3.1. Real-Time PCR for Integron *intI* Gene. Lane 1: DNA Marker, Lane 2: *Salmonella* Typhimurium CVM 786 (Positive Control), Lane 3: Negative Control, Lane 4: 61-4, Lane 5: 74-4, Lane 6: 78-2-2, Lane 7: 87-3, Lane 8: 95-1, Lane 9: 47-2-1, Lane 10: 108-2, Lane 11: 100-3, Lane 12: 103-3, Lane 13: 107-2, Lane 14: 108-1-2.

Table 3.1. Primers and Control Strains for Polymerase Chain Reactions.

Gene[1]	Primer Name	Primer Sequence (5'-3')	Control Strain	Reference
intI	INTI-F	CCTCCCGCACGATGATC	*Salmonella*	Bass et al. (1999)
	INTI-R	TCCACGCATCGTCAGGC	Typhimurium CVM 786	
CS	Integron5-CS	GGCATCCAAGCAGCAAG	*Salmonella*	
	Integron3-CS	AAGCAGACTTGACCTGA	Typhimurium CVM 786	
*stx*1	VT1A	GACTGCAAAGACGTATGTAGATTCG	*Escherichia coli*	Lang et al. (1994)
	VT1B	ATCTATCCCTCTGACATCAACTGC	O157:H7 F07M-020-1	
*stx*2	VT2A	ATTAACCACACCCCACCG	*E. coli* O157:H7 F07M-020-1	
	VT2B	GTCATGGAAACCGTTGTCAC		
gad	GADRT-1	GCGTTGCTGAAATATGGTTGCCGA	*E. coli* O157:H7 F07M-020-1	Chen et al. (2006)
	GADRT-2	CGTCACAGGCTTCAATCATGCGTT		
*chu*A	ChuA1	GACGAACCAACGGTCAGGAT	*E. coli* ATCC 25922	Clermont et al. (2000)
	ChuA2	TGCCGCCAGTACCAAAGACA		
*yja*A	YjaA1	TGAAGTGTCAGGAGACGCTG	*E. coli* ATCC 25922	
	YjaA2	ATGGAGAATGCGTTCCTCAAC		
TSPE4.C2	TspE4C2-1	GAGTAATGTCGGGGCATTCA	*E. coli* ATCC 25922	
	TspE4C2-2	CGCGCCAACAAAGTATTACG		

[1]: *intI*, integrase; CS, integron gene cassette; *stx*1 and 2, shiga-toxins 1 and 2; *gad*, glutamate decarboxylase; *chuA*, *yjaA*, and TSPE4.C2, phylogentic group markers

Table 3.2. Characterization of *Escherichia coli* Isolates from Organic Fertilizers.

Source (n)	*E. coli* (%/range/ Avg. log CFU/g)	Number of *E. coli* Isolates picked/confirmed[1]	Phylogenetic Groups (%)				Number of *E. coli* Isolates with Antibiotic Resistance[2]
			A	B1	B2	D	
Cow Manure Compost (15)	13/<1-4.02/3.62	10/10	10	60	10	20	1
Horse Manure Compost (10)	67/<1-6.34/3.04	10/8	88	13	0	0	1
Spent Mushroom Compost (11)	53/<1-3.74/1.76	16/12	0	83	0	8	1
Poultry Manure (11)	50/<1-4.45/2.97	10/10	30	40	0	0	3
Vermi-compost (5)	80/<1-2.00/1.46	17/13	8	85	0	0	2
Rabbit Manure Compost (3)	67/<1-5.01/4.27	6/5	40	20	0	40	2
Alpaca Manure Compost (2)	100/2.25-2.40/2.33	3/3	0	67	0	33	1
Mixed Animal Compost (6)	50/<1-4.53/3.44	8/4	50	0	25	25	0
Mixed Source Fertilizers (20)	20/<1-3.30/3.36	8/8	88	0	13	0	0

[1]: Confirmed by Gad PCR

[2]: Resistant to at least 1 of 16 antibiotics tested

Table 3.3. Antibiotic Resistance and Integrons in *Escherichia coli* from Organic Fertilizers.

E. coli Isolate	Source	Phylogenetic Group	Resistance Profile	Integron[3]
47-2-1	Horse manure-based compost	A	AMP[2]	-
61-4	Vermi-compost	B1	AMP, GEN, TET	+
74-4	Spent mushroom compost	B1	CHL	+
78-2-2	Alpaca manure	B1	AMP, AUG, AXO, CHL, SXT, FOX, KAN, NAL, STR, TET, TIO, CTX	-
87-3	Vermi-compost	N/A[1]	FOX	-
95-1	Rabbit manure	D	FOX	-
100-3	Cow manure-based compost	A	STR	+
103-3	Rabbit manure	D	NAL, STR, TET	+
107-2	Poultry manure-based compost	B1	TET	-
108-1-2	Poultry manure-based compost	N/A	AMP, AUG, AXO, SXT, FOX, KAN, NAL, STR, TIO, CTX	-
108-2	Poultry manure-based compost	N/A	AUG, FOX	+

[1]: N/A, Gad profile could not be assigned

[2]: AMP, ampicillin; AUG, amoxicillin/clavulanic acid; AXO, ceftriaxone; CHL, chloramphenicol; SXT, trimethoprim/sulfamethoxazole; FOX, cefoxitin; GEN, gentamicin; KAN, kanamycin; NAL, nalidixic acid; STR, streptomycin; TET, tetracycline; TIO, ceftiofur; CTX, cefotaxime

[3]: +, Presence of *int1* gene

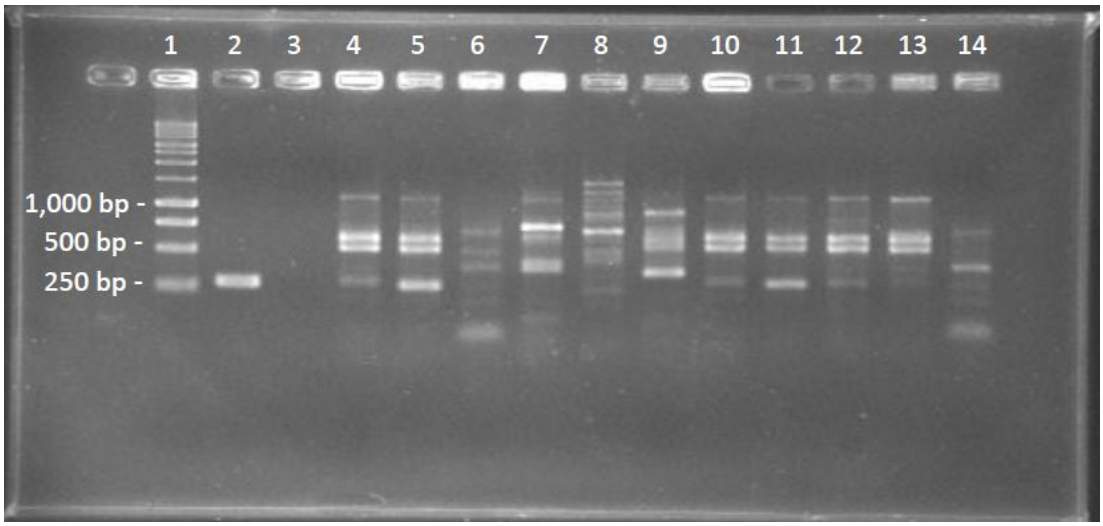

Figure 3.1

CHAPTER FOUR

CONCLUSIONS

In this study, a survey of organic fertilizers was conducted in order to evaluate the fertilizer quality. The microbiological quality of organic fertilizers varies greatly, and each sample is unique based on its specific biotic and abiotic characteristics. Overall, *E. coli* was present in nearly 30% of the samples tested, while 14% of samples exceeded the EPA limits for fecal coliforms. However, no sample was positive for *E. coli* O157:H7, *Salmonella*, or *L. monocytogenes*. These results provide some baseline data on the quality of these products.

Based on our study, growth of *E. coli* O157:H7 and *Salmonella* spp. is more likely to occur in organic fertilizers containing low levels of indigenous microorganisms as compared to fertilizers with high levels of indigenous mircoflora. However, all animal waste-based composts tested in this survey failed to support the growth of these two pathogens. All of the fertilizers tested for growth potential met the EPA guidelines for compost.

Antimicrobial resistance is a growing concern worldwide, and the results of this study demonstrate that antibiotic genes are present in nonpathogenic *E. coli* isolated from organic fertilizers. Resistant isolates were found in fertilizers that exceeded EPA guidelines as well as fertilizers which were within the EPA limits. Many of the isolates that contained antimicrobial resistance were from animal manure-based fertilizers indicating that the use of antibiotics for treatment of animals may introduce resistant enteric microorganisms into the environment.

Good quality organic fertilizers are important in limiting the spread of pathogens to agricultural crops, and the EPA has set up guidelines on bacterial limits in compost. However, the results from this study demonstrate that even if the EPA guidelines are met pathogens may

recolonize the fertilizer and/or antimicrobial resistant microorganisms may be present. Therefore, further research on reducing microbial contamination during production and storage of organic fertilizers is critical to ensure the safety of produce production.