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Evaluation of Animal Manure-based Compost as an Environmental Source of *Clostridium difficile*

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EVALUATION OF ANIMAL MANURE-BASED COMPOST AS AN ENVIRONMENTAL SOURCE OF CLOSTRIDIUM DIFFICILE

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
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by
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ABSTRACT

The number of *Clostridium difficile* infection (CDI) cases associated with healthcare and community is increasing. Increased isolation of this pathogen from environmental samples of soil, water, livestock animals and their feces, and produce suggests its dissemination in the environment. Identification, molecular characterization, and revealing the relationship of those isolates with human pathogenicity would be very helpful in understanding the epidemiology of CDI. Livestock manure is a rich source of nutrients for the growth of many crops, and composted manure is widely used as a fertilizer in organic farming. Microbial activities increase the temperature of composting materials, providing sufficient heat to kill vegetative cells of most pathogenic bacteria. *C. difficile*, is an endospore-forming pathogen present in livestock manure and its fate during composting is not well-understood. However, the survival of *C. difficile* in compost poses a microbial safety threat to the post-harvest environments of crops. The objectives of this study were to 1) to improve the culture media for isolation of *C. difficile* endospores and vegetative cells from compost, 2) to isolate and characterize *C. difficile* and *Clostridium perfringens* from commercially available compost and manure, 3) to study the thermal resistance of *C. difficile* endospores on wet and dry heat exposure in composted dairy cattle manure, and 4) to study the survival of *C. difficile* in composted dairy cattle manure under controlled storage conditions.

The detection methods for the isolation of *C. difficile* from the animal manure-based composts were optimized. Both autoclaved and unautoclaved dairy composts were inoculated with a 12-h old suspension of a non-toxigenic *C. difficile* strain (ATCC
43593), and then plated on selected agar for vegetative cells and endospores separately. Six types of enrichment broths supplemented with taurocholate and L-cysteine were assessed for detecting a low level of artificially inoculated *C. difficile* (ca. 5 spores/g) from dairy composts. The efficacy of selected enrichment broths was further evaluated by isolating *C. difficile* from 29 commercial compost samples. Our results revealed that using heat-shock was more effective than using ethanol-shock for inducing endospore germination, yielding the the highest endospore count at 60°C for 25 min treatment. *C. difficile* agar base, supplemented with 0.1% L-cysteine, 7% defibrinated horse blood, and cycloserine-cefoxitin (CDA-CYS-H-CC agar) was the best medium (p < 0.05) for recovering vegetative cells from compost. *C. difficile* endospore populations from both types of composts enumerated on both CDA-CYS-H-CC agar supplemented with 0.1% sodium taurocholate (CDA-CYS-H-CC-T agar) and brain heart infusion agar supplemented with 0.5% yeast extract, 0.1% L-cysteine, cycloserine-cefoxitin, and 0.1% sodium taurocholate (BHIA-YE-CYS-CC-T agar) media were not significantly different from each other (p > 0.05). Overall, enrichment of inoculated compost samples in broths containing moxalactum-norfloxacin (MN) produced significantly higher (p < 0.05) spore counts than in non-selective broths or broths supplemented with CC. Enrichment in BHIB-YE-CYS-MN-T broth followed by culturing on an agar containing 7% horse blood and 0.1% taurocholate provided a more sensitive and selective combination of media for detecting a low population of *C. difficile* from environmental samples with high background microflora.
As live-stock animals carry and shed *C. difficile* and *C. perfringens* in the feces, animal manure-based composts may play an important role in disseminating both of these toxigenic clostridia species into agricultural environment. A survey study was performed to isolate *C. difficile* and *C. perfringens* contaminations from commercially available composts and livestock animal manure (n=142) using the optimized media. Presumptive *C. difficile* and *C. perfringens* isolates were confirmed by testing for the tpi housekeeping gene and 16S rRNA gene, respectively, in addition to staining methods. The confirmed *C. difficile* isolates were further tested for toxigenicity and PCR ribotyping. Both clostridia isolates were tested for susceptibility to selected antibiotics. A total of 58 *C. difficile* strains and 11 *C. perfringens* strains were identified from 142 compost/manure samples and the majority of *C. difficile* isolates were toxigenic with 63.8% isolates (n=37) positive for toxin A (*tcdA*) while 67.2% isolates (n=39) positive for toxin B (*tcdB*). Only 3 isolates (5.17%) were positive for the binary toxins. However, all *C. perfringens* isolates were negative for enterotoxin gene. There were 38 different PCR ribotypes among 58 *C. difficile* isolates, and ribotype 106 was the most prevalent followed by ribotypes 020, 412, and 251 among the toxigenic isolates. All clostridia isolates were susceptible to the selected antibiotics, but > 50% of *C. difficile* isolates were resistant to clindamycin by agar dilution method. Our results emphasize that compost used in agricultural production may be a reservoir of epidemic *C. difficile* strains.

Thermal responses of *C. difficile* endospores in finished dairy compost were compared at 55 and 65°C under wet and dry heat conditions using a three-strain cocktail of *C. difficile* endospores at a final concentration of ca. $3 \times 10^5$ CFU/g and the moisture
content (MC) of the compost was adjusted to be 20, 30, and 40%. For the wet heat treatment, compost samples were placed in a metal tray submerged in a water bath whereas for the dry heat treatment, samples were placed in an environmental chamber. The come-up times for wet heat treatments were dependent on the targeted temperature and the MC of compost, but for the dry heat treatment, the come-up time was programmed to be 2 days by ramping the temperature from room to target temperatures (55 or 65°C) to simulate the early phase of composting. Thicknesses of endospore cortex and coat were measured using transmission electron microscope. The MCs of compost were maintained well throughout the wet heat treatment while the dry heat treatment reduced the MC of compost to <10% by the end of come-up time. During the holding time, endospore counts were reduced slightly by <0.5 log CFU/g at 55 and 65°C of dry heat treatment, whereas 0.7-0.8 and 0.6-3.04 log CFU/g reductions were observed at 55 and 65°C in wet heat treatment, respectively. However, no temperature provided by either heat treatment eliminated the endospore counts to undetectable level (5 CFU/g) at the MC condition used in this study. Ribotype 596 endospores, having the thickest cortex and coat survived well after heat treatments at all temperature-MC levels. Our results demonstrated that the extrinsic factors such as temperature of the thermophilic phase and MC of feedstock materials and intrinsic factors such as the thickness of endospore cortex and coat have significant (p < 0.05) effects on the thermal resistance of *C. difficile* endospores during composting.

Survival of *C. difficile* vegetative cells and endospores was compared in autoclaved and unautoclaved dairy composts with different MCs. Both types of
composts adjusted with MCs of 20, 30, and 40% were inoculated with *C. difficile* at a final concentration of ca. 5-6 log CFU/g of vegetative cells and ca. 5 log CFU/g of endospores. The inoculated composts were then stored at room temperature (22°C) for 1 year in a humidity controlled chamber. The MCs of both types of composts were very stable during the year-long storage and no significant changes (p>0.05) in MC were observed except for the autoclaved compost with 30% MC. The level of indigenous microflora was very stable during the storage after day 7 in both types of compost. The greatest reductions of *C. difficile* vegetative cell counts were observed during the first 24 h of aerobic storage for all samples, which were 4.7 and 5.51 log CFU/g with 20% MC, 1.85 and 2.13, log CFU/g with 30% MC, and 2.32 and 1.31 log CFU/g with 40% MC, respectively, for autoclaved and unautoclaved compost. The level of MC of compost and the duration of storage have significant (p < 0.05) effects on the survival of vegetative cells, which was considered in the first 120 days of storage. The better survival of *C. difficile* in compost with 30 and 40% MCs during the initial aerobic exposure was supported by the modeling data as well. The reduction of endospore counts during the year-long storage for both types of compost at all MCs was in the range 0.1-0.8 log CFU/g compost. There were no significant changes (p>0.05) in the endospore counts in both types of composts except the autoclaved compost with 30% MC. Decaying of vegetative cells was not affected by the type of compost used at higher MC levels. This study reported the survival of highly resistant *C. difficile* endospores for more than a year while vegetative cells died off exponentially upon the initial aerobic exposure, but leaving more survivors during tailing in drier compost.
Our results suggest that the optimized media could be used to culture *C. difficile* in compost within 24 h anaerobic incubation. The survey for *C. difficile and C. perfringens* in commercially available composts of a variety of feedstocks revealed a high prevalence. Based on our thermal inactivation results, the minimal recommended composting regulations may not be sufficient to lower the level *C. difficile* endospores to an undetectable level in composting. Therefore, the extended thermophilic phase is required if the composting feedstock is moistened well. As both vegetative cells and endospores of *C. difficile* will survive for an extended period of time in the compost, the transmission of the pathogen into fresh produce at pre- and post-harvest conditions may increase the risk of community associated *C. difficile* infections.
DEDICATION

I would like to dedicate this work to my family. First, my parents for making the person who I am today with their endless love. To my beloved husband, for his tremendous support along this long path sacrificing his own career to encourage and support me. To my loving sons for enlighting my world, being my sun and moon. To my sister, brother and in-laws for their continuous great patience and encouragement. Without all your support, this hard work would not have been possible.
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CHAPTER ONE
LITERATURE REVIEW

Introduction

*Clostridium difficile* is an anaerobic, gram-positive and spore-forming bacillus, which causes enteric diseases commonly known as *C. difficile* infection (CDI) in humans and animals. CDI causes variable symptoms ranging from mild diarrhea to life-threatening toxic megacolon and pseudomembranous colitis (PMC) in humans (Arroyo et al. 2005; Rodriguez-Palacios et al., 2009). This bacterium was first identified four decades ago (1978) from the patients who underwent clindamycin treatments for PMC, which is caused by *C. difficile* (Voth and Ballard, 2005). CDI is a well-known hospital or healthcare-acquired infection in most industrialized countries including the USA (Voth and Ballard, 2005, Denève et al., 2009). *C. difficile* is responsible for nearly 20% of reported antibiotic-associated diarrhea (CDC, 2012). In 2011, the number of cases due to CDI was approximately 453,000 and the number of deaths was ca. 29,300 (95% CI, 16, 500-42, 100) in the United States (Lessa et al., 2015). With the emergence of extremely virulent (hypervirulent) strains, the cost of infection control is increasing, affecting the economy of the country. According to the data available for 2008, CDI costs exceed $ 4.8 billion, only in acute care facilities, but 20-30 % of patients reported the recurrence of CDI, which extends the cost further (Dubberke and Olsen, 2012). A recent study emphasized the increase of CDI associated with non-healthcare environments, which was approximately 35% (Lessa et al., 2015). Consequently, more information regarding the
environmental sources of *C. difficile*, and how this pathogen circulates within the community are needed to be broadened.

This thesis reviews the potential of compost as a source of *C. difficile*, its long-term survival in compost, survival of resistant endospores during the simulated composting process, and its resistance to disinfections on surfaces. The knowledge gathered here will be helpful to reconsider the current guidelines of composting in controlling community-associated *C. difficile* infection (CA-CDI).

**Community-associated *C. difficile* infection (CA-CDI)**

CA-CDI is defined as presence of *C. difficile* in feces from a patient within 3 days after hospitalization, who did not have an overnight stay in a healthcare facility for 12 weeks before (Lessa et al, 2015). Rupnik et al (2009) reported that the rate of CA-CDI is rarer than hospital-acquired CDI. However, this rate has been changing during the last 5 years as 76% of healthcare-associated CDI cases did not have a hospital-onset cause and 35% of total CDI cases were community-associated, suggesting that the numbers of CA-CDI cases are becoming significant (Lessa *et al.*, 2015). CA-CDI has been identified in even younger individuals, with less comorbidity, and less exposure to healthcare compared to HA-CDI infected persons (Kuntz et al, 2011). However, deaths and recurrent rates are less among the CA-CDI patients (Lessa et al, 2015).

Although there is no strong evidence on the sources of *C. difficile* regarding CA-CDI, previous studies have reported the ubiquitous survival of this pathogen in environmental sources such as water, soil, meat, vegetables, pets, livestock animals, and compost (Rupnik et al, 2009; Xu et al, 2014; Usui et al, 2017). These possible
environmental sources may bring the pathogen from pre-harvest environments to the food chain. Only one doubtful CDI case that implies a foodborne transmission is reported so far (Gurian et al., 1982). A 78-year old lady without any antibiotic exposure was infected with PMC caused by *C. difficile*. Although the facts to determine the exact source of the pathogen were scarce, the suggestion was consumption of contaminated canned salmon.

The level of *C. difficile* contamination in foods may be low; however, regarding the increasing numbers of CA-CDI cases, foodborne transmission of *C. difficile* should not be ignored. Consequently, more raw and cooked food items, especially animal products, are gaining attention as sources of *C. difficile* (Songer et al., 2009; Rodriguez-Palacios et al., 2007; Rahimi et al., 2015a; Kouassi et al., 2014).

**Risk factors for CDI**

*C. difficile* is a very common pathogen in nosocomial environments, and acquiring the pathogen from healthcare facilities is well-documented. According to the Emerging Infections Program in CDC, 94% of CDI cases were related to healthcare exposures (CDC, 2012). Therefore, the acute care hospitalization within 30 days before CDI diagnosis has been confirmed as a risk factor of CDI based on the laboratory results (Henrich et al., 2009). In addition to the hospitalization and lengthy hospital stays, ages above 65 years, antibiotic usage, comorbidities that leads increased use of antibiotics, the use of proton pump inhibitors (PPIs), and diet are risk factors associated with CDI.

Age is a very critical risk factor for antibiotic-associated diarrhea (AAD). Age greater than 70 years confers risk for antibiotic-associated diarrhea caused by *C. perfringens* and *S. aureus*, whereas being older than 65 years is a risk factor for CDI.
However, age greater than 70 is more associated with severe CDI (Henrich et al., 2009). According to Lessa et al (2015), age groups older than 65 have a 15-fold increase in developing CDI compared to the age group of 1-17 years. The same study further revealed that the ratios of having CDI among these 2 age groups is 44:1 and 5:1, respectively, in healthcare associated CDI (HA-CDI) and CA-CDI incidents.

Antibiotics, which are sometimes overprescribed and misused, play a major role in CDI. The majority of hospitalized patients in the US are prescribed antibiotics and generally, half of them do not present with any sign of an infection (Safdar et al., 2002). Prescription of broad-spectrum antibiotics is a major risk factor in any AAD including CDI (Larcombe et al., 2016). Antibiotic treatments change the diversity of indigenous bacterial flora in colon of patients and allow opportunistic pathogens such as *C. difficile* to colonize and produce toxins that cause diarrhea. Colonic bacteria such as *Clostridium scindens* convert primary bile acids such as cholate into secondary bile acid, deoxycholate, which are inhibitory to *C. difficile* endospore germination. When antibiotics remove the gut microflora including *C. scindens*, *C. difficile* endospores meet the requirements to germinate in the presence of primary bile salts: a germination factor for *C. difficile* endospores (Vishwanathan et al., 2010; Monaghan et al., 2015). Thus, with less colonization resistance from the indigenous microflora, *C. difficile* colonization is not restricted. Having multiple antimicrobials simultaneously and using antibiotics for an extended time significantly enhance CDI (Bignardi, 1998). Immunized patients who undergo the antibiotic therapy may not be infected; they instead may become asymptomatic carriers that disseminate endospores, causing CA-CDI after being
discharged from the hospital (Borriello, 1998; Wilcox et al., 2008). Previous exposures to CDI could also be a risk factor for a severe CDI infection at a later time due to this asymptomatic carrying of *C. difficile* (Dubberke and Olsen, 2012).

Comorbidity is the condition having simultaneous health issues such as inflammatory bowel diseases (IBDs), immunodeficiency, HIV, malnutrition, low serum albumin level, neoplastic diseases, cystic fibrosis, antibiotic-associated diarrhea caused by other bacteria, and diabetes. Such health situations may increase the contraction of CDI due to the attenuated immunity of the patient (Larcombe et al., 2016).

According to a recent study in the USA, sex and race also affect CDI (Lessa et al, 2015). Females had higher incidence rates for both CA-CDI and HA-CDI compared to males. When white and non-white patients were compared, the rate for both CA-CDI and HA-CDI was 6 times higher among white patients (Lessa et al, 2015).

Use of proton pump inhibitors (PPI) is also a potential risk increasing the cases of CDI among adults. According to Larcombe et al (2016), consumption of PPIs significantly increases the likelihood of acquiring CDI. *C. difficile* endospore germination occurs well between pH 6.32-7.53 and moving the pH of the medium to both basic and acidic ends suppresses the endospore germination (Wheedleton et al, 2008). PPIs increase the gastric pH and this could increase survival of even vegetative cells and endospores of *C. difficile* better, which will eventually increase the opportunities for germination and colonization.
Pathogenicity and virulence of *C. difficile*

*C. difficile* is transmitted via the fecal-oral route. If vegetative cells of *C. difficile* are ingested by a healthy individual, these cells may die off rapidly due to susceptibility to gastric acid in the stomach, but the endospores can tolerate the harsh conditions successfully during the passage to the colon. However, the intestinal microflora defends the host from pathogen colonization in healthy individuals. Upon the administration of antibiotics, indigenous microflora is excluded and primary bile acids such as cholic acid and taurocholic acid that enter the ileum will pass to colon, promoting endospore germination of *C. difficile*. Therefore, *C. difficile* colonization is usually observed in the terminal ileum and cecum of the colon (Borriello, 1998). *C. difficile* colonizes easily by adhering and then penetrating the mucus layer of enterocytes with the aid of its flagella and proteases (Deneve, et al., 2009; Vishwanathan et al., 2010; Monaghan et al., 2015). Next, the pathogen can adhere to the enterocytes by means of its multiple adhesins, resulting in the first phase of the pathogenic process.

Figure 1.1 Pathogenesis of *Clostridium difficile* infection (CDI) - reffered from Ibrahim S. 2011.
Colonization of toxigenic \textit{C. difficile} produces 2 major toxins during the late log and stationary phases of its growth cycle. They are toxins A and B, which are large clostridial toxins and glucosyltransferases that catalyze the monoglucosylation of threonine of cytidine triphosphate (CTP) binding proteins Rho, Rac, and Cdc42 in targeting enterocytes leading strong physiological reactions in cells (Drudy \textit{et al}., 2007; Rupnik \textit{et al}., 2003; Rupnik \textit{et al}., 2009). The enzymatic activity is in the N-terminal of the enzyme while the C-terminal is responsible for receptor binding (Drudy \textit{et al}., 2007). Toxin A is an enterotoxin that internalizes the enterocytes and destroys the cytoskeleton of cells in combination with the enzymes such as collagenase, hyaluronidase and some other proteases (Borriello, 1998). This damages the tight junctions of epithelial cell barrier, resulting in cell death or the production of inflammatory substances (Rupnik \textit{et al}., 2009). Collectively, all those effects cause the accumulation of fluid, leading to watery diarrhea. Damages to tight junctions allow the passage of both toxins to the epithelium. Toxin B, which is a cytotoxin, binds to the basolateral cell membrane. Both toxins stimulate monocytes and macrophages to release immunomodulatory mediators, such as interleukin 8. This leads to the penetration of toxins more and more in the intestinal tissue and accumulate neutrophils (Rupnik \textit{et al}., 2009; Tonna and Welsby, 2005) (Figure 1.1).

Toxins A and B are found in a single open reading frame within a 19.6 kb pathogenicity locus (PaLoc) in the genome of toxigenic \textit{C. difficile} (Figure 1.2) (Voth and Ballard, 2005; Vishwanathan \textit{et al}, 2010; Monaghan \textit{et al}, 2015). The two toxin genes show 66% of overall similarity to each other and the similarities at N-terminals of
both genes imply that those two genes are a result of gene duplication (Voth and Ballard, 2005). Other than the genes encoding toxins A (tcdA) and B (tcdB), PaLoc harbors 3 more genes: tcdC, tcdR, and tcdE, which are responsible for the regulation of toxin production and release of toxins from the vegetative cell (Voth and Ballard, 2005). tcdB is 1,000 times more toxigenic than tcdA (Tonna and Welsby, 2005; Keessen et al., 2011). Toxin B has been proven to be the essential virulence factor in CDI by studying the isogenic mutant activity for toxins A and B using a hamster model (Lyras et al., 2009). Lyras et al. (2009) reported that tcdB mutants have shown longer survival, whereas tcdA and wild-type strain (a derivative of C. difficile 630) have shown similar mortality rates in hamsters. A deletion of 5.9 kb in PaLoc has been identified in some C. difficile isolates and those variants are unable to produce a detectable amount of tcdA generating, only toxin B positive strains (A-B+) (Drudy et al., 2007). However, A-B+ strains also cause similar symptoms as A+B+ and are responsible for several C. difficile outbreaks (Drudy et al., 2007). This confirms again that tcdB by its own can lead to CDI in an infected individual. C. difficile strain with A+B- is very rare (Lyras et al., 2009). A recent study reported toxigenic A+B- strain isolated from a diarrheic patient in France (Monot et al., 2015).

Toxin production is positively controlled by tcdR, a transcriptional regulator. tcdC encodes an anti-sigma factor for tcdR and negatively controls the toxin expression. The role of tcdE is assumed to be related to toxin release from the bacterial cell, which is homologous to “holin” protein in bacteriophages. Variations of the PaLoc due to
insertions, deletions, polymorphic restriction sites in genes have generated 28 toxinotypes (Drudy et al., 2007).

Most epidemiological observations were explained using animal models and the interpretations of the contributions of each toxin in CDI are dependent on the sensitivity of different animals to each toxin (Lyerly et al., 1985). Most importantly, there is an increase in CDI severity and outbreaks linked to the hypervirulent strains due to alterations in the regulatory genes for both toxins A and B (Wiegand et al., 2012). The most common hypervirulent ribotypes 027 and 078 have deletions of some nucleotides in the regulatory gene, tcdC (Keessen et al., 2011). In ribotype 027, the regulating gene in PaLoc is truncated and the production of the toxin is continuous. Consequently, it produces 16 times more toxin A and 23 times more toxin B compared to the reference strain (C. difficile 630) (Deneve et al., 2009). Genes encoding toxins A and B are absent in non-toxigenic C. difficile strains and instead, a 115 bp genomic fragment has been detected (Monaghan et al., 2015).

Figure 1.2 Pathogenicity Locus (PaLoc) of Clostridium difficile (referred from Elliott et al., 2017).

Some C. difficile strains produce a third toxin, known as binary toxin (CDT), which is an actin modifying ADP-ribosyltransferase (Rupnik et al., 2003; Voth and
Ballard, 2005). CDT consists of two subunits: cdtA and cdtB, which are separately encoded by cdtA and cdtB genes, respectively, in the 6.2 kb CdtLoc. The third gene in CdtLoc, cdtR, regulates binary toxin expression. cdtB is responsible for receptor binding, endosome membrane insertion, pore formation, and oligomerization whereas cdtA has the enzyme (ADP-ribosyltransferase) activity (Monaghan et al., 2015). In contrast to toxins A and B, both components of the binary toxin are required for the toxigenicity (Perelle et al., 1997). Binary toxins are produced by toxigenic, only toxin B positive, and non-toxigenic strains of C. difficile. However, it is commonly found among toxigenic variants isolated from animals (Gerding et al., 2014). Ribotype 033 that harbors only the genes for binary toxins has been identified in asymptomatic carriers (Gerding et al., 2014).

**Presence of C. difficile in the environment**

The primary reservoirs for C. difficile are mainly the infected patients and contaminated environments, especially within the nosocomial environments and long-term care facilities. Environmental sources outside the nosocomial environment, such as water, soil, produce, and meat are identified as vectors for many foodborne pathogens. Previous studies reported the ubiquitous nature of endospores of C. difficile in environmental samples taken outside the nosocomial environment in last 2 decades (Rodriguez-Palacios et al., 2009; Thitaram et al., 2011; al Saif and Brazier, 1996). Although the vegetative form of C. difficile loses its viability rapidly upon aerobic exposure, the endospores are aero-tolerant and can survive for an extended period of time; for more than 5 months in the nosocomial environment aerobically (Kim et al.,
1981) and for at least four years outside the nosocomial environment (Båverud et al., 2003). In order to control CDI, it is critical to determine the reservoirs for human pathogenic C. difficile strains. After analyzing 786 C. difficile isolates from humans, livestock animals and pets, soil, and water from the vicinity of a poultry farm, Janezic et al. (2012) revealed that there were many shared genotypes of C. difficile between humans and animals, in addition to hypervirulent ribotype 078. Therefore, sources outside healthcare environments may play an important role in harboring and transmitting C. difficile in the community.

**Animal feces and compost**

The fecal matter of livestock animals is the most studied environmental source for the presence of C. difficile in many countries. Most studies reported the prevalence of C. difficile among young animals. According to Bignardi (1998), the reduction of C. difficile in the adult animal is because of the development of complex gut microflora that increases the colonization resistance to C. difficile. For example, CDI is the most prevalent enteritis in neonatal pigs of 1-7 days old. Songer et al. (2004) discovered that the prevalence of toxins A and B of C. difficile was 47%, ranging from 0-97% for some selected herds in North Carolina. Some asymptomatic piglets also carried the toxins in stools (Songer et al. 2004). Another study also reported the significantly higher prevalence (73-87.5%) of C. difficile among young swine from the selected farms in Ohio and North Carolina (Thakur et al., 2010). Moreover, the prevalence of C. difficile in the feces of conventionally raised pigs with antimicrobial treatments and pigs in antibiotic-free farms did not show significant differences (Susick et al., 2012). In 2007, the USDA’s
National Animal Health Monitoring System analyzed the fecal matter of dairy cows in 17 states and reported the prevalence rate as nearly 2% (APHIS, Technical brief, 2011). Zidaric et al. (2008) isolated this pathogen in 63% of fecal samples obtained from laying hens in a farm in Slovenia. Fresh fecal matter of the farmed white-tailed deer was also identified as a source of pathogenic *C. difficile*. About 37% of farms were positive for *C. difficile* with 64% of positive isolates confirmed as toxigenic. Further analysis revealed that the majority of the toxigenic *C. difficile* was human epidemic ribotype 078 (French et al. 2010). Thitaram et al. (2011) analyzed fecal samples obtained from some healthy food animals from 35 states, and the prevalence of *C. difficile* was 16, 6.4, and 2.4% in the fecal samples of swine, beef cattle, and dairy cattle, respectively. Evidently, *C. difficile* is carried by food-producing animals and shed in their feces to the environment. Usually infected humans shed vegetative cells and endospores approximately $10^4$-$10^7$ CFU/g feces (Foster and Riley, 2012). Any subsequent step, where animal waste or treated animal waste is utilized may potentially contaminate the surrounding with *C. difficile*.

Animal waste is rich in nutrients, which can be readily utilized by a variety of agricultural crops such as fresh produce. Due to the fact of animal wastes being the major source of human pathogens, composting is used routinely for on-site waste treatment to inactivate human pathogens and the finished compost products are an excellent soil amendment widely used by both organic and conventional farming worldwide. Composting is a biological decomposition process driven primarily by microbial activities. The elevated temperatures (50 to 70°C) within the compost heap are generally regarded as the most important factor resulting in pathogen abatement in compost.
According to the United States Environmental Protection Agency (EPA) composting guidelines, a minimum composting temperature of 55°C for 3 days in aerated static piles or in-vessel systems, or 15 days with 5 turnings in windrow systems is sufficient to sanitize the pathogens of biosolids (USEPA, 1999). Although active composting effectively inactivates gram-negative pathogens such as *Escherichia coli* O157:H7 and *Salmonella* species (Shepherd et al. 2011; Singh et al., 2011), studies on the behavior of gram-positive endospore forming pathogens during composting are scarce (Wichuk and McCartney, 2007). In a challenge study, biosolid waste inoculated with toxigenic *C. difficile* ribotype 078 was composted by windrow composting and *C. difficile* level was decreased from 3·7 log CFU g$^{-1}$ to 0·3 log CFU g$^{-1}$ with the greatest observed reduction in the curing phase (Xu et al., 2016). This study demonstrates that the level of *C. difficile* in finished compost could be very low. However, a recent study reported an abundance of 36% of *C. difficile* in finished composted swine manure sampled from 14 farms (Usui et al., 2017). The majority of the isolates (82%) in their study was toxigenic and ribotype 078 was the most abundant. Moono et al. (2017) detected *C. difficile* in 59% of lawns in Western Australia, where the lawns were assumed to be contaminated by humans, pets or biosolids used in the preparation of the lawns. Their study isolated many toxigenic *C. difficile* ribotypes from the lawn samples. As organic farming of agriculture relies on organic fertilizers such as manure-based compost for crop growth, risk of *C. difficile* contaminations is expected. However, there is scarce research on animal waste used in agriculture contaminated with *C. difficile*. 
Soil

There have been few studies on the presence of *C. difficile* in soil and characterization of the isolates to understand their genetic relatedness to human pathogenic strains (Janezic et al., 2016). Yard soil samples taken outside the CDI-associated homes were positive for *C. difficile* (Kim et al., 1981), suggesting the ability of endospores to disseminate when there is a source of the pathogen in the vicinity. A large study conducted in Cardiff, UK reported 21% prevalence of *C. difficile* in soil samples (al Saif and Brazier, 1996). Additionally, the same study reported the presence of the pathogen in both sea and fresh water sediments. Later, some studies confirmed the presence of *C. difficile* in soil near the livestock farm areas (Båverud et al. 2003; Janezic et al. 2016). Some soil samples (n = 598) tested for *C. difficile* near pastures and paddocks at two stud farms, public parks, playgrounds, gardens, and cultivated fields, confirmed 4% positive results, with the majority of those samples harboring toxigenic genes (Båverud et al. 2003). A prevalence rate of 37% of *C. difficile* was reported for soil samples in a rural village in Zimbabwe, where poultry was an income of the villagers (Simango 2006). The majority of the chicken fecal sample and soil sample isolates were toxigenic and were resistant to some antibiotics such as cefotaxime, gentamicin, ciprofloxacin, norfloxacin and nalidixic acid (Simango, 2006). A recent study reported the same rate of persistence (37%) of *C. difficile* in soil and most of the isolates were new ribotypes which were non-toxigenic (Janezic et al. 2016). However, some isolates characterized in their study were resistant to clindamycin, erythromycin, and imipenem. Some soil samples collected from public parks and playgrounds of elementary schools
also had a 6.5% positive rate of toxigenic \textit{C. difficile}, which could be due to animal fecal contamination (Higazi et al. 2011).

**Water**

 Aside from soil, water can also be contaminated with \textit{C. difficile} vegetative cells or endospores either directly from the contaminated animal or human excreta, or indirectly, due to water runoff from contaminated lands or soil (Båverud et al. 2003; Janezic et al. 2016). \textit{C. difficile} was isolated from water samples (6\%) from a rural village in Zimbabwe (Simango et al., 2006), puddle water samples (14\%) in Slovenia (Janezic et al., 2016), and river water (87.5\%), seawater (44\%), lake water (46.7\%), swimming pool water (50\%), and tap water (5.5\%) samples in the Cardiff area, UK (al Saif and Brazier, 1996). Most \textit{C. difficile} isolates from water samples in Slovenia were pathogenic to humans and animals (Zidaric et al., 2010; Janezic et al., 2016). The presence of \textit{C. difficile} in river water was associated with high population densities nearby, suggesting the contamination due to human excreta (Zidaric et al., 2010).

 Therefore, the positive rate of \textit{C. difficile} in wastewater is justifiably higher than other water sources mentioned earlier. The oyster harvest water along the Louisiana Gulf Coast and the neighboring municipal water treatment plant in New Orleans depict how the food chain carries this pathogen (Montazeri et al., 2015). All influent and effluent samples from the municipal water treatment plant were positive with toxigenic \textit{C. difficile}, while the harvest water (37\%) and oysters (47\%) had the same toxigenic ribotypes, indicating that those filter feeders accumulate pathogenic \textit{C. difficile} from the harvest waters (Montazeri et al., 2015). According to the information gathered,
wastewater treatment plant (WWTP) influent should be a potential source of toxigenic \textit{C. difficile}. Several studies confirmed the presence of pathogenic strains in WWTPs (Romano et al. 2012; Xu et al., 2014; Steyer et al., 2015). All the influent and effluent samples collected from 9 WWTPs in Switzerland were positive (100\%) for \textit{C. difficile} and 44\% of those isolates were positive for toxins A and B (Romano et al. 2012). Further support for these results came from the 100\% prevalence of \textit{C. difficile}, which was observed in 12 concentrated water samples from WWTPs in Slovenia (Steyer et al., 2015). \textit{C. difficile} positive rates were 92 and 96\% in a raw sludge and an anaerobically digested sludge from 2 WWTPs from Canada, respectively (Xu et al, 2014). All three studies recovered human pathogenic strains, which are associated with CA-CDI.

**Produce**

Although \textit{C. difficile} is not reported as a foodborne pathogen, there is an increasing attention to the contamination of fresh produce with \textit{C. difficile}. Being an enteric pathogen, the possible sources of this pathogen would be the same sources that contribute as the sources of pathogens in produce, such as soil, water, manure and compost (Beuchat, 2002). Additionally, food handling by the symptomatic and asymptomatic workers could also be a source. The rate of abundance of \textit{C. difficile} in produce seems lower compared to that of manure, soil or water (Table 1.1). \textit{C. difficile} was isolated in 2.3 \% of unwashed raw vegetable samples in Cardiff and the majority of the positive samples were root vegetables (al Saif and Brazier, 1996). However, \textit{C. difficile} contaminated cucumbers were also detected in the same study, which is above ground and usually consumed raw, suggesting the possible cross contaminations with other
contaminated source. Another study in Canada reported a 4.5 % prevalence of pathogenic 
*C. difficile* in raw retail vegetables exported from China, the European Union, UK and
USA, and all of those isolates were identified as ribotype 078 (Metcalf *et al*., 2010a).
More CA-CDI strains such as, ribotypes 001 and 014, in addition to *C. difficile* ribotype
078 have been isolated from produce (Eckert *et al*., 2013). A study from Scotland
revealed that 7.5 % of packaged, ready-to-eat salads contained toxigenic *C. difficile*
(Bakri *et al*., 2009). When the produce samples are ready-to-eat, *C. difficile* endospores
will be ingested directly (Bakri *et al*., 2009; Eckert *et al*., 2013; Rahimi *et al*., 2015b).
Vegetables are usually washed with clean tap water before consumption. However, clean
tap water does not remove *C. difficile* endospores from the skin, a biological surface
(Edmonds *et al*. 2013). Probably, cleaning the surfaces of vegetables with clean tap water
may not remove all the attached endospores from the produce surfaces as well.

Several more studies were conducted on the presence of *C. difficile* in fresh
produce from different countries. All studies selected vegetables and ready-to-eat salads.
Leafy greens including spinach and lettuce, tomatoes, and seed sprouts are the most
popular produce types that cause outbreaks in the USA due to being contaminated with
enteric pathogens (Olaimat and Holley, 2012). Most studies have assessed those popular
vegetables related with foodborne outbreaks for *C. difficile* in addition to the root
vegetables, which may contain fine particles of soil. Accordingly, root vegetables such as
carrots, onions, potatoes, radish, parsnip, ginger, and beat and salad vegetables such as
lettuce, spinach, mixed vegetables, pea and pea sprouts were tested in addition to packed
salad meals (Table 1.1). The prevalence of *C. difficile* in vegetables was < 5% for every
except for 2 most recent studies (Lim et al., 2017; Han et al., 2018). Lim et al (2017) isolated *C. difficile* from 30% of the root vegetables from Australia while Han et al., isolated 13.8% of *C. difficile* from ready to eat lettuce in the USA. CDI is spreading rapidly in the developed countries and the results of these recent studies could be an evidence for increasing trend of *C. difficile* contaminations in the environment.

The study by Eckert et al. (2013) performed a quantitative detection of contamination level of *C. difficile* in vegetables (carrots, mushroom, radish, broccoli, celery roots, red cabbage, cauliflower, soya bean, pea sprouts, and lettuce salads) and revealed that 20g of contaminated vegetable contained 6-15 spores and 1-3 vegetative cells. Interestingly, most studies revealed that the majority or all of the isolated *C. difficile* strains from fresh produce harbored toxigenic genes. However, there were some studies, which isolated non-toxigenic strains of *C. difficile* as well from the vegetables (al Saif and Brazier, 1996; Yamoudy et al., 2015; Lim et al., 2017; Han et al., 2018). Majority of the studies further characterized the ribotypes of *C. difficile* isolates and there were outbreak-causing ribotypes such as 078, 027, 017, and 020 among those isolates.
Table 1.1: Prevalence of *C. difficile* isolated from fresh produce or related products\(^a\)

<table>
<thead>
<tr>
<th>Product</th>
<th>Country</th>
<th># of samples</th>
<th>Positive rate % (# of positives)</th>
<th>Toxigenicity (%)</th>
<th>PCR ribotypes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetables</td>
<td>United Kingdom</td>
<td>300</td>
<td>2.3 [7-potato (2), onion (1), cucumber (1), tomato (1), mushroom (1), cabbage (1), lettuce (1), carrot (1), radish (1)]</td>
<td>71 for Toxin A (5)</td>
<td>ND(^b)</td>
<td>al Saif and Brazier, 1996</td>
</tr>
<tr>
<td>Ready to eat salads</td>
<td>Scotland</td>
<td>40</td>
<td>7.5 [3-leaf spinach (1), org. mixed salad (1), organic lettuce (1)]</td>
<td>100 (3)</td>
<td>017 and 001</td>
<td>Bakri et al., 2009</td>
</tr>
<tr>
<td>Vegetables</td>
<td>Canada</td>
<td>111</td>
<td>4.5 [5-Ginger (3), carrot (1), eddoes (1)]</td>
<td>100 (5)</td>
<td>3 isolates were 078</td>
<td>Metcalf et al., 2010a</td>
</tr>
<tr>
<td>Ready to eat salads and raw vegetables</td>
<td>France</td>
<td>104</td>
<td>2.9 [3-lettuce salads (2), pea sprouts (1)]</td>
<td>100 (30)</td>
<td>001, 014/020/077 and 015</td>
<td>Eckert et al., 2013</td>
</tr>
<tr>
<td>Vegetables</td>
<td>USA</td>
<td>125</td>
<td>2.4 [3-Iceberg lettuce (1), green pepper (1), and eggplant(1)]</td>
<td>100 (3)</td>
<td>2 isolates were 027</td>
<td>Rodriguez-Palacios et al., 2014</td>
</tr>
<tr>
<td>Ready to eat salads (cabbage, corn, pea, carrots, tomato, lettuce)</td>
<td>Iran</td>
<td>106</td>
<td>5.7 [6]</td>
<td>16.6 (1)</td>
<td>ND</td>
<td>Yamoudy et al., 2015</td>
</tr>
<tr>
<td>Food Category</td>
<td>Country</td>
<td>Number</td>
<td>Mean (SD)</td>
<td>CFU/100g (N)</td>
<td>Source</td>
<td></td>
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<td>----------------------------</td>
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<td></td>
</tr>
<tr>
<td>Ready to eat salads</td>
<td>Iran</td>
<td>368</td>
<td>1.36 [5]</td>
<td>80 (4)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>(Fasl, Falafel, Olovyeh,</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Rahimi et al., 2015b</td>
<td></td>
</tr>
<tr>
<td>Maccaroni</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vegetable</td>
<td>Saudi</td>
<td>200</td>
<td>1.5 [3-potato (2), parsnip (1)]</td>
<td>100 (3)</td>
<td>078</td>
<td>Bakri, 2016</td>
</tr>
<tr>
<td>Arabia</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Root vegetables</td>
<td>Australia</td>
<td>300</td>
<td>30 [100 pooled-organic and non-organic potatoes, organic beet, organic onion, organic carrots]</td>
<td>51.2 (NA&lt;sup&gt;c&lt;/sup&gt;)</td>
<td>051, 056, 014/020, 101, QX ribotypes (145, 393, 049, 142, 545)</td>
<td>Lim et al. 2017</td>
</tr>
<tr>
<td>Ready to eat lettuce</td>
<td>USA</td>
<td>297</td>
<td>13.8 (41)</td>
<td>100 (41)</td>
<td>027</td>
<td>Han, 2018</td>
</tr>
</tbody>
</table>

<sup>a</sup>Publications including full text of peer-reviewed articles published in English were used for data collection.
ND-Not detected.
NA-Not available.
Meat

Due to increasing numbers of CA-CDI cases, more and more environmental sources are being explored as sources of *C. difficile*. As the animals can carry *C. difficile*, more studies reported the prevalence of human pathogenic *C. difficile* strains in retail meat samples worldwide (Rodriguez-Palacios et al, 2007; Songer et al, 2009; Weese et al., 2009) (Table 1.2). The contamination of retail meat could be due to the contaminated surfaces of processing plants or carcasses of slaughtered animals with *C. difficile* positive in gastrointestinal tract/fecal matter (Harvey et al., 2011; Hopman et al., 2011; Rahimi et al, 2014; Wu et al., 2017). Wu et al. (2017) confirmed that feces and scalding tank water are the main sources of cross contaminations of pork in slaughterhouses. The survival of *C. difficile* endospores for longer periods of time might increase the chances of contaminations of meat or any food item processed in that environment.

Aside from raw meat, meat-related products, such as hamburgers (Rahimi et al., 2015b), may also get contaminated with *C. difficile*. The possible sources of contamination in beef hamburger patties were confirmed by analyzing 211 samples in Iran, and positive samples were obtained from the processing plant, beef meat before processing, and molded and frozen hamburger samples (Esfandiari et al., 2014a). Interestingly, isolating the same ribotype of *C. difficile* from raw meat, environmental samples, and molded and frozen hamburger samples suggests raw beef meat as a possible source of contamination in the final product. For that study, non-meat ingredients were negative for *C. difficile* (Esfandiari et al., 2014a). In contrast to above studies, testing 471 carcass samples and 956 commercial ground beef samples across the USA proved that the prevalence of pathogenic *C. difficile* strains in the US beef processing is rare (Kalchayanand et al., 2013). In supporting that study, a pork processing plant certified for HACCP reported that although the holding area for pigs before slaughtering was a reservoir of *C.
difficile, slaughtered pigs did not carry the pathogen to the processing line (Hawken et al., 2013). These findings illustrate that the hygienic plan in slaughterhouses also determines whether or not the pathogen is present in the final meat product.

Although it is difficult to confirm the sources of C. difficile in meat, most studies reported a low prevalence of C. difficile in all kinds of raw meat poultry, pork, beef, ground meat and meat products (Table 1.2). Most studies isolated toxigenic strains of C. difficile isolates more often whereas limited studies isolated more non-toxigenic isolates (Jöbstl et al., 2010; Guran and Ilhak, 2015). The dissemination of pathogenic ribotypes varies geographically and some pathogenic ribotypes have been isolated from meat samples as well. For example, ribotypes 078 and 027 were the major ribotypes isolated from meal types from the USA and Canada suggesting meat as a potential vector for C. difficile. Similarly, ribotypes 078 and 014 isolated from pork and beef in Belgium are two of the most prominent ribotypes isolated from humans in Europe (Rodriguez et al., 2014). In contrast, a study from Iran revealed that there was no relationship between clinical and meat C. difficile isolates (Esfandiari et al., 2014b).

Early studies on C. difficile prevalence in meat were reported from from Canada and USA, and were higher than 10% (Rodrigues-Palacois et al, 2007; 2009; Songer et al. 2009; Weese et al, 2009; 2010; Harvey et al., 2011). Interestingly, some studies performed later with a large number of samples in the USA did not report positive isolates even after an enrichment method (Limbago et al., 2012; Curry et al., 2012) suggesting that retail meat is not a common contaminant of C. difficile.

Cooked meat also has been assessed for the presence of C. difficile and 12.4% of samples of cooked beef bought from street vendors were contaminated with C. difficile in Ivory coast (Kouassi et al., 2014). The rate of contamination in these cooked beef products is much higher.
than most other raw meat products tested for the presence of *C. difficile* elsewhere (Table 1.2).

Several laboratory studies confirmed that currently recommended meat cooking temperatures are not sufficient to kill all the endospores in meat (Rodrigues-Palacois et al. 2011a; Redondo-Solano et al., 2016). In fact, the temperatures used in low-cooking may induce the germination of *C. difficile* endospores.
<table>
<thead>
<tr>
<th>Product</th>
<th>Country</th>
<th># of samples</th>
<th>Positive rate % (# of positives)</th>
<th>Toxigenicity (%) (^b)</th>
<th>PCR ribotypes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retail ground meat</td>
<td>Canada</td>
<td>60</td>
<td>20 (12)</td>
<td>92</td>
<td>077, 014, M26, M31</td>
<td>Rodrigues-Palacois et al., 2007</td>
</tr>
<tr>
<td>Retail meat (Ground beef and veal chops)</td>
<td>Canada</td>
<td>214</td>
<td>6.01 (14)</td>
<td>77</td>
<td>027, 077, 014</td>
<td>Rodrigues-Palacois et al., 2009</td>
</tr>
<tr>
<td>Retail meat (both uncooked and RTE meat)</td>
<td>USA</td>
<td>88</td>
<td>42 (37)</td>
<td>100</td>
<td>027 and 078</td>
<td>Songer et al., 2009</td>
</tr>
<tr>
<td>Retail ground meat</td>
<td>Sweden</td>
<td>82</td>
<td>2.4 (2)</td>
<td>100</td>
<td>NT(^c)</td>
<td>von Abercron et al., 2009</td>
</tr>
<tr>
<td>Ground beef ground pork</td>
<td>Canada</td>
<td>230</td>
<td>12(28)</td>
<td>100</td>
<td>078, 027 and C, E, Y</td>
<td>Weese et al., 2009</td>
</tr>
<tr>
<td>Ground beef</td>
<td>France</td>
<td>105</td>
<td>1.9 (2)</td>
<td>NA</td>
<td>012</td>
<td>Bouttier et al., 2010</td>
</tr>
<tr>
<td>Ground meat</td>
<td>Austria</td>
<td>100</td>
<td>3 (3)</td>
<td>33</td>
<td>053, AI57</td>
<td>Jöbstl et al., 2010</td>
</tr>
<tr>
<td>Retail pork (ground and chops)</td>
<td>Canada</td>
<td>393</td>
<td>1.8 (7)</td>
<td>85.7</td>
<td>027, V, Y, OCV B</td>
<td>Metcalf et al, 2010b</td>
</tr>
<tr>
<td>Retail chicken</td>
<td>Canada</td>
<td>203</td>
<td>12.8 (23)</td>
<td>100</td>
<td>078</td>
<td>Weese et al., 2010</td>
</tr>
<tr>
<td>Food Type</td>
<td>Location</td>
<td>N</td>
<td>Percentage</td>
<td>Salmonellae</td>
<td>Reference</td>
<td></td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>----------------</td>
<td>-------</td>
<td>------------</td>
<td>-------------</td>
<td>------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Retail meat</td>
<td>Netherlands</td>
<td>500</td>
<td>1.6 (8)</td>
<td>62.5</td>
<td>de Boer et al., 2011</td>
<td></td>
</tr>
<tr>
<td>Retail meat (poultry)</td>
<td>USA</td>
<td>32</td>
<td>21.9 (7)</td>
<td>100</td>
<td>Harvey et al., 2011</td>
<td></td>
</tr>
<tr>
<td>Ground meat and sausages</td>
<td>USA</td>
<td>102</td>
<td>2 (2)</td>
<td>100</td>
<td>Curry et al., 2012</td>
<td></td>
</tr>
<tr>
<td>Ground beef and pork</td>
<td>Canada</td>
<td>48</td>
<td>6.3 (3)</td>
<td>100%</td>
<td>Visser et al., 2012</td>
<td></td>
</tr>
<tr>
<td>Meat (Beef, pork, poultry)</td>
<td>Costa Rica</td>
<td>200</td>
<td>2 (4)</td>
<td>100%</td>
<td>Quesada-Gómez et al., 2013</td>
<td></td>
</tr>
<tr>
<td>Ground meat</td>
<td>Iran</td>
<td>200</td>
<td>4 (8)</td>
<td>100%</td>
<td>Esfandiari et al., 2014b</td>
<td></td>
</tr>
<tr>
<td>Cooked beef</td>
<td>Ivory Coast</td>
<td>395</td>
<td>12.4 (49)</td>
<td>NT</td>
<td>Kouassi et al., 2014</td>
<td></td>
</tr>
<tr>
<td>Raw meat</td>
<td>Iran</td>
<td>660</td>
<td>2 (13)</td>
<td>54</td>
<td>Rahimi et al, 2014</td>
<td></td>
</tr>
<tr>
<td>Raw meat (Beef and pork)</td>
<td>Belgium</td>
<td>133 beef and 107 pork</td>
<td>2.3 (3) beef and 4.7% (5) pork</td>
<td>100% beef and 80% pork</td>
<td>Rodrigues et al., 2014</td>
<td></td>
</tr>
<tr>
<td>Retail chicken parts</td>
<td>Turkey</td>
<td>310</td>
<td>8.06 (25)</td>
<td>36%</td>
<td>Guran and Ilhak, 2015</td>
<td></td>
</tr>
<tr>
<td>Meat products and raw meat</td>
<td>Iran</td>
<td>570</td>
<td>1.2(6)</td>
<td>83.3</td>
<td>NT</td>
<td>Rahimi et al, 2015a</td>
</tr>
</tbody>
</table>

*a* Any kind of publication including full text of peer-reviewed articles published in English were used for data collection.

*b* Toxogenic with either tcdA or tcdB.

*c* NT-Not tested.

*d* Collected from three culture methods.
Antibiotic resistance of environmental C. difficile isolates.

C. difficile is naturally resistant to most antibiotics used to treat many bacterial infections (CDC, 2013). Consequently, CDI is developed as a comorbidity in most patients due to antimicrobial exposure. The resistance to antibiotics increases the selective pressure of C. difficile, providing more opportunities to be virulent in the presence of other bacteria. The ability of C. difficile to acquire antimicrobial resistance could be another reason for increasing numbers of CDI cases in addition to the wide variety of possible C. difficile sources in the environment. Not only clinical human isolates but also environmental isolates of C. difficile harbor resistance to antimicrobials (Pirš et al., 2013). Antimicrobial resistance has been reported in C. difficile strains isolated from livestock fecal samples (Thakur et al., 2010; Keessen et al., 2011; Avberšek et al., 2014; Thitaram et al., 2016; ), retail meat (Songer et al., 2009; Mooyottu et al., 2015), ready-to-eat salads (Rahimi et al., 2015b; Bakri et al., 2009), etc. Hypervirulent C. difficile strains such as ribotypes 027 and 078 were much more resistant to fluoroquinolones and clindamycin, which are commonly used to treat bacterial infections in humans (Rodriguez-Palacios et al., 2006; Denève et al., 2009). Fluoroquinolone resistance is observed not only in clinically isolated ribotypes 027 and 078 but also in the same ribotypes isolated from the environmental samples. Ribotypes 027 RT 078 isolated from both cooked and uncooked retail meat were resistant to levofloxacin, moxifloxacin, gatifloxacin, and clindamycin (Songer et al. 2009). More environmental studies isolated ribotypes with clindamycin, gentamycin, erythromycin, and levofloxacin resistance from meat samples (Rodriguez-Palacios et al., 2007; Quesada-Gómez et al., 2013; Rahimi et al., 2015a). Antimicrobial resistance has been observed in both toxigenic and non-toxigenic C. difficile strains (Pirš et al., 2013).
Most environmental isolates are susceptible to first-line antibiotics used in treating CDI, such as vancomycin and metronidazole (Quesada-Gómez et al., 2013; Bouttier et al, 2010; Rodrigues-Palacios et al, 2007). However, environmental isolates resistant to first-line antibiotics have been reported as well. For instance, two non-toxigenic *C. difficile* isolates from retail ground pork samples, whose ribotypes were unclassified, were resistant to vancomycin, in addition to ciprofloxacin and moxifloxacin (Mooyottu et al, 2015). Further analysis of the genome revealed that these two isolates possessed 5 vancomycin-resistant genes and some multidrug resistance genes. Horizontal gene transfer of *C. difficile* can convert these antimicrobial resistant non-toxigenic strains to toxigenic strains, and antimicrobial resistance can be transferred from non-toxigenic strains to toxigenic strains (Mooyottu et al., 2015). A recent study reported the highest prevalence of vancomycin, metronidazole, and erythromycin resistant *C. difficile* from processed retail lettuce in the USA (Han et al., 2018). The circulation of these antimicrobial resistant toxigenic *C. difficile* in the environment is a threat to the community. This threat will be exacerbated when the antibiotic resistance is associated with the virulent *C. difficile* isolates. Thakur et al. (2010) revealed that there is an association between carrying antimicrobial resistant genes and toxigenicity of *C. difficile* by analyzing the strains isolated from piglets and sows in North Carolina and Ohio. Pathogenic *C. difficile* isolates to humans are usually clindamycin resistant and emerging hypervirulent strains such as ribotype 027 have acquired resistance for many antibiotics, which is different from its historic genotype (Rupnik et al., 2009). Fortunately, even though the environmental isolates are antimicrobial resistant, they may not always exhibit multi-drug resistance (Janezic et al., 2012; Avberšek et al., 2014).
Thermal resistance of *C. difficile* endospores

Heat resistance is a unique feature of bacterial endospores. Like *C. perfringens* and *C. botulinum*, the endospores of *C. difficile* are indeed highly resistant to heat. However, the different strains of the same species may have variable heat resistant capabilities, though all cultures were maintained under the same conditions (Wells-Bennik et al., 2016). Generally, the sporulation temperature of bacteria is associated with the wet heat resistance of the endospores (Wells-Bennik et al., 2016), suggesting the endospores formed at higher temperature-generated processes such as composting should possess a high wet heat resistance. Usually, most endospores are resistant to dry heat 1000 fold more than wet heat (Nicholson et al., 2000). In addition to the sporulation temperature, the wet heat resistance is correlated with the lower content of endospore core water, high degree of muramic acid cross-linking in cortex, protection of spore DNA by α/β type small acid soluble proteins (SASP), and endospore core mineralization (Nicholson et al., 2000; Wells-Bennik et al., 2016). Wet heat damages the endospores by denaturation of endospore core proteins and as the DNA is protected by α/β type SASPs (Nicholson et al., 2000). Interestingly, the heat shock proteins in vegetative cells are not associated with the wet heat resistance of endospores (Nicholson et al., 2000; Wells-Bennik et al., 2016). In contrast to wet heat resistance, the dry heat damages the endospore core DNA and DNA repair mechanisms, saturation of endospore core DNA by α/β type SASPs, and endospore core mineralization play an important role in endospore dry heat resistance (Nicholson et al., 2000). Therefore, endospores with mutant α/β type SASPs are more vulnerable to the exposure of dry and wet heat (Nicholson et al., 2000).

Several studies reported the thermal resistance of *C. difficile* endospores belonging to different strains (Lawley et al., 2009; Rodriguez-Palacios and LeJeune 2011; Redondo-Solano et
Endospores of *C. difficile* strain 630 were not affected by the treatment at 60°C for 24 hours, while 70°C for 24 hours resulted in 2 log reduction after culturing (Lawley et al., 2009). This observation questions the thermal resistance of *C. difficile* endospores at cooking temperatures. Rodriguez-Palacios and LeJeune (2011) determined the heat resistance of toxigenic *C. difficile* endospores at temperatures (63-85°C) recommended for cooking meats. Their study reported that several toxigenic *C. difficile* ribotypes had a complete reduction of endospores (5 to 6 logs) in phosphate buffered saline within 15 min at 85°C. The *D* value at 85°C was estimated as 6-8 min for both fresh (1 week old) and aged (20 weeks old) endospores; the inhibitory effect was reversible and retarded the cell division, but not the endospore germination. The same study detected a 6 log complete reduction of *C. difficile* endospores in 1-2 min at 96°C.

The properties of the matrix such as fat content and heat penetration all over the food item also influence the survival rate of *C. difficile* endospores during heat exposure. When the thermal resistance of *C. difficile* endospores was compared in peptone water and meat, *C. difficile* endospores in meat had higher *D* values (Redondo-Solano et al., 2016). Other than the composition of the matrix, this study reported the differences of *D* values on the strain of *C. difficile* and the recovery method used. For example, the average *D* values at 85 °C were 7.65 min and >2 h in pork matrix as compared with the 5.45 min and 10.7 min in peptone water respectively, by taurocholate and lysozyme recovery methods. The *z*-values by taurocholate method were 11.68 and 7.27 and lysozyme method were 6.68 and 9.7 respectively, for pork matrix and peptone water. The same study further reported that thermal resistance is not correlated with hyper-virulence of *C. difficile*. All conclusions of the thermal studies were based on the efficacy of the recovery after the thermal treatment. Endospores are able to germinate only
upon meeting the suitable conditions. Therefore, there will be discrepancies on the values obtained by each study.

Heat activation of endospores is related with the superdormancy of endospores. Superdormancy is not a genetic effect and are unable to germinate with regular endospores in the absence of high levels of nutrients and heat activation (Ghosh and Setlow 2008). According to Rodriguez-Palacios and LeJeune (2011), leaving endospores of *C. difficile* for 20 weeks at room temperature will induce the superdormancy. Treating these aged endospores at 63°C for 30 min resulted in increased counts by 30% as compared with the non-heat shocked endospores suggesting the sub-lethal temperature reactivate superdormant endospores. Superdormant endospores germinate at extraordinarily slow rates and need special treatments such as sub-lethal heat-shock or a mixture of nutrients in the medium for germination (Ghosh and Setlow 2008). Heat treatments that reach the sub-lethal temperatures (63°C) such as composting, lower cooking temperatures, and pasteurization temperatures will enhance the germination of *C. difficile* endospores. Therefore, the composting process and pasteurization may inactivate most vegetative cells of pathogens while activating *C. difficile* endospores in the matrix. In composting, if the germination occurs near the end of the thermophilic composting phase, those endospores may be able to grow and survive inside the composting heaps during the curing phase.

**Detection methods for the isolation of *C. difficile* from the environmental samples**

**Culturing methods.** The original source of contamination of *C. difficile* in the environment should be feces of symptomatic or asymptomatic humans or animals. Vegetative cells are vulnerable to aerobic and low pH conditions (Jump et al., 2007), whereas resistant endospores
can dissipate, and germinate in various environments upon meeting the growth requirements. As there are no established standard methods for detecting \textit{C. difficile} from environmental samples, the sample size, incubation time, and the composition of the enrichment or selective plating media are variable among different research groups and it is therefore hard to compare and conclude the true prevalence of a variety of samples (Table 1.3). Most studies followed an enrichment procedure, alcohol- or heat-shock, and then selective plating to isolate low levels of \textit{C. difficile} endospores from environmental samples (Bakri \textit{et al.}, 2009; Medina-Torres \textit{et al.}, 2011; Weese \textit{et al.}, 2010). In order to enumerate \textit{C. difficile}, how to initiate the germination of endospores is critical. Endospore germination can also triggered by heat- or ethanol-shock and nutrient availability in the environment. There are strain-specific nutrients that activate the germination receptors in the inner spore membrane. Active germination receptors then release calcium dipicolinic acid in endospore core, which triggers the release of cortex lytic enzymes responsible for the degradation of thick peptidoglycan layer (Ghosh and Setlow, 2008; Paredes-Sabja \textit{et al.}, 2011). The availability of nutrient germination receptors is not clear in \textit{C. difficile} endospores, but germination is accelerated in the presence of some germinants such as taurocholate and glycine. Taurocholate is an emulsifier and may be supportive in weakening the endospore membranes, whereas glycine, an amino acid, is a co-germinant, which is not effective when used alone (Sorg and Sonenshein, 2008). However, germination receptors sensitive to taurocholate and glycine have not been detected in \textit{C. difficile} (Paredes-Sabja \textit{et al.}, 2011). Some non-nutrient germinants such as calcium dipicolinic acid, cationic surfactant dodecyl amine, lysozyme, peptidoglycan fragments bryostatin and high-pressure have also been recognized in inducing the germination of some \textit{Bacillus} species (Paredes-Sabja \textit{et al.}, 2011; Wei \textit{et al.}, 2010; Ghosh and Setlow, 2008). Heat activation is not required for most of these alternative

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germinants; high pressure application and using peptidoglycan fragments with bryostatin for pure dormant and superdormant endospore suspensions have resulted in identical germination rates (Wei et al., 2010). As nutrient germinant receptors are not involved in above methods in inducing germination of heat-injured *C. difficile* endospores, these methods could have a potential of recovering more of them.

According to Weese et al. (2009), 10 spores/g is the minimum detection level in meat samples. Enrichment of a sample seems to be a promising method to isolate a low level of *C. difficile* from the environment. For instance, the enriched fecal swab samples from pigs resulted in more than 4 fold increase in rate as compared to the direct plating after an ethanol shock (Blanco et al., 2013).

The composition of enrichment broth and length of incubation are also variables in the enrichment method (Table 1.3). Enrichment is carried out for 2~15 days at 37°C anaerobically (de Boer et al., 2011; Weese et al., 2010; Rogriguez-Palacious et al., 2011b). However, Rodriguez et al. (2013) reported that 30-day incubation with the enrichment broth resulted in more isolates. The most commonly used enrichment medium is *Clostridium difficile* broth supplemented with cycloserine, cefoxitin, fructose, and 0.1% sodium taurocholate (CCFB-T) (Zidaric et al., 2010; Thakur et al., 2010; Metcalf et al. 2010a; 2010b; Weese et al., 2010; Medina-Torres et al., 2011). In addition to that, brain heart infusion broth supplemented with 0.5% yeast extract, 0.05% L-cysteine and 0.1% sodium taurocholate has been used (Songer et al., 2009). Usually, both enriched and non-enriched samples (direct plating) are subjected to heat or alcohol shock to minimize the background microflora and to activate endospores (Metcalf et al., 2010; Zidaric et al., 2010). The treated sample is then cultured on a pre-reduced agar medium such as CDMN agar, brain heart infusion agar, or cycloserine cefoxitin fructose agar
supplemented with antibiotics and taurocholate (CCFA-T) (Lawley et al., 2009; Weese et al., 2009). Zidaric et al. (2010) used lysozymes (5µg/ml) to enhance the digestion of endospore coat. To recover heat- or alkali-injured *C. difficile* endospores, Kamiya et al. (1989) reported that thioglycollate-lysozyme method was more effective than the taurocholate method. Thitaram et al. (2011) applied two isolation methods (single and double alcohol shock) to isolate *C. difficile* from livestock feces and reported that swine samples are more likely positive for *C. difficile* than dairy or beef cattle. Their study suggested isolation methods might vary among different animal species due to the differences in diet, digestive systems, and background microflora. Since the prevailing conditions in the composting environment are much more different from those of animal feces, food products or other environmental samples, it is anticipated that *C. difficile* endospores may be heat-injured. Therefore, the current detection methods for *C. difficile* from fecal or environmental samples need to be optimized to include an injury-repair step in order to accurately assess the effectiveness of composting on the inactivation of *C. difficile*.

**Molecular-biological methods.** In addition to culturing methods, some studies have applied PCR methods to confirm the persistence of *C. difficile*. Alverez-Perez (2009) proved that detection of the housekeeping gene *tpi* in DNA extracted from fecal samples directly by PCR is a more convenient alternative to direct plating after ethanol shock in isolating *C. difficile* in fecal samples of domestic animals. In contrast, fecal samples isolated from slaughter-ready pigs reported that 28% of samples were positive for *C. difficile* after enrichment, but no positive samples were detected by real-time PCR for DNA extracted directly from fecal samples (Hopman et al., 2011). Similar results were reported by Lipman et al. (2011) after comparing enrichment of pig rectal fecal samples in CDMN broth to real-time PCR directly on fecal
samples. Additionally, toxigenic genes (tcdA, tcdB, and cdtB for binary toxin) of the isolated C. difficile from culture-based methods can be confirmed by PCR (de Boer et al., 2011; Thakur et al., 2010; Rodriguez-Palacios et al., 2011b).

**Typing methods.** Rapid toxin identification methods such as cytotoxicity assay, enzyme immune assay, and real-time PCR can be used in clinical diagnosis of C. difficile (Keessen et al., 2011). As C. difficile toxins degrade after storage at room temperature, the detection of toxins in environmental samples will not indicate the presence of C. difficile accurately.

Phenotypic methods such as lysotyping, serogrouping, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and genotyping methods such as PCR ribotyping, arbitrarily primed PCR (AP-PCR), and pulsedfield gel electrophoresis (PFGE) are very useful tools for further confirmation of the similarities exists between environmental and human pathogenic isolates (Bidet et al. 2000). Due to the requirements of specific reagents and lack of standardization of phenotypic methods, genotypic methods are more popular in the analysis of unknown C. difficile isolates. PCR ribotyping is based on the differences of intergenic spacer region of rRNA operon, which is located between 16S and 23S genes (Bidet et al. 2000; Rupnik et al., 2009). Primers for PCR are selected from each region of rRNA operon (first primer from the 3’ end of 16S gene and the second primer from the 5’ end of 23S gene) to amplify the variable length intergenic spacer region (Bidet et al. 2000). The sizes of the bands generated are between 225-700 bp and the bands can be visualized by gel electrophoresis. The band pattern is called the “ribotype”, which can be used to identify epidemic C. difficile strains (Rupnik et al., 2009). PCR ribotyping resulted in 100% typeability in several studies as compared with the AP-PCR (Bidet et al. 2000; Wullt et al. 2003). AP-PCR is based on
nonspecific random amplifications of the bacterial chromosome by PCR of using short primers under low-stringency conditions (Bidet et al. 2000; Wullt et al. 2003). PFGE is based on the digestion of chromosomal DNA with a restriction endonuclease enzyme that chops the chromosomal DNA producing high molecular weight fragments (Bidet et al. 2000). Usually smal enzyme used in the cleavage of C. difficile chromosome (Bidet et al. 2000). Compared to the PCR based genotyping methods, PFGE is more labor intensive and typeability is less than the PCR based methods. Complete bacterial genome chopped with a restriction endonuclease is usually separated in a polyacrylamide gel with continuous switching of voltage allowing the separation of fragments according to its size. The resulting band pattern is called the “pulsotype” or “pulsovar” (Rupnik et al., 2009). Restriction endonuclease analysis (REA) is also similar to PFGE, but cuts the bacterial genome into smaller segments creating a more complex band pattern in an agarose gel after electrophoresis (Rupnik et al., 2009). Table 1.3 Isolation methods of C. difficile from environmental samples using different enrichment broths.
Table 1.3 Isolation methods of *C. difficile* from environmental samples using different enrichment broths

<table>
<thead>
<tr>
<th>Method of isolation</th>
<th>Source</th>
<th>No of samples</th>
<th>Culture method Media and temperatures</th>
<th>Positives (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct plating</td>
<td>Environmental samples, stools, soil, sewage, foods</td>
<td>910</td>
<td>Suitable dilutions or directly the sample on CCFA-egg yolk agar</td>
<td>85 (2.6%)</td>
<td>Kim et al., 1981</td>
</tr>
<tr>
<td></td>
<td>Fecal swab samples from pigs</td>
<td>36</td>
<td>Swabs were treated with 70% ethanol for 20 min at room temperature and plated on CC containing selective agar</td>
<td>8 (22.2%)</td>
<td>Blanco et al., 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>16 (44.4%) - ten plates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enrichment in cycloserine ceftoxitin fructose broth (CCFB) anaerobically</td>
<td>Fecal swab samples from pigs</td>
<td>40</td>
<td>Swabs were incubated with 5 ml of CCFB supplemented with 0.1% taurocholate for 8 days at 37°C and plated on CC containing selective agar after treating with absolute ethanol for 1 h</td>
<td>19 (47.5%)</td>
<td>Blanco et al., 2013</td>
</tr>
<tr>
<td></td>
<td>Fresh fecal samples obtained by rectal swabs (neonatal piglets aged 7 days)</td>
<td>229</td>
<td>Direct culture-on ChromeID agar and enrichment in CCFB supplemented with gentamycin for 48h. 1 ml of enriched broth was treated with 96% ethanol for 1h and plated onto CCFA-T</td>
<td>120 (52.4%) - Chrom ID agar and 154 (67.2%) - enrichment</td>
<td>Knight et al., 2015</td>
</tr>
<tr>
<td>Sample Type</td>
<td>Methodology</td>
<td>Results</td>
<td>Reference</td>
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<tr>
<td>Bed rails, toilets and floors beside beds and toilets surfaces were sampled by rubbing cotton swabs moistened with CCFB</td>
<td>Incubated the swabs for 48 h at 37°C in CCFB and sub-cultured to CCFA (w/o blood) and anaerobic blood agar. Compared CCFB incubation vs CCFA imprint technique.</td>
<td>126 (6%) on CCFA 55 (3%) in CCFB</td>
<td>Clabots et al., 1991</td>
<td></td>
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<tr>
<td>Fecal samples or rectal swabs of sheep and goats</td>
<td>Fecal samples were homogenized in distilled water (1:1) and 1 ml of that was enriched for 7 days in CCFB-T and plated onto selective agar supplemented with CC following the alcohol shock</td>
<td>Culture based methods 6.4% goats and 1.9% sheep were positive. Together with molecular biological methods 9.2% goats and 5.7% sheep</td>
<td>Avberšek et al., 2014</td>
<td></td>
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<tr>
<td>Fecal samples from pigs and dogs</td>
<td>Ca. 1 g of sample was added to 9 ml of CCFB-T and incubated for 10 days at 37°C. After alcohol shock streaked on CCFA-T plates</td>
<td>62 porcine and 39 dogs (No information of sample size)</td>
<td>Spigaglia et al., 2014</td>
<td></td>
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</tr>
<tr>
<td>Retail vegetables</td>
<td>Ca. 15 g of edible parts of produce were enriched in 50 ml CCFB-T for 7 days at 37°C. Samples were treated with 99% ethanol for 50 min</td>
<td>3 (2.4%)</td>
<td>Rodriguez-Palacios et al., 2014</td>
<td></td>
<td></td>
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<tr>
<td>South American coati</td>
<td>Incubated 6 days at 37°C and plated on CCFA-T</td>
<td>3 (6.5%)</td>
<td>Silva et al., 2014</td>
<td></td>
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</tr>
<tr>
<td>Sample Type</td>
<td>Sample Description</td>
<td>Methodology</td>
<td>Result</td>
<td>Reference</td>
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<tr>
<td>Pigs and cattle intestinal (carcasses and intestinal samples)</td>
<td>402 Each swab from carcass in 50 ml of CCFB-T and 1g of intestinal samples in 9 ml of CCFB-T were enriched 3-30 days at 37°C and 10 µl was spread on CCFA-T.</td>
<td>18 (8.9%) cattle 8 (4%) pig</td>
<td>Rodriguez et al., 2013</td>
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<tr>
<td>Food and surface samples in a Belgian nursing home (environmental surfaces and food samples, n=188)</td>
<td>246 CCFB to enrich samples</td>
<td>1(0.5%) Only food</td>
<td>Rodriguez et al., 2015</td>
<td></td>
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<tr>
<td>Retail beef and pork (beef, n=133 and pork, n=107)</td>
<td>240 Ca.10 g of minced meat was enriched with 90 ml CCFB-T for 3 days at 37°C and 10 µl of broth was spread on CCFA-T</td>
<td>3/133 (2.3%) and 5/107 (4.7%)</td>
<td>Rodriguez et al., 2014</td>
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<tr>
<td>Cycloserine-cefoxitin mannitol broth with taurocholate and lysozyme (CCMB-TALY)</td>
<td>Uncooked ground meat</td>
<td>102 Ca.10 g of meat were enriched in CCMB-TALY for 5 days anaerobically at 37°C and plated on to pre-reduced Trypticase soy agar with 5% sheep blood.</td>
<td>2 (2%)</td>
<td>Curry et al., 2012</td>
<td></td>
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<tr>
<td>C. difficile broth with 0.1% TAU and w/o antimicrobial</td>
<td>Retail pork samples</td>
<td>393 Ca.15 g of pork were enriched with 50 mL of broth and incubated anaerobically for 7 days at</td>
<td>7 (1.8%)</td>
<td>Metcalf et al., 2010b</td>
<td></td>
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<tr>
<td>Supplements</td>
<td>37°C. Cultures were alcohol shocked plated onto Columbia Blood agar</td>
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<tr>
<td><strong>BHIB anaerobic incubation</strong></td>
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<tr>
<td>Ready to eat salads and ready to eat raw vegetables from retail stores</td>
<td>Ca.25 g of each vegetable was crushed in 75 ml of pre-reduced BHIB-CC-T and enriched for 3 days and tenfold dilutions of the enriched broth were plated on BHIA-CC-T supplemented with 5% defibrinated horse blood</td>
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<tr>
<td>(Ready to eat salads and ready to eat raw vegetables)</td>
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<tr>
<td>Retail Meat Products</td>
<td>Ca.1 g of meat was incubated in 10- mL of prereduced BHIB supplemented with 0.5% yeast extract, 0.05% L-cysteine and 0.1% taurocholate 37°C for 3 days. Sub cultured on CCFA-T</td>
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<tr>
<td>88</td>
<td>3 (2.9%)</td>
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<td>Eckert et al., 2013</td>
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<tr>
<td><strong>C. difficile broth supplemented with moxalactam, norfloxacin and taurocholate (CDMN broth)</strong></td>
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<tr>
<td>Sea food and fish</td>
<td>Ca. 15 g of sea food were inoculated into 50 ml of CDMN-T broth and incubated for 7 days anaerobically at 37°C.</td>
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<tr>
<td>119</td>
<td>5 (4.8%)</td>
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<tr>
<td>Metcalf et al., 2011</td>
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<tr>
<td>Fecal samples from farm-raised deer (farms)</td>
<td>Homogenized 20 g of sample in 180 ml of UPB and 15 ml of homogenate</td>
<td></td>
<td></td>
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<tr>
<td>30</td>
<td>11 (36.7%)</td>
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<tr>
<td>French et al., 2010</td>
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</tbody>
</table>
were centrifuged and sediments were enriched in 15 ml of CCFB and CDMN broths for 7-10 days anaerobically at 37°C and plated on CDMN agar followed by 5 day incubation.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Samples</th>
<th>Results</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh fecal matter of horses</td>
<td>742</td>
<td>1 ml aliquot of feces was inoculated into 9 ml of CDMN-T and enriched for 7 days at 37°C</td>
<td>Medina-Torres et al., 2011</td>
</tr>
<tr>
<td>Rectal and environmental samples (piglets, n=72, sows ante partum, n=12, and sows post-partum, n=6)</td>
<td>90</td>
<td>Ca.1 g of piglet samples and sow samples or tip of swab was incubated 37°C in 24 h in 9 ml of CDMN broth. Environmental samples and teat samples were incubated at 37°C for 7 days.</td>
<td>Hopman et al., 2011</td>
</tr>
<tr>
<td>Chicken (thighs, n=111, wings, n=72, and legs, n=20)</td>
<td>203</td>
<td>1 ml of hand massaged suspension (entire piece was hand massaged in 50 ml of PBS) was inoculated into 9 ml of CDMN-T and incubated for 2 days anaerobically at 37 °C followed by plating on CDMN agar.</td>
<td>Weese et al., 2010</td>
</tr>
<tr>
<td>Retailed meat (Beef, n=145, pork, n= 63, calf, n=19, lamb, n=16, chicken, n=257)</td>
<td>500</td>
<td>Ca. 5 g of each sample were inoculated into 20 ml of CDMN-T-5% horse blood broth and incubated for 10-15 days anaerobically at 37°C.</td>
<td>8/500 (1.6%)</td>
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</tr>
<tr>
<td>Retail ground beef and pork (115 from each)</td>
<td>230</td>
<td>25 ml of ground meat were hand massaged in 25 ml of PBS and 1 ml of the mixtures was transferred to CDMN-T and incubated for 2 days anaerobically at 37°C, and followed by plating on CDMN agar</td>
<td>14/115 (12.2%) each</td>
</tr>
<tr>
<td>Retail meat (Ground beef, n=149 and chopped veal, n=65)</td>
<td>214</td>
<td>Enriched in CDMN-T broth or CCFB-T broth for 7 days anaerobically at 37 °C.</td>
<td>10/149 (6.7%) 3/65 (4.6%)</td>
</tr>
<tr>
<td>Meat purchased from meat packaging plants (Beef, n=81, and mutton, n=119)</td>
<td>200</td>
<td>Ca. 5 g of a sample was inoculated to 25 ml of CDMN broth supplemented with 0.05% L-cysteine for 7 days at 37 °C and plated on CDMN agar</td>
<td>8 (4%)</td>
</tr>
<tr>
<td>Retail meat (beef, n=72, pork, n=78, turkey, n=76, and chicken, n=77)</td>
<td>303</td>
<td>Ca.10 g of a sample was enriched in 50 ml of CDMN-T broth supplemented with 5% horse blood for 10 days at 37°C and streaked onto</td>
<td>31 (10.2%)</td>
</tr>
<tr>
<td>Sample Type</td>
<td>Sample Size</td>
<td>Enrichment Method</td>
<td>Results</td>
</tr>
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</tr>
<tr>
<td>Retail meat (Chicken parts)</td>
<td>310</td>
<td>Ca. 10 g of each sample was rinsed with 10 ml of phosphate buffered saline and 1 ml of that was enriched in CDMN-T for 2-3 days at 37°C. After an alcohol shock, plated on CDMN agar supplemented with 7% horse blood.</td>
<td>25 (8.06%)</td>
</tr>
<tr>
<td><em>Clostridium difficile</em> in ready-to-eat foods</td>
<td>368</td>
<td>Ca. 5 g of a sample was enriched in 20 ml of CDMN broth supplemented with 5% de-fibrinated sheep blood for 10-15 days at 37°C and streaked onto CDMN agar supplemented with 7% (v/v) defibrinated sheep blood.</td>
<td>5 (1.36%)</td>
</tr>
<tr>
<td><strong>Thioglycollate broth</strong></td>
<td></td>
<td>For fecal samples, 3–4 g of feces were incubated at 35±2°C for 12 days in thioglycollate broth and 5 g of meat were in 20 ml of broth after stomaching. After alcohol shock plated on <em>C. difficile</em> agar.</td>
<td>8/187 (4.3%)</td>
</tr>
</tbody>
</table>

*aNot available.*
Control Strategies for C. difficile

The prevention or minimization of CDI is an obstacle due to its highly resistant and transmissible C. difficile endospores. In order to control CDI, the ingestion of endospores and CDI development after ingestion of endospores should be minimized. The use of broad spectrum of disinfectants and antiseptics can decontaminate biotic and abiotic surfaces to prevent the transmission of C. difficile endospores in the environment. Hence, the probabilities of endospore ingestion would be minimized. To prevent the development of CDI due to ingested endospores, the best strategies would be managing unnecessary consumption of antimicrobials and adhere to the prescribing antimicrobials (Gerding et al., 2008; Dubberke et al., 2014).

C. difficile is a burdensome nosocomial pathogen, and therefore most environmental cleanliness practices and barrier precautions to prevent further dissemination are mostly healthcare associated (Gerding et al., 2008; Dubberke et al., 2014). Due to high contaminations on the surfaces such as floors, commodes, toilets, bedpans, and bed frames using both detergents and chlorine-based sporicidal agents are important in reducing the numbers of endospores (Vonberg et al., 2008). The efficacy of the disinfection process depends on the concentration of the disinfectant, contact time, the presence of organic matter on the surface, and C. difficile ribotype and chlorine-based disinfectants are more effective in controlling C. difficile endospores (Vonberg et al., 2008; Dawson et al., 2011).

Previous studies reported that 3% hydrogen peroxide (H₂O₂) vapor decontamination followed by regular cleaning was effective in reducing C. difficile endospores from surfaces (Shapey et al, 2008). However, regular 3% H₂O₂ does not achieve complete eradication of C. difficile endospores (Lawley, et al., 2010). Several disinfectants are combined to have more effective decontamination and deep cleaning with a chlorine-based disinfectant followed by
hydrogen peroxide ($\text{H}_2\text{O}_2$) decontamination has also been reported as an efficient way of cleaning $\text{C. difficile}$ infected patients rooms (Best et al., 2014). Using 5.25% sodium hypochlorite and accelerated $\text{H}_2\text{O}_2$ gel have shown complete elimination of $\text{C. difficile}$ endospores with 10 min exposure (Best et al., 2014). When using aqueous disinfections on hydrophobic surfaces, a uniform layer of disinfection may not be applied and due to evaporation, the disinfectant would have to be reapplied before completion of the expected contact time (Omidbakhsh, 2010). Moreover, peroxygens are also very effective oxidizers, which damage the endospore coat and all internal components such as DNA, proteins, and lipids. Peracetic acid containing peroxygens (Perasafe®) has exhibited a 5 log reduction of endospores of ribotypes 012, 017, and 027 for a liquid culture (Dawson et al., 2011).

Hand washing with soap and water has been proven to be more effective than using alcohol-based hand rubs in removing $\text{C. difficile}$ endospores from contaminated hands. Lack of biocide activity of 70% ethanol or isopropanol has been previously reported by other studies where complete inactivation of endospores was observed for 1% sodium hypochlorite, 10% hydrogen peroxide (Wult et al., 2003). Alcohol-based hand sanitizers are effective in killing only vegetative cells of most pathogenic bacteria including $\text{C. difficile}$, but not for endospores. Therefore, disinfection of hands with sanitizers first kills vegetative cells and then washing with soap and water to reduce the endospores in hands is recommended (Kampf et al, 2009).
Summary

The number of *C. difficile* infected individuals is increasing in both healthcare facilities and community. Increased isolation of this pathogen from the environmental samples outside of healthcare settings could be a possible solution for reducing CDI. Identification and molecular characterization, and revealing the relationship of those isolates with human pathogenicity would be very helpful in understanding the epidemiology of CDI. Animal manure is a rich source of nutrients for the growth of many crops, and composted manure is widely used as a fertilizer in organic farming. Composting converts the animal waste into a stable humus-like compound by microbial activities. Microbial activities increase the temperature of composting materials, providing sufficient heat to kill vegetative cells of most pathogenic bacteria. Animal manure is a source of pathogenic *C. difficile*, but the fate of this endospore-forming pathogen during composting is not well-understood. The currently used culture media, may not be effective enough to isolate the pathogen from a matrix with a high background flora. The survival of *C. difficile* in compost poses a threat to the post-harvest environments of crops. Therefore, currently using minimal composting conditions should be evaluated to assess the endospore inactivation of this pathogen.

The objectives of this study were,

1. To optimize the culture media for isolation of *C. difficile* endospores and vegetative cells from compost.

2. To evaluate the prevalence of endospore-forming *C. difficile* and *C. perfringens* in commercially available compost and manure and molecular characterization of those isolates.

3. To monitor the persistence of *C. difficile* in dairy compost during long storage at room
temperature.

4. To assess the thermal resistance of *C. difficile* endospores in simulated early stages of composting conditions using dry and wet heat treatments.
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CHAPTER TWO

IMPROVING CULTURE MEDIA FOR THE ISOLATION OF CLOSTRIDIUM DIFFICILE FROM COMPOST

ABSTRACT

This study was to optimize the detection methods for Clostridium difficile from the animal manure-based composts. Both autoclaved and unautoclaved dairy composts were inoculated with a 12-h old suspension of a non-toxigenic C. difficile strain (ATCC 43593) and then plated on selected agar for vegetative cells and endospores. Six types of enrichment broths supplemented with taurocholate and L-cysteine were assessed for detecting a low level of artificially inoculated C. difficile (ca. 5 spores/g) from dairy composts. The efficacy of selected enrichment broths was further evaluated by isolating C. difficile from 29 commercial compost samples. Our results revealed that using heat-shock was more effective than using ethanol-shock for inducing endospore germination, and the highest endospore count (p < 0.05) was yielded at 60°C for 25 min. C. difficile agar base, supplemented with 0.1% L-cysteine, 7% defibrinated horse blood, and cycloserine-cefoxitin (CDA-CYS-H-CC agar) was the best medium (p < 0.05) for recovering vegetative cells from compost. C. difficile endospore populations from both types of composts enumerated on both CDA-CYS-H-CC agar supplemented with 0.1% sodium taurocholate (CDA-CYS-H-CC-T agar) and brain heart infusion agar supplemented with 0.5% yeast extract, 0.1% L-cysteine, cycloserine-cefoxitin, and 0.1% sodium taurocholate (BHIA-YE-CYS-CC-T agar) media were not significantly different from each other (p > 0.05). Overall, enrichment of inoculated compost samples in broths containing moxalactum-norfloxacin (MN) produced significantly higher (p < 0.05) spore counts than in non-selective broths or broths supplemented with CC. Enrichment in BHIB-YE-CYS-MN-T broth followed by culturing on an
agar containing 7% horse blood and 0.1% taurocholate provided a more sensitive and selective combination of media for detecting a low population of *C. difficile* from environmental samples with high background microflora.

**MEDIA ABBREVIATIONS**

BHIA-YE-CYS agar: Brain heart infusion agar supplemented with 0.5% yeast extract, and 0.1% L-cysteine

BHIA-YE-CYS-T agar: BHIA-YE-CYS agar supplemented with 0.1% sodium taurocholate

BHIA-YE-CYS-CC agar: BHIA-YE-CYS agar supplemented with cycloserine-cefoxitin

BHIA-YE-CYS-CC-T agar: BHIA-YE-CYS-CC agar supplemented with 0.1% sodium taurocholate

BHIA-YE-CYS-MN-T agar: BHIA-YE-CYS-T agar supplemented with moxalactum-norfloxacin

CDA-CC-E agar: *C. difficile* agar base supplemented with cycloserine-cefoxitin and 2.5% egg yolk

CDA-CYS agar: *C. difficile* agar base supplemented with 0.1% L-cysteine

CDA-CYS-H-CC agar: CDA-CYS agar supplemented with 7% horse blood, and cycloserine-cefoxitin

CDA-CYS-T agar: CDA-CYS agar supplemented 0.1% sodium taurocholate

CDA-CYS-H-T agar: CDA-CYS-T agar supplemented with 7% horse blood

CDA-CYS-H-CC-T agar: CDA-CYS-H-CC agar supplemented 0.1% sodium taurocholate

BHIB-YE-CYS-T broth: Brain heart infusion broth supplemented with 0.5% yeast extract, 0.1% L-cysteine and 0.1% sodium taurocholate
BHIB-YE-CYS-MN-T broth: BHIB-YE-CYS-T broth supplemented with moxalactam-norfloxacin

BHIB-YE-CYS-CC-T broth: BHIB-YE-CYS-T broth supplemented with cycloserine-cefoxitin.

CDB broth: *C. difficile* broth

CDB-CYS-T broth: CDB broth supplemented with 0.1% L-cysteine and 0.1% sodium taurocholate

CDB-CYS-MN-T broth: CDB-CYS-T broth supplemented with moxalactam-norfloxacin

CDB-CYS-CC-T broth: CDB-CYS-T broth supplemented with cycloserine-cefoxitin

INTRODUCTION

*Clostridium difficile* is an anaerobic, gram-positive and spore-forming bacillus, causing *C. difficile* infection (CDI) in both humans and animals. In the United States, the mortality rate due to *C. difficile*-associated diarrhea has increased in the last decade. For example, *C. difficile*-associated deaths from 1999-2004 were > 20,000 [1] as compared to ca. 29,000 deaths in 2011 [2]. Furthermore, Lessa *et al.* [2] reported in their study that 76% healthcare associated CDI cases were not hospital onset and approximately 35% of those non-healthcare associated CDI cases were community-associated.

Toxigenic *C. difficile* strains have been isolated from various environments outside health care environments such as water, soil, livestock animals and their manure, and fresh produce [3-6]. Consequently, the land application of fresh animal manure, contaminated with *C. difficile* may also transfer endospores and/or vegetative cells of the pathogen into above environments. Sanitizing animal manure by aerobically decomposing the organic matter into compost can reduce the populations of most human pathogens to undetectable levels. However, transfer of
human pathogens from the contaminated compost or soil to fresh produce has been reported [7]. It is well-known that endospores are more resistant to the environmental stresses compared to their vegetative counterparts, suggesting the resistant endospores could be found in compost too. However, the fate of bacterial endospores during composting has not been fully studied [8]. Furthermore, endospores of bacteria, including \textit{C. difficile}, may be heat-injured during composting of contaminated animal manure. As a result, currently available methods for detecting this pathogen in manure-based composts may need further improvements.

Due to the low level of contamination, various enrichment media and selective plating media have been used to maximize \textit{C. difficile} endospore recovery from diverse environmental samples. Among the many evaluated culture media with different selective components, \textit{C. difficile} agar base supplemented with cycloserine-cefoxitin and 2.5% egg yolk (CDA-CC-E agar) improved the isolation of \textit{C. difficile} from environmental samples with a detection limit of 2 x $10^3$ CFU/g [9]. Later on, 2.5% egg yolk in CDA-CC-E agar was replaced with 0.1% taurocholate, a spore germinant [10], and 7% horse blood [11]. \textit{C. difficile} broth supplemented with cycloserine-cefoxitin and 0.1% taurocholate (CDB-CC-T broth) was used as an enrichment broth for fecal samples, but the heavy growth of background flora was a disadvantage [11]. An alcohol-[12] or a heat-[13] shock followed by enrichment controlled the growth of numerous contaminants effectively. According to Aspinall and Hutchinson [14], the addition of moxalactam-norfloxacin (MN) as alternatives to CC yielded 20% more recovery of \textit{C. difficile} and less interfering microbial species after enrichment. Songer et al also used brain heart infusion broth supplemented with 0.5% yeast extract, 0.05% L-cysteine and 0.1% sodium taurocholate to isolate \textit{C. difficile} from environmental samples, instead of using CDB broth [15]. Since animal-waste based compost is high in background micro-flora, the detection media for \textit{C.}
difficile endospores may need to be optimized further. Therefore, the objectives of this study were to improve enrichment and plating media to recover C. difficile endospores from artificially inoculated dairy compost and then analyze commercial compost products for C. difficile contaminations.

MATERIALS AND METHODS

Endospore preparation and induction of germination by sub-lethal treatments: C. difficile ATCC 43593 stock culture stored at -80°C was streaked on brain heart infusion agar (Becton & Dickinson, Sparks, MD) supplemented with 0.5% yeast extract (Hardy Diagnostics, Santa Maria, CA, USA), 0.1% L-cysteine (Alfa Aesar, Ward Hill, MA, USA) and 0.1% sodium taurocholate (BHIA-YE-CYS-T agar) plates and incubated anaerobically at 37°C for 24 h. After 2 transfers, C. difficile biomass was transferred on to BHIA-YE-CYS-T agar plates and incubated anaerobically at 37°C for 7 days. Then plates were sealed with parafilm and left aerobically at room temperature for another 7 days. At the end of the incubation, each plate was washed with 5 ml of 0.01 M phosphate buffered saline (PBS) containing 0.1% tween 80 while sweeping the colonies with sterile cotton tipped applicators (Puritan, MA, USA). The collected spore suspension was washed with sterile cold ice water followed by centrifuging (5 times at 7,000 x g for 15 min). Three separate batches of endospores were prepared and stored at 4°C until used.

One milliliter aliquots of each spore suspension were induced with sub-lethal treatments (Table 2.1) and ten-fold serial dilutions were prepared from each treated endospore suspension. Selected dilutions were plated on BHIA-YE-CYS-T agar in duplicate and the plates were then incubated anaerobically at 37°C for 24 h for endospore enumeration. Additionally, endospores
were enumerated using Petroff-Hausser counting chamber (Hausser Scientific Company, Horsham, PA) under the phase contrast microscope (Model no. DM750, Leica, Microscope Central, Feasterville, PA).

**Inoculum preparation for the recovery study:** Brain heart infusion agar supplemented with 0.5% yeast extract, and 0.1% L-cysteine (BHIA-YE-CYS agar) plates were pre-reduced overnight in gas pack jars using anaerobic gas pouches (Becton & Dickinson, Sparks, MD, USA). *C. difficile* was grown on pre-reduced BHIA-YE-CYS agar and incubated at 37°C for 12 h anaerobically to yield more vegetative cells from the culture. Twelve-hour old biomass on BHIA-YE-CYS agar was collected by washing the plates with sterile 0.85% saline. The suspension was washed with sterile saline followed by centrifuging twice at 7,000 x g for 5 min and resuspended in saline to an optical density (OD) of approximately 0.5 at 600 nm to be used as the inoculum. This inoculum contained both vegetative cells and endospores of *C. difficile*.

**Compost preparation:** Commercial dairy compost was purchased directly from the farm (Wallace Farm Soil Product Inc., Huntersville, NC). As listed on the labels, the compost samples contained 0.5% total nitrogen, 0.5% available phosphate, 0.5% soluble potash, and no more than 1% chlorine. The compost was air-dried under the fume hood to reduce the moisture content to < 10% and then sieved using a screen (sieve pore size, 3 by 3 mm) to reduce matrix heterogeneity for a uniform distribution of the inoculum. A sufficient amount of compost was prepared for the entire study and stored in sealed containers at 4°C until used. Initial moisture contents (MC) were determined using a moisture analyzer (model IR-35, Denver Instrument, Denver, CO). The compost was autoclaved at 121°C for 20 min each day, for 3 consecutive days to reduce the indigenous microflora.
Optimization of culture media for recovering vegetative cells and endospores of *C. difficile* from dairy compost: Media optimization for the isolation of vegetative cells and endospores was performed as described in Figure 2.1. *C. difficile* suspension prepared as described above was added into 100 g of autoclaved and unautoclaved compost separately at 1:10 vol/wt. The MC of compost was adjusted to 30% with sterile tap water. Each inoculated compost sample was mixed well for 5 min by wearing sterile gloves. To enumerate vegetative cells, 5 g of the inoculated compost were homogenized in 45 ml of 0.85% saline for 1 min at the medium speed (230 rpm) of a stomacher (Model 400, Seward Laboratory Systems Inc., Bohemia, NY) and ten-fold serial dilutions were prepared. Selected dilutions were plated on the following pre-reduced media to assess the recovery of vegetative cells: *C. difficile* agar base (CDA) (CM0601, Oxoid, Basingstoke, UK) supplemented with 0.1% L-cysteine (CDA-CYS agar), CDA-CYS agar supplemented with 7% horse blood and cycloserine-cefoxitin (SR0096E, Oxoid) (CDA-CYS-H-CC agar), BHIA-YE-CYS agar, and BHIA-YE-CYS agar supplemented with cycloserine-cefoxitin (BHIA-YE-CYS-CC agar) (Table 2.2). For the endospore enumeration, 1-ml aliquots of $10^{-1}$ dilution of inoculated compost were transferred to 1.5 ml microcentrifuge tubes and sealed with parafilm. Those aliquots were subjected to a sub-lethal heat treatment at 60°C for 25 min, and serial dilutions were plated on culture media supplemented with 0.1% (w/v) sodium taurocholate: CDA-CYS-H-CC-T agar, CDA-CYS-T agar, and BHIA-YE-CYS-CC-T agar (Table 2.2). In all experiments agar plates were incubated anaerobically at 37°C for 24 h unless otherwise stated. The recovery rates of *C. difficile* vegetative cells or endospores were calculated by dividing the population of vegetative cells or endospores recovered from compost by the population of vegetative cells or endospores inoculated into compost and multiplying that by 100%.
Compost inoculation and simulation of early stages of composting: The endospore suspensions used for recovery experiments were diluted in 0.85% saline to yield ca. 50 spores/ml. Two hundred grams of unautoclaved dairy compost at 30% MC were artificially inoculated with *C. difficile* endospores at a final concentration of ca. 5 spores/g compost (1:10, vol/wt) in a sterile aluminum foil tray. Approximately 50 g of inoculated compost were packed in sterile Tyvek® pouches (size, 3.5” × 9.0”; SPS Medical, Rush, NY) and kept in a single layer (ca. 0.5-0.6 cm in depth) on a shelf of an environmental chamber (Model no. EC2047N, Thermo Scientific, Barnstead International, Dubuque, IA). Tyvek pouches provide sterile packaging allowing free oxygen and moisture exchange of the sample to mimic the conditions inside the composting pile [16]. The target temperature was set at 55°C, which was monitored constantly using type-T thermocouples (DCC Corporation, NJ, USA) with two cords inserted inside the Tyvek pouches, and another was kept on the chamber shelf. The temperature rise of the environmental chamber was programmed to ramp stepwise from 25°C to the target temperature in 2 days and then the target temperature was maintained for 3 days. The final temperatures of environmental chamber were set as 0.5°C higher than the target temperature (55°C). Sample bags were removed from the chamber at the end of the treatment, and placed in an ice cold bath analyzed for the presence of viable endospores by enrichment method as described below. Humidity inside the chamber was maintained by placing a metal tray containing distilled water (size: 12” × 21” × 3.5”) and recorded with a Dual Zone electronic thermometer (VWR, Suwanee, GA, USA). The experiment was performed in three separate trials.

Selection of enrichment media for isolating artificially inoculated *C. difficile* endospores during composting: Following the thermal treatment as described above, suitability
of BHI broth and CD broth as enrichment broths was assessed with 6 different combinations of supplements: BHI broth supplemented with 0.5% yeast extract, 0.1% L-cysteine and 0.1% sodium taurocholate (BHIB-YE-CYS-T broth), CD broth supplemented with 0.1% L-cysteine and 0.1% sodium taurocholate (CDB-CYS-T broth), BHIB-YE-CYS-T broth supplemented with moxalactam (32 mg/l) and norfloxacin (12 mg/l) (Alfa Aeser, Ward Hill, MA, USA) (BHIB-YE-CYS-MN-T broth), BHIB-YE-CYS-T broth supplemented with cycloserine-cefoxicitin (Oxoid, Lenexa, OH, USA) (BHIB-YE-CYS-CC-T broth), CDB-CYS-T broth supplemented with moxalactam-norfloxacin (CDB-CYS-MN-T broth), and CDB-CYS-T broth supplemented with CC (CDB-CYS-CC-T broth) (Table 2.3).

*C. difficile* in the compost was enriched and enumerated before and after the thermal treatment as described in Figure 2.2. Briefly, 1 g portions of compost were mixed with 4 ml of each enrichment broth in a 15 ml conical tube (Corning, NY, USA) and homogenized by vortexing gently. Tubes were then anaerobically incubated at 37°C for 7 days. Following the incubation, 1 ml of each enriched broth was heat-shocked at 60°C for 25 min and the selected ten-fold serial dilutions were plated in duplicate. Compost enriched in non-selective enrichment broths (BHIB-YE-CYS-T broth and CDB-CYS-T broth) were plated separately on BHIA-YE-CYS-CC-T agar or BHIA-YE-CYS-MN-T agar. All the replicates in enrichment broths supplemented with CC or MN were plated on BHIA-YE-CYS-CC-T agar or BHIA-YE-CYS-MN-T agar, respectively. The detection limit after enrichment was 1.4 log CFU/g compost.

**Comparison of enrichment broths for *C. difficile* detection from commercial compost samples:** Twenty nine commercial compost samples (collected in 2011-2012) were enriched with three selective enrichment broths: CDB-CYS-CC-T broth, BHIB-YE-CYS-CC-T broth, and BHIB-YE-CYS-MN-T broth. Five grams of each compost sample were enriched in 20
ml of each enrichment broth anaerobically at 37°C for 7 days. One milliliter of each enriched sample was then heat-shocked at 60°C for 25 min and centrifuged at 4,000 x g for 10 min. The resulting pellet was resuspended in 50 µl of saline and streaked on CDA-CYS-H-CC-T agar and CDA-CYS-H-MN-T agar plates from the samples enriched in broths containing CC and MN, respectively. After anaerobic incubation at 37°C for 24-48 h, the suspected colonies were isolated as pure cultures and confirmed if those were *C. difficile* through colony morphology, Gram’s staining, endospore staining, PRO disc test (Remel, Lenexa, KS, USA), the presence of *tpi* house-keeping gene by PCR [17], and latex agglutination test (DR 1107, Oxoid).

**Statistical Analysis:** Each experiment was repeated thrice. *C. difficile* counts on different plating media, sub-lethal treatments, and enrichment broths were compared by converting plate counts into log CFU/g in dry weight of compost. The counts were then subjected to analysis of variance with a test criterion (F statistic) and type I error controlled at a P value of 0.05. The Tukey multiple-comparison procedure of the Statistical Analysis System (JMP® Pro 12, SAS Institute Inc., Cary, NC, 1989-2007) was used to compare the least square differences among different media/treatments.

**RESULTS**

In this study, we initially evaluated selective media for recovering artificially spiked *C. difficile* endospores and vegetative cells from dairy compost, and then an enrichment broth medium was optimized to detect a low level of *C. difficile* endospore contamination in compost during simulated composting process. Finally, the sensitivity of enrichment broths was validated by detecting *C. difficile* from commercial compost products.
Sub-lethal treatments for recovering *C. difficile* endospores: Our study compared 4 commonly used temperature-time combinations of heat-shock method with ethanol-shock method (Table 2.1). There were no significant differences (p > 0.05) on endospore recovery when heat treatments were performed at 60°C for 25 min, 65°C for 10 min, and 70°C for 20 min. Those treatments enumerated ca. 1.2 logs higher endospore counts, which were significantly higher (p < 0.05) than the treatments at 80°C for 10 min, ethanol-shock, and untreated spore suspensions. The heat treatment at 60°C for 25 min was selected for following experiments of endospore enumeration due to the highest endospore yield after the treatment.

Optimization of culture media for recovering vegetative cells and endospores of *C. difficile* from dairy compost: Dairy compost (finished product) used in this study was confirmed to be free of *C. difficile*. Vegetative cells of inoculated *C. difficile* grew on all tested culture media. The highest count was observed on CDA-CYS-H-CC agar for both autoclaved compost (in the presence of ca. 3~5 logs of compost microflora) and unautoclaved compost (ca. 7 logs of compost microflora) detecting significantly (p < 0.05) high *C. difficile* counts compared to other plating media (Table 2.2). The recovery rates of vegetative cells on BHIA-YE-CYS agar and BHIA-YE-CYS-CC agar were not significantly different (p > 0.05) for both pure culture suspension and autoclaved compost. In recovering vegetative *C. difficile* cells from unautoclaved compost, media supplemented with CC showed significantly higher colony counts (p < 0.05) than the respective media without supplement. However, the vegetative cell counts on CDA-CYS agar were not significantly different (p > 0.05) from the counts on BHIA-YE-CYS-CC agar for both types of composts. The highest recovery rates for vegetative cells were observed on BHIA-YE-CYS-CC for both autoclaved and unautoclaved composts, which were 28.18 and 16.98% respectively.
Although the highest yield of *C. difficile* endospores (from the pure culture and both types of composts) were obtained on CDA-CYS-H-CC-T agar, the recovery rates on CDA-CYS-H-CC-T agar, for autoclaved and unautoclaved composts were 5.37 and 3.89%, respectively as compared with pure culture. There were no significant differences (p > 0.05) in the endospore counts on CDA-CYS-H-CC-T and BHIA-YE-CYS-CC-T for both types of composts. In the absence of antibiotics CC, it was difficult to distinguish *C. difficile* colonies from interfering colonies of background microflora on CDA-CYS-T medium.

**Selection of enrichment media for isolating artificially inoculated *C. difficile***

*endospores during composting:* The relative humidity inside the environmental chamber at 55°C was recorded as 88%. Among the 6 enrichment broths assessed, BHIB-based broths were more effective in enriching *C. difficile* endospores in compost than CDB-based broths (Table 2.3). When the 2 non-selective enrichment media were compared, BHIB-YE-CYS-T broth yielded significantly higher counts (p < 0.05) than CDB-CYS-T broth on all recovery media for both control and treated compost samples. The same trend was also observed for CC supplemented enrichment broths, BHIB-YE-CYS-CC-T broth and CDB-CYS-CC-T broth, for both the control and treated samples. Both enrichment broths containing MN (CDB-CYS-MN-T broth and BHIB-YE-CYS-MN-T broth) yielded the highest populations of endospores (> 7 logs/g compost) on BHIA-YE-CYS-MN-T agar, and the counts were not significantly different (p > 0.05) from each other for control and treated samples. However, BHIB-YE-CYS-MN-T broth yielded the highest endospore count in treated compost sample, which was ca. 0.6 log spores/g compost higher than the spore count that on CDB-CYS-MN-T broth. CDB-CYS-CC-T broth showed the lowest recoveries of *C. difficile* from dairy compost for both control and simulated composting samples on corresponding solid agar media (2.31 and 1.91 log CFU/g
compost, respectively). The counts for both control and simulated composting in CDB-CYS-T broth and CDB-CYS-CC-T broth enrichments were not significantly different from each other (p > 0.05) when recovered on BHIA-YE-CYS-CC-T plates.

**Comparison of enrichment broths for detecting C. difficile in commercial compost samples:** The sensitivity of the selected enrichment broths for detecting C. difficile in dairy compost was evaluated using 29 commercial compost samples. Three enrichment broths, i.e. CDB-CYS-CC-T broth, BHIB-YE-CYS-CC-T broth and, BHIB-YE-CYS-MN-T broth were used for the isolation of C. difficile from compost. CDB-CYS-CC-T broth yielded only 3 compost samples as positive for C. difficile as compared with 7 positive samples in BHIB-YE-CYS-CC-T broth enrichment. Interestingly, when the samples were enriched in BHIB-YE-CYS-MN-T broth, 9 compost samples were positive suggesting BHIB-YE-CYS-MN-T broth was more sensitive in detecting C. difficile from environmental samples. The overall positive rate was 31.03% (9/29).

**DISCUSSION**

Increasing reports on community associated CDI cases have suggested that the sources of C. difficile are broad. Therefore, it is important to understand the potential reservoirs of C. difficile and optimize conditions for isolating C. difficile from the environment. Vegetative cells of C. difficile are vulnerable to aerobic and low pH conditions [18], whereas endospore is the most essential form of C. difficile in persistence, dissipation, and germination in various environments, including guts of human and animal. Therefore, this study focused mainly on optimizing the culturing methods for recovering the endospores from animal waste-based composts.
Being more resistant to the environmental stresses, most environmental samples may contain *C. difficile* endospores, which are commonly enumerated on solid media, following a sub-lethal heat/ethanol-shock. Temperatures ranging from 56 to 80°C are applied for 10-30 min in the heat-shock method [4, 19] whereas absolute or 95% ethanol mixed with the sample in 1:1 (v/v) ratio is left for 50-60 min at room temperature in the ethanol-shock method [20]. Resistance to such stress treatments selects endospores in the matrix by inactivating *C. difficile* vegetative cells and other background microflora substantially [21, 22]. Although the exact mechanisms for endospore activation by the sub-lethal treatments are yet to be discovered, a possible explanation for the mechanism of the heat-shock method is partial conformational changes of the proteins responsible for endospore dormancy [23]. These conformational changes affect the tertiary structure of the germination receptor proteins of endospores, increasing the accessibility to germinants [24]. Our study demonstrated that the heat-shock is more effective than alcohol-shock in recovering *C. difficile* endospores on solid media (Table 2.1). Similar to our results, the effectiveness of heat-shock over the ethanol-shock was demonstrated in recovering *C. difficile* from stool samples and fecal swabs [20], and intestinal contents and carcass samples [13].

Activated endospores can germinate rapidly in the presence of germinants such as glucose, amino acids, and/or purine ribosides [23,25]. The germinants bind to germination receptors in the inner membrane of the spore core and stimulate the release of Ca-DPA from the spore core, which triggers the activation of cortex lytic enzymes digesting the peptidoglycan layer of cortex [25,26]. The function and abundance of germination receptors are dependent on the bacterial species [25,27]. Pseudoproteases of *C. difficile* respond to cholic acid derivatives of bile salts [28,29], which are conjugated with taurin or glycine resulting in taurocholate or
glycocholate, respectively [27]. Supplementing BHIA with 0.1% taurocholate has been found to increase the endospore recovery of *C. difficile* by $10^5$ folds [22]. In agreement with this finding, our results showed no *C. difficile* colonies on pre-reduced BHIA-YE-CYS agar compared to > 6 log endospores/ml on BHIA-YE-CYS-T agar after the heat-shock (results not shown). Enhanced recovery of *C. difficile* from fecal samples in the presence of taurocholate was also confirmed by other studies [4,30,31]. In contrast, Parades-Sabja *et al.* [19] demonstrated that BHI broth was a nutrient rich medium and supplementing with taurocholate is not required for endospore germination. Similarly, Heeg *et al.* [21] reported that 6 of 15 tested strains of *C. difficile* germinated in BHIB without taurocholate. Therefore, the spore germination responses may vary among the strains of *C. difficile*.

Being an anaerobe, *Clostridium* requires a redox potential (Eh) of -200 mV or less for the growth in a culture medium [32]. Addition of L-cysteine to a medium would bring its Eh to -175 ~ -200 mV, while the presence of agar in the medium stabilizes the reduced Eh [32]. Our preliminary studies revealed that media without L-cysteine had no or few colonies of *C. difficile* after anaerobic incubation for 48-72 h. After supplementing all broth and agar types used in this study with 0.1% L-cysteine a visible growth of *C. difficile* was observed after overnight incubation anaerobically. However, for enriched samples, incubation time was used as 24-48 h in considering the slow growing *C. difficile* populations.

The origin of *C. difficile* in compost could be the contaminated feedstock, but after composting the level of remaining *C. difficile* endospores should be lower. Xu *et al.* [33] reported a reduction of *C. difficile* endospore level from 3.7 to 2.9 log CFU/g in the thermophilic phase of windrow composting of bio-solid waste. In their study, the level of *C. difficile* was reduced approximately to 2 spores/g compost after curing. The low level of *C. difficile*
contamination in compost was reported by Berry et al. [34], who could not detect C. difficile in minimally managed bovine feedlot manure-based compost by direct plating the 10⁻¹ dilution on C. difficile agar base supplemented with 0.5 g/l of cysteine hydrochloride, 7% horse blood, and norfloxacin-moxalactam after 5 days of anaerobic incubation. In order to detect a low population of C. difficile endospores in an environmental sample, the sample usually needs to be enriched anaerobically in a suitable enrichment broth at 37°C for 2~15 days [35-37]. By mimicking the low level of contamination by C. difficile in compost, the compost was inoculated to have ca. 5 spores/g compost in this study. CDB-CYS-CC-T broth, the most widely used selective enrichment medium (usually reported as CCFB-T without adding L-cysteine) in isolating C. difficile from the environment [38], was one of the selected enrichment broths used in this study. According to our results, CDB-CYS-CC-T broth was the least sensitive broth among the enrichment broths used in isolating C. difficile from compost (Table 2.3). Enrichment broths supplemented with antibiotics MN, i.e. BHIB-YE-CYS-MN-T broth and CDB-CYS-MN-T broth, improved the growth of C. difficile more effectively with a colony count of >7 log CFU/g compost and the counts were not significantly different from each other (p > 0.05) for both treated and untreated composts (Table 2.3).

Numerous interfering colonies were also observed for compost samples enriched in CDB-CYS-CC-T broth after plating on selective agar. Diaz et al., [38] reported that CDB-CC-T broth was less selective since it recovered 24 other bacterial species, including Lactobacillus, Clostridium, and Enterococcus, from environmental and food samples. Commercially available CC (SR0096E, Oxoid) and MN (SR0173, Oxoid) supplements contain lower concentrations of antibiotics than the minimal inhibitory concentrations (MIC) of C. difficile [3,8]. However, the antibiotic sensitivities of C. difficile isolated from different environments could be variable under
the culture conditions. In this study, when the compost was inoculated with a higher initial titer of *C. difficile* endospores, the endospores were successfully recovered on CC supplemented media. In the recovery of a low titer of *C. difficile* endospores after a simulated thermophilic phase in composting, enrichment broths supplemented with MN were more desirable than the enrichment broths supplemented with CC. Therefore, considering the consistently higher *C. difficile* counts in supplemented BHI based broth as compared with the corresponding CDB based broth from simulated composting samples (Table 2.3), BHIB-YE-CYS-MN-T broth was identified as the best enrichment broth for commercial compost analysis.

The sensitivities of enrichment broths observed for inoculated and simulated composting samples were confirmed by the enrichment of commercial compost products. When BHIB-YE-CYS-MN-T broth was used for the enrichment, 9 commercial compost samples were detected as positive. Distinguishing the typical swarming colony morphology of *C. difficile* on blood agar was not challenging after the enrichment in BHIB-YE-CYS-MN-T broth.

The colony counts on selective agar (BHIA-YE-CYS-CC-T and BHIA-YE-CYS-MN-T) were not significantly different (p > 0.05) from each other for the compost after the enrichment in each non-selective broth, except for the simulated composting samples in CDB-CYS-T broth enrichment (Table 2.3). This suggests that the composition of the enrichment broth is more crucial in isolating *C. difficile* from the environment than the composition of subsequent plating media. Previous studies have reported contradictory results regarding selective enrichment of samples for isolating *C. difficile*. Chai et al., [39] reported the disadvantages of using selective enrichment broths compared to non-selective enrichment broths in isolating *C. difficile* from chopped meat samples. According to their findings, indigenous microflora of chopped beef is resistant to the selective agents but *C. difficile* strains are sensitive. On the contrary, Hink et al
[20] reported that the recovery of *C. difficile* from stool samples using selective enrichment broths was better than using non-selective broths. However, the media in their study were supplemented with CC, which were observed to be less effective compared to the enrichment broths supplemented with MN and the non-selective enrichment broths in this study.

To the best of our knowledge, compost has not been well-studied as a source of *C. difficile*. In our study, *C. difficile* contamination rate was 31% among 29 compost samples after BHIB-YE-CYS-MN-T anaerobic enrichment. Usui *et al* [40] recently isolated *C. difficile* from 36% of finished pig manure compost samples (n=14) after 10-15 day anaerobic incubation in CDB supplemented with MN, 5% horse blood, and 0.1% sodium taurocholate. In their analysis, every pig manure based compost pile had piglet feces. *C. difficile* prevalence in piglets is usually higher than that in adult pigs [41]. Although none of the compost samples used in our study was pig manure-based, our findings revealed a considerably high rate of *C. difficile* contaminations in compost products used in agriculture suggesting that *C. difficile* endospores available in finished compost could serve as the source of contaminations of this pathogen in produce agricultural environment.

**CONCLUSION**

*C. difficile* in dairy compost can be cultured successfully within 24 h by using nutrient-rich media supplemented with horse blood, selective antibiotics, and L-cysteine. Pre-reduced CDA-CYS-H-CC agar was the most effective medium in the recovery of *C. difficile* vegetative cells, whereas CDA-CYS-H-CC-T agar and BHIA-YE-CYS-CC-T agar were equally appropriate for enumerating *C. difficile* endospores from unautoclaved compost following the heat-shock at 60°C for 25 min. BHIB-YE-CYS-MN-T broth was found to be the most sensitive enrichment
broth to detect a low titer of *C. difficile* endospores from compost. Furthermore, the presence of *C. difficile* in commercial compost samples after enrichment implies that the current processing may not guarantee a complete inactivation of *C. difficile* endospores. Although optimizing *C. difficile* growth and enrichment media using a single ATCC strain was a limitation, our study has provided some useful information on the isolation of *C. difficile* from environmental samples with a high level of background microflora, such as compost. A larger survey of compost is needed to understand the effectiveness of composting process and identify the environmental sources of pathogenic *C. difficile*.

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Table 2.1 Inducing germination of *C. difficile* endospores with sublethal treatments

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<tr>
<th>Sublethal Treatment</th>
<th>Log spore count/ml&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>6.42 ± 0.12B</td>
<td>[21]</td>
</tr>
<tr>
<td>60°C-25 min</td>
<td>7.98 ± 0.08A</td>
<td>[42]</td>
</tr>
<tr>
<td>65°C-10 min</td>
<td>7.73 ± 0.21A</td>
<td>[43]</td>
</tr>
<tr>
<td>70°C-20 min</td>
<td>7.95 ± 0.19A</td>
<td>[19]</td>
</tr>
<tr>
<td>80°C-10 min</td>
<td>6.52 ± 0.09B</td>
<td>[6]</td>
</tr>
<tr>
<td>23°C-95% Ethanol-50 min</td>
<td>&lt; 5.00</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Plate count data are expressed as means ± standard deviation for triplicate trials. Means with the same letter in the same column are not significantly different (p > 0.05).
Table 2.2 Optimizing culture media for the recovery of *C. difficile* vegetative cells and endospores

<table>
<thead>
<tr>
<th>Culture</th>
<th>Recovery medium&lt;sup&gt;a&lt;/sup&gt;</th>
<th>log CFU/ml or g</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Pure culture</td>
<td>Autoclaved compost</td>
<td>Un autoclaved compost</td>
</tr>
<tr>
<td>Vegetative</td>
<td>CDA-CYS-H-CC</td>
<td>8.10±0.05&lt;sup&gt;b&lt;/sup&gt;A</td>
<td>6.83 ± 0.06A</td>
<td>6.69 ± 0.11A</td>
</tr>
<tr>
<td></td>
<td>CDA-CYS</td>
<td>7.18±0.22B</td>
<td>6.12 ± 0.15B</td>
<td>6.08 ± 0.10B</td>
</tr>
<tr>
<td></td>
<td>BHIA-YE-CYS-CC</td>
<td>6.67±0.11C</td>
<td>6.12 ± 0.13B</td>
<td>5.90 ± 0.20B</td>
</tr>
<tr>
<td></td>
<td>BHIA-YE-CYS</td>
<td>6.74±0.10C</td>
<td>6.16 ± 0.08B</td>
<td>5.09 ± 0.17C</td>
</tr>
<tr>
<td>Endospores</td>
<td>CDA-CYS-H-CC-T</td>
<td>6.52±0.04A</td>
<td>4.49±0.07A</td>
<td>4.52 ± 0.09A</td>
</tr>
<tr>
<td></td>
<td>BHIA-YE-CYS-CC-T</td>
<td>6.31±0.07B</td>
<td>4.45±0.10A</td>
<td>4.37 ± 0.13A</td>
</tr>
<tr>
<td></td>
<td>CDA-CYS-T</td>
<td>4.03±0.15C</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>ND</td>
</tr>
</tbody>
</table>

<sup>a</sup> Recovery media used for vegetative cells: CDA-CYS-H-CC - *C. difficile* agar base supplemented with 0.1% L-cysteine, 7% horse blood, and cycloserine-cefoxitin, CDA-CYS - *C. difficile* agar base supplemented with 0.1% L-cysteine, BHIA-YE-CYS-CC - Brain heart infusion agar supplemented with 0.5% yeast extract, 0.1% L-cysteine, and cycloserine-cefoxitin, and BHIA-YE-CYS- Brain heart infusion agar supplemented with 0.5% yeast extract, and 0.1% L-cysteine. Recovery media used for endospores: CDA-CYS-H-CC-T - *C. difficile* agar base supplemented with 0.1% L-cysteine, 7% horse blood, cycloserine-cefoxitin, and 0.1% sodium taurocholate, BHIA-YE-CYS-CC-T Brain heart infusion agar supplemented with 0.5% yeast extract, 0.1% L-cysteine, cycloserine-cefoxitin, and 0.1% sodium taurocholate, and CDA-CYS-T - *C. difficile* agar base supplemented with 0.1% L-cysteine, and 0.1% sodium taurocholate.

<sup>b</sup> Plate count data are expressed as means ± standard deviation for triplicate trials. Means with the same letter in the same column are not significantly different (p > 0.05).

<sup>c</sup> Not detected, but plates were overgrown with indigenous bacteria.
Table 2.3 Comparing the effectiveness of enrichment broths in recovering artificially inoculated *C. difficile* endospores following thermal treatment.

<table>
<thead>
<tr>
<th>Enrichment Brotha</th>
<th>C. difficile log CFU/g compost on selective mediab</th>
<th>Without thermal treatment (23°C - 3 days)</th>
<th>With thermal treatment (55°C - 3 days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDB-CYS-T</td>
<td>3.33±0.36E,F</td>
<td>3.12±0.15E,F,G</td>
<td>3.67±0.26D,E,G</td>
</tr>
<tr>
<td>CDB-CYS -MN-T</td>
<td>7.49±0.43A</td>
<td>NTd</td>
<td>7.09±0.10A</td>
</tr>
<tr>
<td>CDB-CYS -CC-T</td>
<td>NT</td>
<td>2.31±1.00F,G,H</td>
<td>NT</td>
</tr>
<tr>
<td>BHIB-YE-CYS-T</td>
<td>4.57±0.14B,C,D</td>
<td>5.43±0.55B</td>
<td>4.98±0.28B,C</td>
</tr>
<tr>
<td>BHIB-YE-CYS-MN-T</td>
<td>7.45±0.72A</td>
<td>NT</td>
<td>7.76±0.34A</td>
</tr>
<tr>
<td>BHIB-YE-CYS-CC-T</td>
<td>NT</td>
<td>5.45±0.70B</td>
<td>NT</td>
</tr>
</tbody>
</table>

*a* Media used for enrichment: CDB-CYS-T = *C. difficile* broth supplemented with 0.1% L-cysteine and 0.1% sodium taurocholate, CDB-CYS -MN-T= *C. difficile* broth supplemented with 0.1% L-cysteine, moxalactam – norfloxacin, and 0.1% sodium taurocholate, CDB-CYS -CC-T= *C. difficile* broth supplemented with 0.1% L-cysteine, cycloserine-cefoxitin, and 0.1% sodium taurocholate, BHIB-YE-CYS-T= Brain heart infusion broth supplemented with 0.5% yeast extract, 0.1% L-cysteine, and 0.1% sodium taurocholate, BHIB-YE-CYS -MN-T= Brain heart infusion broth supplemented with 0.5% yeast extract, 0.1% L-cysteine, moxalactam-norfloxacin, and 0.1% sodium taurocholate, and BHIB-YE-CYS -CC-T= Brain heart infusion broth supplemented with 0.5% yeast extract, 0.1% L-cysteine, cycloserine-cefoxitin, and 0.1% sodium taurocholate

*b* Media used for recovery: BHIA-YE-CYS -MN-T= Brain heart infusion agar supplemented with 0.5% yeast extract, 0.1% L-cysteine, moxalactam-norfloxacin, and 0.1% sodium taurocholate and, BHIA-YE-CYS -CC-T= Brain heart infusion agar supplemented with 0.5% yeast extract, 0.1% L-cysteine, cycloserine-cefoxitin, and 0.1% sodium taurocholate

*c* Plate count data are expressed as means ± standard deviation for triplicate trials. Means with the same letter in the table are not significantly different from each other (p > 0.05).

*d* Not Tested
Figure 2.1 Flow chart for the media selection for isolation of vegetative cells and endospores of *C. difficile* from compost.

*C. difficile* (ATCC 43593) culture grown on BHIA-YE-CYS-T agar and washed with 0.85% saline and adjusted to OD$_{600nm}$ to 0.5.

- Adjusted MC of compost to 30% with sterile tap water and mixed
- Autoclaved and unautoclaved compost (100 g)
- Recovered on taurocholate (0.1%) free media for vegetative cells (CDA-CYS-H-CC, CDA-CYS, BHIA-YE-CYS-CC, BHIA-YE-CYS)
- Heat-shocked at 60°C for 25 min
- Recovered on media supplemented with taurocholate (0.1%) for endospores (CDA-CYS-H-CC-T, BHIA-YE-CYS-CC-T, CDA-CYS-T)
Figure 2.2 Experimental design for the optimization of the isolation of *C. difficile* from finished compost.

Un autoclaved compost with 30% MC was inoculated with 5 endospores/g and placed 50 g of compost in Tyvek bags.

Programmed the temperature to reach from RT-55°C in 2 days with a 3-day holding time.

1 g of treated compost was enriched with BHIB and CDB based enrichment broths for 7 days at 37°C anaerobically.

Heat-shocked (60°C-25 min) 1 ml of enriched broth.

Plated on BHIA-YE-CYS-CC-T agar or BHIA-YE-CYS-MN-T agar.

Enrichment broth with the highest endospore yield (BHIB-YE-CYS-MN-T broth).

Screened 29 commercial compost samples.
CHAPTER 3

ISOLATION AND CHARACTERIZATION OF CLOSTRIDIUM DIFFICILE AND CLOSTRIDIUM PERFRINGENS IN ANIMAL MANURE AND COMPOSTS

ABSTRACT

The well-known nosocomial pathogen Clostridium difficile, recently has been recognized as a community associated pathogen. As livestock animals carry and shed C. difficile and Clostridium perfringens in the feces, animal manure-based composts may play an important role in disseminating both of these toxigenic clostridia species into agricultural environment. The present study surveyed C. difficile and C. perfringens contaminations of commercially available composts and livestock animal manure (n=142). Presumptive C. difficile and C. perfringens isolates were confirmed by testing for the tpi house-keeping gene and 16S rRNA gene, respectively, in addition to staining methods. The confirmed C. difficile isolates were further tested for toxigenicity and PCR ribotyping. Both C. difficile and C. perfringens isolates were tested for susceptibility to selected antibiotics. A total of 58 C. difficile strains and 11 C. perfringens strains were identified from 142 compost/manure samples. The majority of C. difficile isolates were toxigenic with 63.8% isolates (n=37) positive for toxin A (tcdA) while 67.2% isolates (n=39) positive for toxin B (tcdB). Only 3 isolates (5.17%) were positive for the binary toxins. However, C. perfringens isolates were not positive for enterotoxin gene. There were 38 different PCR ribotypes among 58 C. difficile manure/compost isolates, and ribotype 106 was the most prevalent followed by ribotypes 020, 412, and 251 among the toxigenic isolates. All C. difficile and C. perfringens isolates were susceptible to the selected antibiotics, but some C. difficile isolates was resistant to clindamycin. Clindamycin resistance, a risk factor for C. difficile infection (CDI), was observed among > 50% of C. difficile isolates by agar
dilution method. This study indicates that compost used in agricultural production may be a reservoir of toxigenic clostridia.

INTRODUCTION

The annual animal manure production in the US is estimated to be 1.37 billion tons including cattle, hog, chicken and turkey manure (1). Due to the high nitrogen and phosphorus content, animal manure, and composted manure are used as crop fertilizers in agricultural production and to improve the soil structure (2). Animal manure is a well-known source of foodborne pathogens such as Salmonella spp., Escherichia coli O157:H7, Campylobacter spp., Listeria monocytogenes, and parasites such as Cryptosporidium parvum (2). Composting is an effective way to eliminate the majority of aforementioned human pathogens in animal manure. During the thermophilic phase of composting, high temperatures generated by microbial activities can reach to ca. 55 to 70°C inside the composting piles/heaps, which is typically held for a few days to several weeks. The elevated temperatures have been proven to inactivate Gram-negative pathogens such as E. coli O157:H7 and Salmonella spp. inside frequently turned heaps of animal manure within 3 weeks of composting (3). However, the fate of endospore forming entero-pathogenic Gram-positives such as Clostridium difficile and Clostridium perfringens is not fully understood in composted animal manure (4).

Both Clostridia species cause enteric diseases in humans and animals. Infected animals/humans shed the vegetative cells and endospores of pathogenic Clostridia with their fecal matter (5,6). Clostridium difficile infection (CDI) is mainly associated with exposure to antibiotics, which decreases the competition of gut micro-flora and allows spore forming C. difficile to proliferate. The symptoms can vary from mild diarrhea to life-threatening toxic
megacolon or pseudomembranous colitis in humans (7). *C. perfringens* is one of the most common causative pathogens of food poisoning in the USA and pathogenic to domestic animals and wildlife due to production of enterotoxins (8).

A few previous studies have reported that composting reduced the levels of both *C. difficile* and *C. perfringens* endospore in feedstocks (5,9). If a high initial load of endospores is present in unprocessed manure, it is possible for a fraction of those endospores to survive in finished compost due to the inherited heat resistance nature of endospores. When contaminated compost is subsequently used on vegetable crops, it can be a source of the pathogen contaminating the edible parts of fresh produce. The ingestion of pathogenic clostridial endospores from toxigenic and antimicrobial resistant strains would result in the symptoms of CDI or *C. perfringens* food poisoning. Although the sources of contamination have not been confirmed, evidence for *C. difficile* contaminations in vegetables and ready-to-eat vegetable salad ingredients have been reported in Scotland, France, Canada, and the USA (10-13). All studies reported a prevalence of <10%, but, all isolates were human pathogenic *C. difficile* strains. An outbreak of *C. perfringens* due to consumption of salad was reported previously (14). Manure has been identified as a source of contamination of *C. perfringens* in greens (15). In fact *C. perfringens* is considered as an indicator microorganism in assessing microbial safety of fresh produce (16) and environmental samples such as compost (17) due to its prolonged survival accompanied by the low inactivation rate.

The objective of this study was to determine the contamination of animal manure/composts by *C. difficile* and *C. perfringens* from the samples collected from multiple states across the United States. Further characterization of isolates was performed to broaden the
current knowledge of potential sources of these endospore forming pathogens especially *C. difficile*, a community-associated pathogen.

**MATERIALS AND METHODS**

**Sample collection.** A total of 142 manure and compost samples of different feedstocks were collected from multiple states in the United States, including California, Colorado, Delaware, Florida, Georgia, Illinois, Iowa, Kentucky, Massachusetts, Michigan, Nevada, New Mexico, New York, Pennsylvania, Philadelphia, South Carolina, South Dakota, Tennessee, Texas, Vermont, Virginia, Washington and Wisconsin. Out of those samples, 113 samples were collected from 2014 to 2015. Twenty one unprocessed manure samples were collected and most of them were from the farms in the vicinity of Clemson University in Clemson, South Carolina, including chicken/turkey litter (n=12), cow manure (n=2), and mixed animal manure (n=7). Commercial finished compost samples (n=91) were generously provided upon the request to the compost makers and an additional sample was purchased from a local supplier. Samples were collected in Ziploc™ bags (S.C. Johnson & Son, Racine, WI), shipped under ambient conditions, and stored under refrigeration conditions (4°C) upon arrival until analyzed. Additionally, 29 finished compost samples collected in 2011-2012 and stored at -20°C from our previous study (18) were characterized further.

**Physical and Microbiological analysis.** The Water activity of each sample was measured using a dewpoint water activity meter (Aqualab Series 3TE, Decagon Devices, Pullman, WA). Moisture content (MC) of each sample was determined using a moisture analyzer (Model IR-35, Denver Instrument, Denver, CO). The pH values in dairy compost were measured
by a multi-parameter benchtop meter (Orion VERSA Star meter, Thermo Fisher Scientific Inc., Fort Collins, CO) according to the test methods described by the U.S. Composting Council (19).

Selected dilutions from the prepared tenfold serial dilution were plated on Tryptic Soy agar (TSA, Becton & Dickinson, Sparks, MD), actinomycetes agar (Becton & Dickinson) and Rose Bengal agar (CM 0549, Oxoid, Lenexa, OH) supplemented with chloramphenicol (100 mg/l) (SR 0078, Oxoid) to enumerate bacteria, actinomycetes and yeast-molds, respectively. Incubation temperatures of 37, 55°C, and room temperature (~22°C) were used for mesophiles, thermophiles, and yeast-molds, respectively. Coliforms and E. coli were enumerated by plating 1 ml of the selected dilutions on E. coli/coliform Petrifilm® (3M, St. Paul, MN) and incubating overnight at 37°C. All samples were tested in duplicate.

**Isolation of C. difficile and C. perfringens from manure and compost samples.**

Experimental procedures used for the isolation of C. difficile and C. perfringens are depicted in Figure 3.1. Qualitative detection of C. difficile from manure/compost samples was performed using brain heart infusion (Becton & Dickinson) enrichment broth supplemented with moxalactam (32 mg/l) (Alfa Aeser, Haverhill, MA), norfloxacin (12 mg/l) (Alfa Aeser), L-cysteine (1 g/l) (Alfa Aeser), and sodium taurocholate (1 g/l) (Alfa Aeser) as previously described (18). Briefly, 5 g of each sample were added to 20 ml of enrichment broth in a sterile 50 ml conical tube (Corning, Tewksbury, MA), mixed by vortexing, and then incubated anaerobically at 37°C for 7 days with loose lids inside an anaerobic jar with anaerobic gas packs (Becton & Dickinson). Following the incubation, 1 ml of each sample was heat-shocked at 60°C for 25 min in a water bath (Model no. DL 30, Haake, Paramus, NJ) and centrifuged at 4,000 × g for 10 min. The supernatant was discarded and the pellet was re-suspended in 50 µl of sterile distilled water and streaked on to C. difficile agar supplemented with 7% horse blood (Remel,
Lenexa, KS), sodium taurocholate (1 g/l), L-cysteine (1 g/l), moxalactum (32 mg/l), norfloxacin (12 mg/l), and cycloheximide (50 mg/l). After the anaerobic incubation of plates at 37°C for 24 h, presumptive positive colonies were identified by non-hemolytic, swarming, flat colony morphology, Gram-staining, and l-proline aminopeptidase activity using PRO discs (Remel).

For *C. perfringens*, 10 g of each manure/compost sample were added to 90 ml of sterile saline (0.85% NaCl) in a sterile Whirl-Pak® Stand-Up Bag (24 oz, Nasco, Inc., Modesto, CA) and homogenized in a stomacher (Model 400, Seward Laboratory Systems Inc., Bohemia, NY) at 230 rpm for 1 min. Tenfold serial dilutions were prepared in saline 1 ml aliquots of selected tenfold serial dilutions were mixed with ca.10 ml of Tryptose Sulphite Cycloserine (TSC) agar (CM0587, Basingstoke Hampshire) supplemented with D-cycloserine (SR 0088, Oxoid, Basingstoke Hampshire) and 2.5% of Oxyrase (Oxyrase Inc., Mansfield, OH) by pour plate method. After solidifying, the plates were overlaid with another ca. 10 ml of the same agar and incubated at 37°C for 24 h aerobically as described previously (20). Presumptive black colonies with a white halo embedded in agar layer were obtained as pure cultures and observed microscopically after Gram-staining.

**PCR analysis for the identification of *C. difficile* and *C. perfringens***. Bacterial genomic DNA was extracted using a microbial DNA extraction kit (MoBio, Carlsbad, CA) and the extracted DNA was stored at -20°C until used. Presumptive *C. difficile* isolates were confirmed by touchdown PCR (21) for species-specific housekeeping gene, *tpi* (22), using previously published primers (Table 3.1) and *C. difficile* ATCC 43593 served as the positive control. PCR-positive isolates were further confirmed by latex agglutination test for *C. difficile* (DR 1107, Oxoid). Presumptive *C. perfringens* isolates were confirmed by conventional PCR (23) using *C. perfringens* ATCC 13124 as the positive control. All PCRIs were performed in an
Eppendorff thermal cycler (Mastercycler Realplex², Eppendorff, Westbury, NY) in a final volume of 25 µl/reaction, which consisted of 10 mM amplification buffer, 2 mM MgCl₂, 0.2 mM of each deoxynucleoside triphosphate, 0.4 µM of each primer, 1 unit of Taq polymerase (Takara Bio Inc, Japan), and 2 µl of template DNA.

**Detection of toxigenic genes.** Genes encoding toxins A (*tcdA*) and B (*tcdB*) of *C. difficile* were detected using the primers described previously (22) (Table 3.1). Binary toxins were detected by screening for *ctdA* and *ctdB* genes as previously described (24) using *C. difficile* ATCC BAA 2155 as the positive control. *C. perfringens* isolates were screened for the presence of enterotoxin gene (*cpe*) as described by Alguilera et al (25) using *C. perfringens* ATCC 12916 as the positive control. PCR products (5 µl each) were resolved on a 1.5 % agarose gel for 60 min at 75 V and visualized after staining with ethidium bromide (10 mg/ml).

**Capillary gel electrophoresis based PCR-ribotyping.** PCR-ribotyping was performed using the primers (26) (Table 3.1) and methods (27) described previously. PCR amplicons were purified using UltraClean™ GelSpin PCR Clean-Up Kit (Mo-Bio) and analyzed using a genetic analyzer (Model no. 3730xl, Applied Biosystems, Foster City, CA) with a 50 cm capillary loaded with a POP4 gel (Applied Biosystems). Samples were injected at 5 kV over 5 s with a total running time of 30 min at 15 kV run voltage. GeneScan 1200 LIZ standard (Applied Biosystems) was used as an internal marker. Fragment analysis results were obtained as .fsa files and uploaded to the web-based database, “Webribo” (http://webribo.ages.at), to identify if *C. difficile* ribotypes distributed in manure/compost were outbreak strains. *C. difficile* ATCC strains 43593 (ribotype 060) and BAA 2155 (ribotype 251) were used to validate ribotyping analysis.

**Antimicrobial resistance (AMR).** All confirmed *C. difficile* and *C. perfringens* isolates were tested for the susceptibility to the selected antimicrobials using the agar dilution method.
C. difficile was tested for metronidazole (MT), vancomycin (VN), moxifloxacin (MX), clindamycin (CL), linezolid (LN), and tigecyclin (TG) (Sigma-Aldrich, St. Lois, MO), while C. perfringens was tested for vancomycin (VN), clindamycin (CL), amoxicillin (AM), moxifloxacin (MX), chloramphenicol (CH) (Sigma-Aldrich, St. Lois, MO), and rifampicin (RF) (TCI, Portland, OR). Brucella blood agar (Himedia, Mumbai) supplemented with 5 µg/ml vitamin K (Sigma-Aldrich) and 1 µg/ml hemin (Sigma-Aldrich) was used as described earlier (28).

Additionally, the medium used for C. difficile isolates was supplemented with L-cysteine (1 mg/ml). After C. difficile and C. perfringens isolates were grown anaerobically on Brucella blood agar for ~24 h, colonies were suspended in 0.85% saline, and the turbidity of each suspension was adjusted to McFarland 0.5 (29). The suspensions were delivered to the supplemented Brucella blood agar plates using a sterile 48 pin replicator (3 mm in diameter) (Sigma-Aldrich) starting from the highest to lowest dilution of each antibiotic. C. difficile ATCC 700057 and Bacteroides fragilis ATCC 25285 were used as quality control microorganisms.

The inoculated plates including the control plates were incubated at 37°C for 48 h anaerobically and 2 additional control plates were incubated at 37°C aerobically to assess aerobic contaminations in the suspensions prepared. Brucella blood agar plates for TG and LN concentrations were prepared on the same day of inoculation whereas the plates for other antibiotic concentrations were prepared 1-2 days in advance. The epidemiological cut off values for antimicrobials (metronidazole R ≥ 32, vancomycin R ≥ 4, tigecyclin R ≥ 8, linezolid R ≥ 8, clindamycin R ≥ 8, moxifloxacine R ≥ 8, and chloramphenicol R ≥ 32) were used as described previously (28). Additionally, C. difficile isolates with clindamycin resistance were further screened for the presence of the corresponding gene ermB by PCR as described previously (30) (Table 3.1).
**Statistical Analysis.** Bacterial counts on different plating media were compared by converting plate counts into log numbers as log CFU/g of compost. Logistic regression of the Statistical Analysis System (JMP® Pro 12, SAS Institute Inc., Cary, NC 1989-2007) was used to reveal the correlations between the presence of *C. difficile* with the physical and microbiological factors tested. A complete model was used to determine the effect of each factor on the presence of *C. difficile* in compost/manure and stepwise approaches were used to confirm the impact of each selected factor on the whole model in each type of composts (p = 0.05).

**RESULTS**

**Physical and microbiological parameter analysis.** For the convenience of reporting, finished compost samples were categorized into 6 types: 1) cow manure-based compost, 2) poultry litter-based compost, 3) plant-based compost (plant, raw and cooked food waste), 4) mixed manure-based compost (mixed-manure, sheep, horse, and goat manure-based compost), 5) biosolid-based compost (both biosolid and municipal solid waste-based compost), and 6) other composts (mushroom, peat moss, and fish emulsion based). The moisture content (MC) of all samples ranged approximately from 7.74 to 97.98% with an overall average of 42.56%. For finished composts, the average MC was 41.60% (Table 3.2). The highest MC (97.98%) was for a composted tea product (spray), the only liquid sample analyzed in this study. Unprocessed manure had the highest average MC (56.20%) while the biosolid-based compost category had the lowest average MC (34.54%). The pH values of the majority of the samples were in a range of 3.88-9.73 with the average as 7.87. Cow manure and mixed-manure samples had pH >8.00. Average water activity (a$_w$) for all compost/manure samples was 0.967, where the range was 0.320-1.011. However, the average a$_w$ for each category was > 0.900.
Thermophilic bacteria counts in samples ranged from approximately 2.8 to 9.5 log CFU/g. The lowest average (6.29 log CFU/g) for thermophilic bacteria was observed for unprocessed manure while the highest average (7.55 log CFU/g) was observed for biosolid waste-based compost (Table 3.2). Mesophilic bacteria counts for all samples varied from 5.21 to 10.09 log CFU/g. Average mesophilic counts of > 8.00 log CFU/g were observed for all categories, except for cow and mixed manure based compost categories. Fecal coliforms were detected in 23.14% (28/121) of finished compost samples, and the highest proportion was from cow manure-based compost and the lowest was for biosolid waste-based compost. Fecal coliform level was below the detection limit (0.7 log CFU/g) for more than 70% (36/51) of C. difficile positive compost/manure samples. Approximately, 15% of C. difficile positive finished compost samples (7/47) were also positive for fecal coliforms, and only one of those (mix manure-based) reported >3 log CFU/g compost. Yeast-mold counts ranged from 4.5 to 6.00 log CFU/g for samples analyzed in all of the categories. MC (p = 0.037), thermophilic count (p = 0.035), and meso-actinomycetes count (p = 0.011) had significant effects on the presence of C. difficile in composts using the complete model. However, according to the stepwise approach, those factors were not contributing to the presence of C. difficile significantly (p > 0.05) in each type of manure/composts used in this study.

**Isolation of C. difficile and C. perfringens.** A total of 58 C. difficile isolates were obtained from 51 samples (35.61% - 47 from finished compost and 4 from manure samples). All categories of compost samples were positive for C. difficile. The highest positive rate for C. difficile was observed in biosolid waste-based compost, 59.09% and the lowest positive rate was in poultry litter-based compost, 14.28%. The positive rate for unprocessed manure samples was 19.05% (Table 3.3).
C. perfringens was isolated from 11 samples (7.75% - 9 from finished compost and 2 from manure samples). The highest C. perfringens contamination rate was observed in cow manure-based compost while the ‘other’ category was negative (Table 3.3). There were only 6 samples positive for both C. difficile and C. perfringens, i.e. 2 cow manure-based, 2 mixed manure-based, 1 poultry litter-based and one plant-based. According to the statistical analysis of results, there was not a significant correlation (p = 0.61) between the presence of C. difficile and C. perfringens in compost/manure samples.

Detection of toxigenic genes. PCR was used to distinguish the toxin types A+B+, A-B+ and A-B- of C. difficile. The majority of the isolates was toxigenic with 62.07% and 65.51% positive rates for toxin A (tcdA) and toxin B (tcdB), respectively. From 2 isolates which were positive for only tcdB, one isolate had a deleted fragment of tcdA (110 bp) while the other isolate had neither tcdA nor deleted tcdA. There were 3 isolates positive for binary toxins. One of them was the isolate negative for both tcdA and deleted tcdA and other two were positive for both toxins A and B. There were no cpe positive C. perfringens isolates in manure/compost samples.

Ribotyping. Among the 58 C. difficile isolates, 38 different ribotypes were identified by sequence-based PCR ribotyping (Tables 3.3 & 3.4). The fragment size of each isolate varied from 231 to 546 bp. The best match of the “Webribo” database was accepted when the band pattern, band sizes, and the genetic analyzer matched to the experimental conditions. Among our isolates, the most common toxigenic (A+B+) ribotype was 106 and it was isolated from all categories of compost, except for the ‘mixed compost’ and ‘other compost types’. Ribotypes 020, 412, and 251 were the next most prevalent toxigenic ribotypes. The most prevalent non-toxigenic (A-B-) ribotype was 009. There were 8 ribotypes (8/38) that could not be assigned to an internationally recognized ribotype. The same ribotype was isolated from multiple compost
samples made from different feedstocks, whereas some compost samples had more than one ribotype. From the 3 isolates positive for binary toxins, one was identified as ribotype 078, which was the isolate from biosolid waste-based compost without both \(tcdA\) and deleted \(tcdA\). Other two positive isolates for binary toxins were identified as ribotype 251 (A+B+CDT+).

**Antimicrobial Resistance (AMR).** All *C. difficile* isolates (n=58) isolates were susceptible to vancomycin (MIC\(_{90}\) = 1 \(\mu\)g/ml), metronidazole (MIC\(_{90}\) = 2 \(\mu\)g/ml), linezolid (MIC\(_{90}\) = 2 \(\mu\)g/ml), tygecycline (MIC\(_{90}\) = 4 \(\mu\)g/ml), and moxifloxacin (MIC\(_{90}\) = 4 \(\mu\)g/ml), suggesting the lack of selected or acquired resistant mechanisms. However, a reduced susceptibility was observed for clindamycin in 53.45% of *C. difficile* isolates (Table 3.5). When all the clindamycin resistant isolates were tested for the *ermB* gene, only 4 isolates had positive bands and all those had a breakpoint of 32 \(\mu\)g/ml for clindamycin as determined by the agar dilution method. Interestingly, those four isolates with reduced clindamycin susceptibility were non-toxigenic and isolated from biosolid-based (2), dairy manure (1), and poultry litter (1) based composts. All *C. perfringens* isolates were susceptible to all the selected antibiotics.

**DISCUSSION**

This study was performed to assess manure/compost as a potential carrier of the endospore forming Gram-positive bacteria, *C. difficile* and *C. perfringens*. The compost samples collected for this study represented a wide variety of feedstocks currently being used for composting in the USA. Having positive results for all categories of compost samples reveals the environmental sources in which *C. difficile* could persist. This study did not investigate how the compost was contaminated. However, the contamination could be from contaminated feedstock, soil, workers, wildlife, or post-processing of the compost.
Prevalence of *C. difficile* in compost feedstocks such as anaerobically digested sludge from waste water treatment plants, dewatered biosolids (31), and farm animal fecal matter (5) have been reported. Composition of feedstock, moisture content, and storage temperature affect the survival of many foodborne pathogens in animal manure and compost (32). In this study, no significant correlations (p > 0.05) were detected between the persistence of *C. difficile* in compost and the physical factors such as pH, moisture content, and water activity. The differences in the whole model and stepwise analysis could be due to either the natural trend that no factor in the matrix has a correlation on the presence of *C. difficile* endospores or small sample size (< 30 samples in each category of compost) for a fair statistical analysis.

According to the information acquired from the compost makers, most of the composting processes were conducted with a thermophilic phase, where the temperatures were >55°C (>131°F) for >3 days in order to comply with US Environmental Protection Agency regulations (33) or USDA organic composting guidelines (34) to reduce the levels of most pathogenic bacteria in feedstocks. The lower level of fecal coliforms in most finished compost samples of the current study implies that the treatments used in composting eliminated most Gram-negative pathogens of *Enterobacteriaceae* family. Fecal coliforms are commonly used as the indicators of fecal contaminations of environmental samples. However, in this study, the presence of fecal coliforms does not correlate with the presence of *C. difficile* suggesting the difference between fecal coliforms and the resistant *C. difficile* endospores when exposed to composting temperatures.

More heat resistant endospores of both *C. difficile* and *C. perfringens* may survive the thermophilic phase of composting and certain cooking temperatures (5,8). Theoretically, high dipicolinic acid and metal ion content, lower spore core water content, and higher thickness of
spore core and protoplast to sporoplast ratio impact the thermal resistance of endospores (8). Windrow composting was reported to reduce the majority of *C. difficile* endospores (> 99%) effectively compared to land application of dewatered biosolids (4). However, previous studies reported the abundances of *C. perfringens* in different composts (96%) (35) and *C. difficile* in swine manure compost (36%) (9). At mesophilic temperatures, *C. difficile* endospores in swine manure were reported to germinate and proliferate the population in 100 folds (9). Upon land application of contaminated compost, anaerobic environments in subsurface soil create suitable conditions for the proliferation of vegetative cells of *C. perfringens* (3636). These studies suggest that compost is a potential environment for the survival and proliferation of both *C. perfringens* and *C. difficile*.

In our study, 40% of the plant and food waste compost samples were positive for *C. difficile*. Songer et al (37) reported the isolation of *C. difficile* from raw meat samples suggesting that raw or under cooked meat as a potential source of *C. difficile* in food waste. That could be source of the pathogen in food waste-based compost samples then. Moreover, *C. difficile* was isolated from compost prepared solely of plant materials too. One of the *C. difficile* positive plant based composts was made from plant leaves collected from curbs in a municipal area, which could be possibly contaminated with the infected/carrier pet animal feces.

The major typing method for *C. difficile* used in this study was capillary gel electrophoresis based PCR ribotyping (27). Each ribotype was matched to the closest ribotype in the “Webribo” database. In some cases, there were slight differences between the experimental and reference band patterns in the database. Indra et al (27) explained that an error margin of ±4 bp existed in creating the database. Hence, the “most likely” match was accepted when the deviations were within ± 4 bp of fragments. Even the two reference strains used in the validation
were also not identified as ribotypes 060 and 251 directly by the database. The “most likely” match was accurate for those two strains too. When the database selection was “most likely”, the band pattern and DNA fragment sizes of the isolate were compared with the database match. For example, the isolate with A(?)B+CDT+ was identified as ribotype 078 and every fragment had a deviation of <0.5 bp compared to the ribotype 078 in the database. If the band pattern for the isolate was apparently different from the database match, that isolate was considered as a new ribotype. The differences in DNA fragment sizes observed in this study could be due to the use of different DNA extraction kits, PCR master mixes, genetic analyzer, column length, and polymer packed in the column, from the ribotypes reported by other researches in the database.

Toxigenic C. difficile strains harbor the main virulence factors: toxin A (tcdA), an enterotoxin and toxin B (tcdB), a cytotoxin in the pathogenicity locus (PaLoc), which is a highly stable and conserved region in the chromosome (38). Presence of toxins in C. difficile can be used to classify toxigenic strains (A+B+), only toxin B producing strains (A-B+), and non-toxigenic strains (A-B-). Previous studies have demonstrated that the diversity of C. difficile strains due to horizontal gene transfer and recombination in the PaLoc determine the variations of toxigenic genes. These variations are commonly found among A+B+ strains from the reference strain, VPI 10463 (38). In this study, 25 A+B+ C. difficile strains were observed among 38 isolates from manure/compost. Based on literature, four A-B+ ribotypes of C. difficile, 036, 017, 047, and 110 have been detected (38). In the present study, the only A-B+ strain present was ribotype 017 from a biosolid waste-based compost sample. The strain that had bands neither for tcdA nor deleted fragment of tcdA should have an altered sequence for tcdA or the primer binding site. Absence of deleted tcdA fragment is an evidence for the existence of alterations in tcdA and so its toxigenic profile can not be determined as A-B+. The third toxin in
C. difficile, binary toxins cause some cytotoxic effect in cell cultures (39) and increase the adherence of C. difficile in to intestinal cells by destructing the actin cytoskeleton (40). Binary toxins are more prevalent among the variant A+B+ strains (39). In agreement with that, 2 out of 3 isolates with binary toxin genes detected in this study were positive for A+B+, the third one positive for binary toxins was the isolate positive for tcdB and negative for both tcdA and deleted tcdA. The presence of binary toxins in non-toxigenic isolates had also been reported in the USA (39). However, no “binary toxin only” strains were identified in the present study.

Toxigenic strains of C. difficile have previously been isolated from fresh retail vegetables (both root and aboveground vegetables) and ready to eat vegetable salads (11-13). A recent study by Rodriguez-Palacios et al (12) isolated three toxigenic C. difficile strains (3/125) from aboveground grown retail vegetables. However, that study (12) selected some vegetables with some visible amount of soil, which could be the source of C. difficile. Although the prevalence is low, all studies confirmed that vegetables also carry toxigenic and antimicrobial resistant C. difficile isolates (11-13). Some ribotypes (001, 014, 017, and 078) isolated from vegetables (10-13) were the same as the ribotypes determined in the present study from compost samples. None of the studies determined the sources of C. difficile in vegetables; but these matches suggest that compost could be a potential source of contamination in fresh vegetables.

Dissemination of AMR in the application of compost contaminated with AMR strains has been suggested with the previous studies. Some phylogenetic groups of E. coli isolated from animal manure based compost have reported to carry resistant genes to multiple antibiotics (41). Although, there is a little information on the antimicrobial resistance of C. difficile in compost (9), strains resistant to multiple antibiotics have been isolated from livestock fecal samples (42). AMR is detected in laboratories using breakpoints to explain the minimal inhibitory
concentration (MIC) of an antimicrobial agent that separates wild–type bacteria from the resistant bacteria (43,44). Usually, the term “breakpoint” is used only if the data represent a therapeutic application and The European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommends use of the term “epidemiological cut off value” for non-clinical applications (43,44). However, regarding all antimicrobials tested against C. difficile, there are no epidemiological cut off values, under Clinical and laboratory standards institute (CLSI) guidelines. The majority of the isolates (31/58) reported reduced susceptibility to clindamycin, which is a risk factor for CDI (44). Clindamycin resistance in C. difficile is due to carrying *ermB* gene (30). In this study, only 4 non-toxigenic strains out of the 31 clindamycin resistant isolates from the agar dilution method were positive for *ermB* gene. The MIC of those 4 strains was recorded as 32 µg/ml and clindamycin breakpoint is 8 µg/ml by the agar dilution method (28). The same discrepancy of clindamycin resistance was reported by Dong et al. (45) by analyzing clinical C. difficile isolates for antimicrobial susceptibilities by agar dilution method and PCR detection of *ermB*. Low-level clindamycin resistance (8 –24 µg/ml) in *ermB* negative isolates have been isolated due to a point mutation in this gene (46). It is difficult to compare our results directly to the previous studies, as other studies have used different methods such as e-strips in determining the antimicrobial resistance of C. difficile isolated from the community (12,41). Due to the presence of many mobile genetic elements in the genome of C. difficile, non-toxigenic strains may become toxigenic, and antimicrobial susceptible strains may become antimicrobial resistant by horizontal gene transfer (47). Therefore, clindamycin resistance in non-toxigenic strains of C. difficile should not be overlooked.

Direct pour-plating and over-laying agar method was used to isolate C. perfringens from compost/manure samples without an enrichment or alcohol/heat shock as described previously.
Craven and Blankenship reported that only 4-6% of *C. perfringens* endospores germinated without a heat or alcohol treatment on TSC whereas the positive rate in the present study was 8% (48). However, Brinton et al (17), reported 70% positive results for *C. perfringens* in organic compost samples by direct spread plating without an alcohol/heat shock on the same agar base with different supplements (Shahidi-Ferguson perfringens cycloserine agar supplemented with egg yolk). Orsburn et al reported that *cpe*+ strains exhibited higher thermal resistance than *cpe*-strains (8). Although feedstocks are subjected to higher temperatures during the thermophilic phase of composting, none of the *C. perfringens* isolates recovered in the present study possessed the *cpe* gene. According to Immerseel et al, intestinal *C. perfringens* isolated from most of the livestock animals is *cpe*- (49). Consequently, *cpe*+ *C. perfringens* in animal manure would also be lower. In agreement with that, Gómez-Govea et al (2012) reported that 20% of retailed parsley samples, which were positive for *C. perfringens* and possibly contaminated by compost, were negative for *cpe* (50).

Brinton et al (17) revealed the presence of the indicator microorganism *E. coli* has a strong positive correlation to *C. perfringens*. However, our statistical analysis proved that there were no correlations between the presence of *C. difficile* with the presence of *C. perfringens* and the presence of both spore formers with the presence of *E. coli*. This suggests that presence or absence of the indicator microorganism *E. coli* does not represent the contaminations by *C. difficile* or *C. perfringens* in composts/manure.

LIMITATIONS

In this study, the samples were collected according to the feasibility of collection and positive responses to our requests for samples. The results reported here may not represent the
prevalence of *C. difficile* and *C. perfringens* in compost produced in the USA due to the limited number of samples examined. Moreover, the data on the seasons of the compost were processed, and the differences of batches of animal manure used in each composting process such as if animals were having some diarrheal symptoms and antibiotic usage, were not available. The limited number of samples collected from each type of compost may not represent the finished compost accurately if finished compost was not homogenous. Therefore, a larger survey of compost products is needed.

**CONCLUSION**

This study demonstrated that 36% of composts used in agro-production were contaminated with *C. difficile*. Even though the foodborne transmission of *C. difficile* has not been clearly established, compost contaminated with *C. difficile* could likely transmit the pathogen to the edible parts of the fresh produce, when it is used as a biological soil amendment. The results of this study indicate that not only unprocessed animal manure, but also properly processed finished composts are a reservoir of toxigenic *C. difficile* ribotypes such as 106, 020, 412, and 251. Our study revealed that antimicrobial resistance is rare among those *C. difficile* isolates from compost. However, clindamycin resistance may be disseminated via compost. Further studies on potential contamination of fresh produce by pathogenic *C. difficile* and *C. perfringens* via compost need to be conducted. Additionally, current regulations on composting might need to be re-evaluated regarding the survival of endospore-forming pathogens in manure/composts.
ACKNOWLEDGEMENTS

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REFERENCES


Table 3.1 PCR primers used in this study.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’-3’)</th>
<th>Product size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>tpi</td>
<td>5’-AAAGAAGCTACTAAGGGTGACAAA-3’ 5’-CATATAATGCTCTATCCTAC-3’</td>
<td>230</td>
<td>(22)</td>
</tr>
<tr>
<td>tcdA</td>
<td>5’-GATTCCCTATATTTACATGACAATAT-3’ 5’-GTATCAGGCAATCA AGATAATATACCTTT-3’</td>
<td>369 (undeleted)</td>
<td>(22)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>110 (deleted)</td>
<td></td>
</tr>
<tr>
<td>tcdB</td>
<td>5’-GGAAAAAGAATGTTTTATTA-3’ 5’-ATCTTTAGTTATAA CTTTGACATCTTT-3’</td>
<td>160</td>
<td>(22)</td>
</tr>
<tr>
<td>cdtA</td>
<td>5’-TGAACCTGGAAAAGGTGATG-3’ 5’-AGGATTATTTACTGGACCATTG-3’</td>
<td>376</td>
<td>(24)</td>
</tr>
<tr>
<td>cdtB</td>
<td>5’-CTTAATGCAAGTAAATACTGAG-3’ 5’-AACGGATCTCTTGCATGTC-3’</td>
<td>510</td>
<td>(24)</td>
</tr>
<tr>
<td>Ribotyping</td>
<td>16S 5’-FAM-GTGCCTGGATCACCCTCC-3’ 23S 5’-CCCTGCAACCTTTAATAACTTGACC-3’</td>
<td>Variable</td>
<td>(26)</td>
</tr>
<tr>
<td>ermB</td>
<td>2980 5’-AATAAGTACACACGTAAGGTTG-3’ 2981 5’-GCTCCTGGAAGCTGTCAGTAG-3’</td>
<td>688</td>
<td>(30)</td>
</tr>
<tr>
<td>16S rRNA</td>
<td>5’-AAAGATGGCATCATCATTCAAC-3’ 5’-TACCGTCATTATCTTCACCAAA-3’</td>
<td>279</td>
<td>(23)</td>
</tr>
<tr>
<td>cpe</td>
<td>5’-TGTTAATACCTTTAAGGATATGTATCC-3’ 5’-TCCATCACCCTAAGGACTG-3’</td>
<td>935</td>
<td>(25)</td>
</tr>
</tbody>
</table>
Table 3.2 Physical and microbiological property analysis of compost/manure samples.

<table>
<thead>
<tr>
<th>Source of compost/ manure</th>
<th>n</th>
<th>Moisture content (%) avg. ± SD</th>
<th>pH avg. ± SD</th>
<th>Water activity avg. ± SD</th>
<th>Mesophilies (Median/avg. log CFU/g)</th>
<th>Thermophiles (Median/avg. log CFU/g)</th>
<th>Yeast and molds (Median/avg. log CFU/g)</th>
<th>Fecal coliforms (%a/Median/avg. log CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow manure based</td>
<td>20</td>
<td>44.14 ± 21.66</td>
<td>8.27 ± 0.74</td>
<td>0.977 ± 0.450</td>
<td>7.91/7.86</td>
<td>6.81/6.70</td>
<td>4.68/4.28</td>
<td>40/2.83/2.37</td>
</tr>
<tr>
<td>Poultry litter based</td>
<td>20</td>
<td>40.79 ± 26.30</td>
<td>7.42 ± 3.42</td>
<td>0.929 ± 0.421</td>
<td>8.14/8.13</td>
<td>6.81/6.61</td>
<td>4.79/4.88</td>
<td>17.86/2.52/2.82</td>
</tr>
<tr>
<td>Plant based</td>
<td>25</td>
<td>40.91 ± 16.90</td>
<td>7.96 ± 0.88</td>
<td>0.981 ± 0.038</td>
<td>7.99/8.03</td>
<td>7.32/7.32</td>
<td>4.73/4.79</td>
<td>12/2.14/2.32</td>
</tr>
<tr>
<td>Mix manure based</td>
<td>26</td>
<td>43.91 ± 13.91</td>
<td>8.20 ± 0.74</td>
<td>0.985 ± 0.020</td>
<td>8.06/7.76</td>
<td>7.17/7.07</td>
<td>5.25/4.85</td>
<td>34.62/2.68/3.04</td>
</tr>
<tr>
<td>Biosolid based</td>
<td>22</td>
<td>34.54 ± 11.05</td>
<td>7.64 ± 0.97</td>
<td>0.940 ± 0.146</td>
<td>8.12/8.39</td>
<td>7.65/7.55</td>
<td>4.59/4.52</td>
<td>4.54/1.46/1.46</td>
</tr>
<tr>
<td>Other composts</td>
<td>8</td>
<td>52.18 ± 16.54</td>
<td>7.14 ± 2.19</td>
<td>0.988 ± 0.027</td>
<td>8.08/8.06</td>
<td>6.83/7.08</td>
<td>5.71/5.46</td>
<td>12.5/1.78/1.78</td>
</tr>
<tr>
<td>Unprocessed manure</td>
<td>21</td>
<td>56.20 ± 31.94</td>
<td>7.87 ± 3.70</td>
<td>0.964 ± 0.495</td>
<td>8.86/8.63</td>
<td>6.06/6.29</td>
<td>5.94/4.88</td>
<td>47.62/5.86/2.58</td>
</tr>
</tbody>
</table>

*aPercent positive among samples tested in each category.
Table 3.3 Summary of the distribution of *C. difficile* ribotypes and *C. perfringens* among different feedstock of compost/manure samples.

<table>
<thead>
<tr>
<th>Manure/Compost type</th>
<th># of samples tested</th>
<th><em>C. difficile</em> positive samples (% positive)a</th>
<th><em>C. difficile</em> ribotypes</th>
<th><em>C. perfringens</em> positive samples (% positive)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cow manure based</td>
<td>20</td>
<td>8 (40.00)</td>
<td>001, 012, 033, 075, 106, 596, 705, PR 13474</td>
<td>3 (15.00)</td>
</tr>
<tr>
<td>Poultry litter based</td>
<td>20</td>
<td>4 (20.00)</td>
<td>009, 039/2, 106, PR 13476</td>
<td>1 (5.00)</td>
</tr>
<tr>
<td>Plant based</td>
<td>25</td>
<td>7 (28.00)</td>
<td>014/0, 034, 039/2, 449, 106, PR11498, PR11692</td>
<td>1 (4.00)</td>
</tr>
<tr>
<td>Mixed manure based</td>
<td>26</td>
<td>12 (36.36)</td>
<td>009, 010, 020, 039/2, 081, 251, 412, 652</td>
<td>1 (3.85)</td>
</tr>
<tr>
<td>Biosolid based</td>
<td>22</td>
<td>13 (59.09)</td>
<td>009, 010, 017,020, 039/02, 078,203, 235, 251, 404, 412, 106, 684, AI 8/0,AI 82/1, AI/83, PR 13475</td>
<td>3 (13.64)</td>
</tr>
<tr>
<td>Other composts</td>
<td>8</td>
<td>3 (37.5)</td>
<td>449, 593, PR11665</td>
<td>NDb</td>
</tr>
<tr>
<td>Unprocessed manure</td>
<td>21</td>
<td>4 (19.05)</td>
<td>115, 412, 446, 705</td>
<td>2 (9.52)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>142</strong></td>
<td><strong>51 (35.92)</strong></td>
<td></td>
<td><strong>11 (7.75)</strong></td>
</tr>
</tbody>
</table>

*a* Percent positive among samples tested in each category.

*b* Not detected
Table 3.4 Analysis of ribotypes and toxigenic genes of *C. difficile* isolated from compost/manure samples.

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>tcdA</th>
<th>tcdB</th>
<th>cdtA</th>
<th>cdtB</th>
<th>Ribotype</th>
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<tr>
<td>ATCC 43593</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>PR11586</td>
</tr>
<tr>
<td>ATCC BAA-2155</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>PR11587</td>
</tr>
<tr>
<td>CPS 5</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>PR11676</td>
</tr>
<tr>
<td>CPS 7</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>PR11677</td>
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<tr>
<td>CPS 10</td>
<td>+</td>
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<tr>
<td>CPS 15</td>
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<td>+</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td>CPS 16</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>PR 11493</td>
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<tr>
<td>CPS 23</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>PR11588</td>
</tr>
<tr>
<td>CD 6</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>PR11653</td>
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<tr>
<td>CD 13</td>
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<td>+</td>
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<td>-</td>
<td>PR11688</td>
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<td>CD 22</td>
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<td>PR11689</td>
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<tr>
<td>CD 24</td>
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† Ribotypes that start with PR was the ID given by WEBRIBO database. The identified number in the parenthesis is the accepted best match given by the same data base. When the band pattern was the same and the differences of fragment sizes were ± 4 bp, the ‘most likely’ ribotype was accepted. Otherwise, it was considered as a new ribotype.

– Negative for toxin

+ Positive for toxin

*Presence of tcdA or deleted tcdA was not observed
Table 3.5 Analysis of antimicrobial susceptibilities of *C. difficile* isolated from compost/manure samples by agar dilution method.

<table>
<thead>
<tr>
<th>Test bacteria</th>
<th>Antimicrobials</th>
<th>Break point (µg/ml)a</th>
<th>MIC rangeb (µg/ml)</th>
<th>MIC50 (µg/ml)c</th>
<th>MIC90 (µg/ml)c</th>
<th>Resistance (%)</th>
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<tr>
<td><em>C. difficile</em></td>
<td>Metronidazole</td>
<td>≥32*</td>
<td>≤0.125-64</td>
<td>&lt;0.125</td>
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<td></td>
<td>Moxifloxacin</td>
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<td>Clindamycin</td>
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<td>Tygecycline</td>
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<td>≤0.125-16</td>
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<td><em>C. perfringens</em></td>
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<td>≤0.125-64</td>
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<td></td>
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<td>&lt;0.125</td>
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*aAntimicrobial resistance breakpoints: CLSI, 2012; Miller et al, 2011; Rodriguez-Palacios et al, 2014*

*bTested concentration range of antibiotics

*cMIC50 and MIC90 are the concentrations of antibiotics at which the 50 and 90% of the isolates were inhibited respectively.

*dNot detected
Figure 3.1 Flow chart for the isolation procedures for *C. difficile* and *C. perfringens* from compost

1. **Manure/compost (n=142)**
   - 10 g
   - 90 ml of saline

2. **Selected dilutions were pour-plated using TSC supplemented with 2.5% oxyrase with agar overlay method for the isolation of *C. perfringens***

3. **Incubated aerobically for 24 h at 37°C**

4. **Tested presumptive black colonies with a white halo embedded in agar layer by PCR (16s rRNA)**

5. **cpe toxin detection (PCR), and Antimicrobial resistance (Agar dilution method)**

6. **20 ml of BHB-YE-CYS-MN-T broth**
   - 5 g

7. **Incubated anaerobically for 7 days at 37°C for the isolation of *C. difficile***

8. **Heat-shocked at 60°C for 25 min and centrifuged for 10 min at x 4,000 g**

9. **Streaked the pellet on CDA-CYS-H-MN-T and incubated anaerobically for 48 h at 37°C**

10. **Tested suspected isolates for PRO disk, *tpi* (PCR), Latex agglutination**

11. **PCR-Ribotyping, Toxin detection (PCR), and Antimicrobial resistance (Agar dilution method)**
CHAPTER 4

THERMAL RESISTANCE OF CLOSTRIDIUM DIFFICILE ENDOSPORES ON WET AND DRY HEAT EXPOSURE IN DAIRY COMPOST

ABSTRACT

Thermal responses of Clostridium difficile endospores in finished dairy compost were compared at 55 and 65°C under wet and dry heat conditions. A three-strain cocktail of C. difficile endospores was inoculated into dairy compost to a final concentration of ca. 3×10⁵ CFU/g and the moisture content (MC) of the compost was adjusted to be 20, 30, and 40%. For the wet heat treatment, the inoculated compost samples were placed in a tray submerged in a water bath whereas for the dry heat treatment, the compost samples were placed in an environmental chamber. The come-up times for wet heat treatments were dependent on the targeted temperature and the MC of compost, but for the dry heat treatment, the come-up time was programmed to be 2 days by ramping the temperature from room to target temperatures (55 or 65°C) to simulate the early phase of composting. The thickness of endospore cortex and coat were measured using transmission electron microscope. The MCs of compost were maintained well throughout the wet heat treatment while the dry heat treatment reduced the MC of compost to < 10% by the end of come-up time. Endospore counts were reduced by 0.2-0.6 log CFU/g at both treatments during the come-up time of dry heat treatment, which was 2 days and the reduction was 0.01-0.70 log CFU/g at both temperatures of wet heat treatment, where the come-up time was < 2 h. During the holding time, endospore counts were reduced slightly by < 0.5 log CFU/g at 55 and 65°C of dry heat treatment, whereas 0.7-0.8 and 0.6-3.04 log CFU/g reductions were observed at 55 and 65°C in wet heat treatment, respectively. However, no temperature of at either heat treatment eliminated the endospore counts to undetectable level (5 CFU/g) at
selected MC condition in this study. Having the thickest cortex and coat, ribotype 596 endospores survived well after heat treatments at the selected temperature-MC levels. Our results demonstrated that the extrinsic factors such as temperature of the thermophilic phase and MC of feedstock materials and intrinsic factors such as the thickness of endospore cortex and coat have significant (p < 0.05) effects on the thermal resistance of \textit{C. difficile} endospores during composting.

\textbf{INTRODUCTION}

\textit{Clostridium difficile} is an endospore-forming bacterium that causes a cascade of symptoms varied from mild diarrhea to life-threatening toxic megacolon (Arroyo \textit{et al.} 2005). Community associated \textit{C. difficile} infection cases are increasing as compared to the last decade, and food and animals have been identified as sources of this pathogen (Gupta and Khanna, 2014). The pathogen is carried by both humans and animals, especially, the livestock animals such as cattle (Thitaram \textit{et al.}, 2011; Bandelj \textit{et al.}, 2016), poultry (Zidaric \textit{et al.}, 2008), and swine (Thitaram \textit{et al.}, 2011) suggesting that livestock manure is a source of pathogenic \textit{C. difficile}.

Composting is an effective way of treating the livestock manure into a stable and microbiologically safe organic fertilizer. As required by the Food Safety Modernization Act (FSMA), FDA established standards to improve the safety of produce for human consumption at pre- and post-harvest conditions by emphasizing the microbial safety of biological soil amendments such as stabilized compost used in growing produce (US FDA, 2015). Although there is a cost associated with composting process, using composted animal manure is always safer on human health as compared with using unprocessed animal manure as a fertilizer.
Conventional farming in the USA uses ca. $2.5 billion excess cost for fertilizer and the excessive use of fertilizer causes soil erosion, which creates more public and environmental health issues (Pimentel et al., 2005). Therefore organic farming using treated animal manure is encouraged to protect the public and environmental health as it produces the same crop yield with less energy input, improved soil organic matter, and higher biodiversity as compared with the conventional farming (Pimentel et al., 2005).

The levels of most human pathogens *Listeria* spp. (Singh et al., 2010), *Escherichia coli* O157:H7 (Shepherd et al., 2011; Singh et al., 2010), *Salmonella* (Singh et al., 2010), *Giardia*, *Cryptosporidium* (Van Herk et al., 2004), avian influenza and Newcastle disease viruses (Guan et al., 2009), can be reduced to lower levels in the composting process. However, the fate of composting on the endospores is different from those of vegetative cells of bacteria. According to Hassen et al. (2001), the starting endospore counts of *Bacillus* in composting feedstock (ca. 7-8 log CFU/g) were reduced to ca. >4 log CFU/g in the finished municipal solid waste compost. Windrow composting of biosolid waste resulted in a 3.4 log reduction of *C. difficile* endospores (Xu et al., 2016) in finished compost within 10 months. Our recent study has confirmed that both unprocessed animal waste and finished compost contain *C. difficile* with a prevalence of 36% (Dharmasena and Jiang, 2018). Limited studies on the fate of *C. difficile* endospores in composting have emphasized the necessity of an extended duration of thermophilic treatment to reduce the population of *C. difficile* endospores (Xu et al., 2016).

Although, the temperature is the main factor that kills foodborne pathogens during composting, clumping of solids, uneven temperature distribution due to incomplete mixing, moisture content, wind, solar radiation, and poor process design contribute to the pathogen survival in large-scale composting operations (Wichuk and McCartney, 2007). Therefore, it is
important to know more about the inactivation of emerging pathogen *C. difficile* endospore as affected by the physical properties of composting feedstock such as the moisture content (MC). During composting, the initial MC of the compost mixture is ca. 50-60% and in finished product, it is around 20-30% (Singh et al., 2011). MC is a factor that determines the temperature tolerance of plant pathogenic microorganisms in composting feedstock (Noble and Roberts, 2004). In this study, we simulated the early stages of composting by raising the temperatures of compost with 20, 30, and 40% MCs to thermophilic range using dry and wet heat treatments to observe the effect of the MC and temperature on the reduction of *C. difficile* endospores during composting.

**MATERIALS AND METHODS**

**Compost preparation.** Commercial dairy compost was purchased directly from the farm (Wallace Farm Soil Product Inc., Huntersville, NC). According to the label, the compost samples contained 0.5% total nitrogen, 0.5% available phosphate, 0.5% soluble potash, and no more than 1% chlorine. The compost was air-dried under the fume hood to reduce the moisture content to < 10% and then sieved (sieve pore size, 3×3 mm) to reduce matrix heterogeneity and stored in sealed containers at 4°C until used.

**Endospore preparation.** Three non-toxigenic *C. difficile* strains; ATCC 43593 (ribotype 060) and two strains (RT 033 and RT 596) isolated from 2 dairy compost samples in our previous study (Dharmasena and Jiang, 2018), were used for the endospore preparation. The strains were maintained in brain heart infusion broth (Becton & Dickinson, Sparks, MD) with 20% glycerol at -80°C. Above 3 *C. difficile* strains were grown separately on brain heart infusion agar supplemented with 0.5% yeast extract (Hardy Diagnostics, Santa Maria, CA), 0.01% of L-cysteine (Alfa Aeser, Ward Hill, MA), and 0.01% sodium taurocholate (Alfa Aeser) (BHIA-YE-
CYS-T agar) anaerobically for 7 days at 37°C and then aerobically at room temperature for another 7 days. Endospores were harvested by washing the surfaces of agar with 0.01 M phosphate buffered saline (PBS) containing 0.1% tween 80. One-milliliter aliquots of each endospore suspension were serially diluted in 0.85% saline followed by heat-shock at 60°C for 25 min, plated on BHIA-YE-CYS-T agar, and incubated anaerobically at 37°C for 24 h to estimate the initial endospore titer. Each suspension was adjusted to be ca. 3×10^6 spores/ml based on viable counts and stored at 4°C until used. Equal volumes of each *C. difficile* strain were mixed and prepared the cocktail for the following experiments with each experiment repeated trice.

**Inoculation of dairy compost.** Two hundred and fifty grams of compost were inoculated with the cocktail of *C. difficile* endospore suspension at a final concentration of ca. 3×10^5 CFU/g (1:10, vol/wt). The inoculated compost was adjusted to moisture contents (MC) of 20, 30, and 40% with sterile tap water while hand mixing for 5 min by wearing sterile gloves. Approximately 50-g portions of inoculated compost were placed in sterile Tyvek® pouches (size, 3.5”×9.0”; SPS Medical, Rush, NY) as a thin layer (ca. 0.5-0.6 cm depth).

**Heat treatment of compost.** Two heat treatments, wet heat and dry heat, were applied to the inoculated compost as presented in Figure 4.1. For dry heat treatment, the compost samples in enclosed pouches were randomly arranged in a single layer on the shelf of an environmental chamber (Thermo Scientific, Barnstead International, Dubuque, IA). For the wet heat treatment, compost samples in open Tyvek pouches were inserted into a gallon size zip-lock bag. Each zip-lock bag contained 3 pouches, one pouch from each trial. The arrangement of 3 pouches inside the zip-lock bag was random. All zip-lock bags were placed in a metal tray (13”×9” × 2”), which was immersed in the water bath (Haake, Paramus, NJ) with the lid on.
For both treatments, the target temperatures were 55 and 65°C, which were monitored constantly using the type-T thermocouples (DCC Corporation, NJ, USA) with two cords inserted inside the Tyvek pouches, and another kept inside the chamber or water bath, respectively, for dry and wet heat treatments. For wet heat treatment, the cords were inserted into the bottom and top pouches. For dry heat treatment the temperature rise of the environmental chamber was programmed to ramp stepwise from room temperature (ca. 25.5°C) to the target temperature (55 or 65°C) of compost in 2 days and then that temperature was maintained at the target temperature for 3 days. In the wet heat treatment, the temperature of the water bath was initially set at 5°C higher than the target temperature to reduce the come-up time and the temperature of the water bath was then decreased gradually to maintain the targeted temperature. The final temperatures of the water bath and environmental chamber were set as 0.5°C higher than the target temperature. Sample bags were withdrawn from the chamber at 24 h time intervals for 5 days and from the water bath, at the come-up time and then at 24 h intervals for 3 days. Withdrawn samples were immediately placed in to an ice-water bath to prevent further thermal inactivation.

**Physical and other microbiological analysis.** The MC of compost was measured on each sampling time using a moisture analyzer (model IR-35, Denver Instrument, Göttingen, Germany). For the assessment of indigenous microflora, 5 g of compost were homogenized with 45 ml of 0.85% saline in a stand-up bag at 230 rpm for 1 min using a stomacher (400 circulator, Seward Ltd., West Sussex, UK). Selected serial dilutions were plated on tripticase soy agar (TSA - Becton & Dickinson, Sparks, MD), and incubated at 37°C for 24 h for mesophilic bacteria enumeration.
Enumeration of thermal resistant *C. difficile* endospores. Five grams of compost were homogenized and 1 ml aliquots of the homogenate (10⁻¹) were heat-shocked as described above. Selected dilutions were plated on BHIA-YE-CYS-T agar supplemented with cycloserine (500 mg/l) and cefoxitin (16 mg/l) (Himedia, Mumbai, India) (BHIA-YE-CYS-T-CC agar) and incubated anaerobically at 37°C for 24 h.

Identification of most heat-resistant *C. difficile* ribotypes. A total of 107 isolates were collected from the plates of samples being held for 3 days at the target temperatures. There were 9 randomly selected colonies per temperature-MC-heat treatment except for 65°C-20% MC of dry heat treatment (n=8). The pure cultures were ribotyped using the primers and PCR conditions as described previously (Bidet et al., 1999). The ribotype of the selected isolates were compared with the ribotype of three *C. difficile* strains used in this study.

Transmission electron microscopy. The ultrastructures of *C. difficile* endospores of three ribotypes were observed using transmission electron microscope (TEM) as described by Lawly et al. (2009) with some modifications. Briefly, pelleted endospores of 3 *C. difficile* ribotypes were resuspended in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at room temperature (20°C) for 10 min before being transferred to an ice bath for 2 h. Next, the endospores were pelleted by spinning (3,000 rpm) and rinsed three times for 10 min in 500 µl of sodium cacodylate buffer (pH 7.4). Endospores were then fixed secondarily with 1% osmium tetroxide in sodium cacodylate buffer at room temperature for 1 h, then rinsed three times in cacodylate buffer over 30 min, and mordanted with 1% tannic acid for 30 min, followed by a rinse with distilled water for 10 min. The samples were dehydrated through an ethanol series of 20%, 30% (staining en bloc with 2% uranyl acetate at this stage), 50%, 70%, 90%, and 95% for 20 min at each step. Finally, the endospores were washed with 100% ethanol for 3 times, for 20
min each time. All the steps were performed on a nurator (Thermo Fisher Scientific, Hampton, NH), which was used to aid infiltration of the spore coat at room temperature. Ethanol was exchanged in 1:1 LR white resin (Polysciences, Inc. Warrington, PA): 100% ethanol for 1 h at room temperature and then transferred to 4°C overnight. In the following day, the endospores of each ribotype were embedded with undiluted LR white resin in embedding capsules and were cured in an oven at 60°C for 24 h. Sections (thickness, ca. 40 nm) were cut on a ultramicrotome (Model. Ultracut E, Leica, Microscope Central, Feasterville, PA) and imaged on a 120-kV H7600 transmission electron microscope (Hitachi, Tokyo) using a charge-coupled device camera (Advanced Microscopy Techniques Corp., Woburn, MA).

**Modeling of endospore survival curves.** The survival curves of *C. difficile* endospores during the holding time were modeled using linear regression model as:

\[
\log_{10} \beta_0 = -\mu t + \log_{10} \beta_1
\]

where, \(\beta_0\) is the log count of surviving *C. difficile* endospores at the limit of detection (LOD) at time \(t\), \(\mu\) is the inactivation rate of endospores under the given treatment, \(\beta_1\) is the estimated initial log count of endospores fit by the regression model, and \(t\) is the time taken to reach the LOD for a given treatment. Separate models were used for each trial-MC-heat treatment-temperature. The estimated model parameters were used to calculate the time, \(t\) (days) to reach the LOD at each heat treatment-temperature-MC. Regression coefficient (R²) were calculated to assess the goodness-of-fit of the model.

**Statistical analysis:** The experiments were conducted in triplicate at each temperature-MC combination. Plate count data were converted to log values on dry weight base of compost and mean log counts were compared among MC and targeted temperature at each sampling time with an analysis of variance (ANOVA). Additionally, the time to reach LOD, initial log count of
endospores, and inactivation rate were compared among each treatment used in the experiment with an ANOVA. All ANOVA comparisons were followed by Student’s t-test least square differences (LSD) procedure and calculations were performed using JMP® Pro 12 (SAS Institute Inc., Cary, NC, 1989-2007). Any p ≥ 0.05 was considered evidence of statistically significant.

RESULTS

**Thermal treatment set-up.** Finished dairy compost used in this study was confirmed to be free of *C. difficile* following the enrichment methods as described previously (Dharmasena and Jiang, 2018). The thermal responses of 3 strains of *C. difficile* endospores were measured at the minimum recommended temperature (55°C) for compost processing and a selected higher temperature (65°C) which may be reached during the thermophilic phase of composting. For dry heat treatment, the come-up time of the environmental chamber was programmed to be 2 days, and for the wet heat treatment, the come-up times were dependent on the target temperature and the MCs of compost. At 65°C, the come-up times were approximately 25, 48, 60 min for compost samples with 40, 30, and 20% MCs, as compared with approximately 30, 60, and 75 min for compost samples with 40, 30, and 20% MC at 55°C, respectively.

**Moisture changes in compost during the thermal treatments.** Upon exposure to dry heat in the environmental chamber for 2 days, the recorded MCs of dairy compost were reduced to 5-7% regardless of the target temperature (55 or 65°C) and initial MC used in the experiment (Figure 4.2, A and B). During the 3-day holding time, slight differences were observed for compost samples in all three MCs at 55°C, but no difference at 65°C of dry heat treatment.
Compost exposed to wet heat treatment kept the initial MC approximately constant throughout the entire experiment (Figure 4.2, C and D). However, there were some significant (p < 0.05) fluctuations from each initial MC values on some sampling days. For example, at 55°C, the dairy compost samples with 20 and 30% MCs were approximately 1% higher than the initial MC, which resulted in significant change in MC (p < 0.05) on day 3. On the contrary, for compost with 40%, the MCs were significantly lower (p < 0.05) than the initial MC. At 65°C of wet heat treatment, the MC in each sample was not changed significantly (p > 0.05) from the initial MC.

**Change of background microflora during the simulated thermophilic phase.** The initial counts of indigenous mesophilic microflora were enumerated for the compost adjusted with MC, just before heat treatment. The initial counts of composts prepared for heat treatments were between 8.0-9.5 log CFU/g (Figure 4.3, A, B, C, & D). At 55°C of the dry heat treatment, indigenous microflora significantly (p < 0.05) increased in compost samples with 20, and 30% MCs and decreased in compost with 40% of dry heat treatment. At 65°C, the indigenous microflora increased in 1.7 and 1.2 logs during the come-up time in compost samples with 30 and 40% MCs, respectively, and decreased in 0.5 log in compost samples with 20% MC.

During the wet heat treatment at 55°C, indigenous microflora was increased in 0.48, 1.62 and 2.59 logs, respectively, in composts with 20, 30, and 40% MC by the end of the holding time. At 65°C of the wet heat treatment, the initial counts of the indigenous microflora in compost samples with 20, 30, and 40% MCs, increased significantly (p < 0.05), not changed significantly (p > 0.05), and decreased significantly (p < 0.05), respectively, by the end of the 3-day holding.
Effect of temperature and MC on the survival of *C. difficile* endospores - dry heat treatment. The log reductions of *C. difficile* endospores treated by dry heat treatment at 55 and 65°C are presented in Table 4.1. At 55°C, during the 2-day come-up time, there were 0.26-0.54 log reductions of endospore counts, but no further significant (p<0.05) reductions of endospore counts were observed during the holding time regardless the changes of MCs. At 65°C, 0.21-0.61 log reduction was observed during the come up time with <0.5 log reduction further at all MCs during the holding time. Although, the log reductions during the come-up time increased with the increasing MC, none of them were significant (p>0.05). The level of temperature was not a significant (p>0.05) factor on the log reduction of *C. difficile* endospores until the day 3 of holding time. The effect of MC was significant (P<0.05) on the log reductions of day 1 in compost with 30% MC.

Effect of temperature and MC on the survival of *C. difficile* endospores - wet heat treatment. The log reductions of *C. difficile* endospores treated by wet heat treatment at 55 and 65°C are presented in Table 4.2. At 55°C, 0.01-0.29 log reduction of *C. difficile* endospores occurred during the come-up time, and additional 0.74-0.80 log reduction was observed further during the 3-day holding time at all MCs. At 65°C, 0.18-0.69 log reduction was observed during the come-up time, with additional 0.47-3.16 log reductions occurred during the hold time. Only temperature had a significant (p<0.05) effect on the reduction of *C. difficile* endospores during the come-up time, and both temperature and MC had significant (p<0.05) effects during the holding time. Although, the highest log reduction was observed in compost with 30% MC at 55°C during the holding time, the counts were not significant (p>0.05) among different MCs. There was a trend of increasing log reduction with the increasing MC level was observed at
65°C, and the reductions of \textit{C. difficile} endospores at MC level were significantly (p<0.05) different from each other.

**Modeling of endospore survival curves.** The survival curves of \textit{C. difficile} endospores during the holding time were simulated by linear regression to compute the time to reach the detection limit at each treatment (Table 4.3). \textit{C. difficile} endospore exposed to the dry heat treatment had ascending counts for some treatments at 55°C (20 and 30% MCs). The inactivation rates of endospores were not significant (p>0.05) at all temperature-MC combinations at 55°C for both heat treatments. At 65°C, compost samples with 40% MC of both heat treatments had significant (p<0.05) inactivation rates of endospores. Both the temperature of composting and the MC of compost were significant (p<0.05) in the inactivation of \textit{C. difficile} endospores during both heat treatments. The R^2 values were acceptable only for the inactivation rates at 65°C-40% MC for both heat treatments.

**Identification of most heat-resistant \textit{C. difficile} ribotypes.** A total of 107 isolates were collected for PCR ribotyping. Majority of the isolates after dry heat treatment were ribotype 596 (n=49) and 3 isolates from ribotype 033 and 2 isolates from ribotype 060 were isolated additionally (Table 4.4). There were 44 isolates of ribotype 596, 8 isolates of ribotype 060, and 1 isolate of ribotype 033 among the randomly selected isolates after wet heat treatment.

**Transmission electron microscopy analysis.** TEM analysis reveals the typical structure of bacterial endospore including coat, cortex, and core. The protein-rich endospore coat was more electron dense (Figure 4.4). Laminating the endospore core, a putative inner membrane was
observed in *C. difficile* ribotypes 596 and 033. The thickness of average endospore coat was highest in ribotype 596 (109.0±27.0 nm), but it was not significantly (P>0.05) different from the endospore average coat thickness of ribotype 060 (89.6±24.6 nm). Ribotype 033 had the least endospore coat thickness (77.3±18.1 nm). The cortex thickness of endospore was also significantly (p<0.05) higher in ribotype 596 (83.0 ± 51.4 nm) as compared to the ribotypes 060 (71.9±31.8 nm), and 033 (47.9±27.3 nm), which were not significantly (p>0.05) different from each other. The outermost layer of *C. difficile* endospores, exosporium, was not observed in our strains.

**DISCUSSION**

Microbial safety of manure-based compost is largely dependent on the effectiveness of thermophilic phase of composting, which inactivates majority of the pathogens. During the thermophilic phase of composting, the temperature could rise to ca. 55 to 72°C and the elevated temperatures should be maintained at least for three consecutive days in aerated static piles or in-vessel systems, or 15 days with five turnings in windrow composting to inactivate the pathogens in biosolids (USEPA, 1999). The temperature elevation is important to denature the proteins of pathogens including enzymes, and finally inactivate most pathogens from the composting feedstock (USEPA, 1999; Bernal et al., 2009). In this study, with two types of heat treatments, wet and dry, an attempt was taken to evaluate the fate of *C. difficile* endospores during composting in a laboratory set-up. Two heat treatments represent the heterogeneous conditions of composting piles. The wet heat treatment was used to maintain the MC, within the levels recommended for finished compost and dry heat was used to represent the surface conditions or dry spots inside the composting pile.
According to our observations, the transfer of temperatures took a longer time when the
MC of compost was lower at both temperatures of wet heat treatment. At lower MCs of compost,
there are more void spaces. Huet et al. (2012) reported that void spaces reduce the thermal
conductivity in compost heaps. By increasing the bulk density of compost, the temperature
transfer can be accelerated in composting feedstocks. However, there are controversial reports
regarding temperature transfer through compost. Abu-Hamdeh and Reeder (2000), agreeing with
our observation, reported that the thermal conductivity was increased with the increased MC in
different soil types, amended with peat moss. On the contrary, Chen et al. (2015) reported that
the change of temperature transfer was higher when the MC of the matrix (chicken litter) was
lower. In this study, dairy compost was tested. Therefore, the composition of feedstock materials
and physical characteristics of the matrix might affect the temperature transferring inside the
composting materials during the come-up time.

A limited number of studies reported the resistance of endospore-forming bacteria such
as *Bacillus* and *Clostridia* in composting. Krogstadt and Gudding (1975) artificially inoculated
5-6 logs of *Bacillus cereus* to compost with initial MC as 60% and detected the inoculated
endospores by direct plating, after 7 days at 60-65°C of composting, whereas no endospores
were detected after 2 days at 70°C. Our study used approximately the same level of initial
inoculum, and we observed a maximum of ca. 3.0 log CFU/g reduction of *C. difficile* endospores
in compost with 40% MC after the 3-day treatment 65°C. The projected survival time to reach
the LOD during the holding period for this treatment was 4.2 days (Table 4.3). However, in a
field study, 47 days were needed to reduce the endospore level of *C. difficile* from 3.7 to 2.9 log
CFU/g in the thermophilic phase of windrow composting of biosolid waste, where the initial MC
of the feedstock mixture was 40-55% and temperatures during thermophilic phase were between
At the end of ca.10 month curing period (with temperatures > 55°C), *C. difficile* endospore count was 0.3 log CFU/g. Therefore, it is difficult to compare the times required for the inactivation of endospores under the laboratory conditions and field. Cumulatively, all studies confirm the necessity of longer duration of thermal treatment in inactivating endospores of pathogenic bacteria.

The temperatures generated inside the composting pile is not evenly distributed in static pile composting. According to Shepherd et al (2007), the MC of composting pile surface was <10% within 24 h, which is similar to the MC of the dry heat treatment of this experiment. Dry spots can occur even inside the composting piles, and inactivation of plant pathogens in the dry pockets has been found to be low despite of the thermophilic temperatures recorded (Noble and Roberts, 2004). As simulated in the dry heat treatment of our study, the dry spots in compost pile contaminated with *C. difficile* endospores will be less affected even at higher temperatures, which can be explained by the osmoregulation feature of endospore cortex as suggested by Gould and Dring (1975). Osmoregulation expands the less cross-linked peptidoglycan in cortex by compressing the endospore core. Multivalent cations in the outside matrix enter the endospore cortex via endospore coat and react with electronegative peptidoglycan in the cortex by changing its existing osmoregulatory properties. In the wet heat treatment, the multivalent cations in dairy compost may be dissolved in available water and enter the endospore cortex and then induce those subsequent changes. When applying dry heat or if the matrix is dry, multivalent cations may not enter the endospore cortex due to less available water. Consequently, endospore may maintain its internal structure better in dry conditions.

The endospores withstand the environmental stresses (nutrient exhaustion, high temperature, oxygen, disinfectants, etc.) better than the vegetative cells due to the presence of
some structural differences such as membranes, cortex and coat, and production of endospore specific dipicolinic acid (Leggette et al, 2012; Wells-Bennik et al., 2016). Orsburn et al. (2008) demonstrated that the thickness of endospore cortex is related with the heat resistance of C. perfringens, and Wells-Bennik et al. (2016) reported that the cortex is a requirement for the protection of endospores from the wet heat. The reason behind could be osmoregulation by the endospore cortex as explained by Gould and Dring (1975). The contribution of the endospore coat on the heat resistance is minor. However, decoated endospores have exhibited a reduced resistance to heat as compared with the endospores with the coat (Marquis et al., 1994). The most heat resistant C. difficile endospore strain in this study had the thickest endospore coat and cortex suggesting the role of endospore structure in enhancing thermal resistance. Exosporium is the loosely attached outermost layer that contributes the adherence to host cells in an endospore (Lawly et al. (2009; Permpoompattana et al. 2011). This loosely attached outermost layer was not observed in the endospores in our study. Exosporium is usually visible in C. difficile endospores prepared in a liquid medium (Permpoompattana et al., 2011), but for our experiment, the endospores were prepared on solid media.

The wet heat denatures some crucial proteins of endospores, which are not the heat shock proteins in vegetative cells, while dry heat damages endospore DNA (Nicholson et al., 2000). The factors such as sporulation temperature, protection of endospore DNA by α/β-type small acid soluble proteins (SASPs), endospore core mineralization, and core dehydration are important in both wet and dry heat resistance of endospores as well (Nicholson et al., 2000; Setlow, 2005). The less survival of endospores upon heat exposure could be due to the inactivation of endospores by protein (wet heat) or DNA (dry heat) damages or inactivation of germinating endospores (Xu et al., 2015). The survival of Bacillus endospores at dry heat is
around 1,000 folds higher than that of the wet heat (Nicholson et al., 2000). Our results also had a trend of higher thermal resistance at the dry heat treatment as compared with wet heat treatment. The endospores assessed in this study were mixed in a matrix, and the composition of the matrix may also affect the heat transfer and protection. A previous study has demonstrated higher thermal resistance of *C. difficile* endospores in meat than in peptone water suggesting the effect of matrix properties on the survival rate of *C. difficile* endospores (Redondo-Solano et al., 2016).

Although the linear regression model did not fit well into the survival of endospores during the holding time, the estimated decaying rates provide some comparable results with a previous study (Xu et al., 2015). The computed inactivation rates (0.276-0.281 log CFU/g/day) at 55°C of the wet heat treatment of this study were comparable to the inactivation rates of more heat resistant *C. difficile* endospores in anaerobic sludge digestion at the same temperature (Xu et al., 2015). The number of days to be treated was estimated as 15-20 in our study in all MCs at 55°C, whereas Xu et al (2015) reported a ca. 5 log reduction of *C. difficile* endospores in 25 days at 55°C. Both studies further reported the heterogeneous survival of different strains of *C. difficile*. Although the initial endospore counts, endospore inactivation rates, log reductions during the holding time, and the number of days required to reach the LOD at each MC were not significantly different (p>0.05) with each other at 55°C in this study, the observed trend was, increasing number of days to reach LOD with the increasing MCs of compost.

As finished dairy compost was used for this experiment, the indigenous microflora in compost may consist of endospore-forming bacteria that may be resistant to the temperatures used in the simulated early stages of composting study. Indigenous microflora had mixed results upon the exposure of heat treatments. After the heat treatment at 55°C of the wet heat treatment,
the growth of indigenous microflora was increased with the increasing MC of compost suggesting the meeting of favorable conditions for certain populations of indigenous microflora, but after the treatment at 65°C, the trend was the reduction of indigenous microflora with the increasing MC of the compost. In the dry heat treatment, the trend was not the same and indigenous microflora decreased in compost with 20% MC at 65°C and with 40% MC at 55°C. These discrepancies could be due to the heterogenous distribution of indigenous microflora in compost. According to Strom (1985) and Hassen et al. (2001), the predominant microflora of finished compost is endospore-forming *Bacillus*. The increases of the counts possibly should be due to induction of germination of those aerobic endospore formers by heat and moisture in the matrix.

**CONCLUSION**

Our thermal inactivation study has demonstrated that *C. difficile* endospores are resistant at temperatures of composting with ≤ 3-log reduction under different temperature-MC combinations. Our study clearly demonstrated that the temperature in thermophilic phase of composting and moisture content in composting feedstock are very important factors in the inactivation of *C. difficile* endospores. Increased inactivation rates of *C. difficile* endospores were observed in compost with 30 and 40% MCs at 65°C of wet heat treatment. Sub-optimal conditions, such as the temperatures <65°C and low MC of compost that can be seen on the surface of the pile and dry pockets inside the pile, will lessen the inactivation of *C. difficile* endospores in composting feedstock. Our results further demonstrated that recommended minimal composting treatments to reduce the pathogens in composting for A-grade biosolid waste composts do not reduce *C. difficile* endospores to undetectable levels in dairy compost.
tested in this study. Therefore, re-considering the holding time of thermophilic phase of composting will produce safer compost if the feedstocks are contaminated with \textit{C. difficile}. Furthermore, heat resistance of \textit{C. difficile} endospore is strain specific and the thickness of endospore cortex might play a role in the survival of \textit{C. difficile} upon heat exposure. However, further research is needed with field trials in identifying the parameters critical for the inactivation of \textit{C. difficile} endospores in composting.

ACKNOWLEDGEMENTS

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REFERENCES


of the composting process. Applied and Environmental Microbiology, 77(12), 4126-4135.


Table 4.1 Log reduction of *C. difficile* endospores in simulated early phase of composting upon the exposure of dry heat.*

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>MC (%)</th>
<th>Initial population log CFU/g*</th>
<th>During the come-up time (Day)†</th>
<th>Population reduction (CFU/g compost) During the holding time (Day)‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>55</td>
<td>20</td>
<td>5.56 ± 0.12A</td>
<td>0.16 ± 0.21A,a</td>
<td>0.31 ± 0.09A,a, 0.18 ± 0.06b,a 0.27 ± 0.09A,a</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>5.57 ± 0.02A</td>
<td>0.15 ± 0.33A,a</td>
<td>0.32 ± 0.10A,b 0.49 ± 0.10A,a 0.38 ± 0.13A,b</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.83 ± 0.34A</td>
<td>0.40 ± 0.02A,a</td>
<td>0.71 ± 0.06A,a 0.47 ± 0.19A,a 0.53 ± 0.02A,b</td>
</tr>
<tr>
<td>65</td>
<td>20</td>
<td>5.32 ± 0.05B</td>
<td>0.12 ± 0.50A,a</td>
<td>0.18 ± 0.19A,a 0.05 ± 0.05B,a 0.29 ± 0.05B,a</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>5.06 ± 0.09C</td>
<td>0.50 ± 0.63A,a</td>
<td>0.70 ± 0.02A,a 0.65 ± 0.20A,B,a 0.76 ± 0.11A,B,a</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.68 ± 0.53A</td>
<td>0.39 ± 0.08A,a 0.61 ± 0.15A,a</td>
<td>0.76 ± 0.11A,a 1.11 ± 0.10A,a 1.08 ± 0.03A,a</td>
</tr>
</tbody>
</table>

*Values are expressed as log mean reduction from initial endospore counts ± standard deviation for triplicate trials. Means with the same upper case letter in the same column are not significantly different (p > 0.05) for a selected temperature of composting at different MC levels.
†Come-up time was programmed to be 2 day.
‡The values represent the log reduction from the count at come-up time. Values are expressed as mean log reduction ± standard deviation for triplicate trials. Means with the same upper case letter in the same column are not significantly different (p > 0.05) for a selected temperature of composting at different MC levels and the means with the same lower case letter in the same column are not significantly different (P > 0.05) for compost with a selected MC level subjected to different temperatures.
Table 4.2 Log reduction of *C. difficile* endospores upon the exposure of wet heat.*

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>MC (%)</th>
<th>Initial population log CFU/g*</th>
<th>Population reduction (CFU/g compost)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>During the Come-up time†</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>55</td>
<td>20</td>
<td>5.22±0.06B</td>
<td>0.15±0.06A,a</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>5.41±0.03A</td>
<td>0.29±0.08A,a</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.40±0.07A</td>
<td>0.01±0.20A,b</td>
</tr>
<tr>
<td>65</td>
<td>20</td>
<td>5.39±0.09A</td>
<td>0.27±0.17A,a</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>5.44±0.05A</td>
<td>0.69±0.08A,a</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.45±0.09A</td>
<td>0.18±0.02A,a</td>
</tr>
</tbody>
</table>

* Values are expressed as log mean reduction from initial endospore counts ± standard deviation for triplicate trials. Means with the same upper case letter in the same column are not significantly different (p > 0.05) for a selected temperature of composting at different MC levels. †Come-up time is dependent on the MC of compost and target temperature. At 55°C, the come-up times were approximately 30, 60, and 75 min for 40, 30, and 20% MCs, respectively, and at 65°C, the come-up times were approximately 25, 48, 60 min, respectively, for 40, 30, and 20% MCs. ‡The values represent the log reduction from the count at come-up time. Values are expressed as mean log reduction ± standard deviation for triplicate trials. Means with the same upper case letter in the same column are not significantly different (p > 0.05) for a selected temperature of composting at different MC levels and the means with the same lower case letter in the same column are not significantly different (P>0.05) for compost with a selected MC level subjected to different temperatures.
Table 4.3 Parameter estimates by the linear inactivation of *C. difficile* endospores under simulated early stages composting in dairy manure at different MC, temperature and heat treatments*.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>MC (%)</th>
<th>Initial endospore count (log CFU/g)</th>
<th>Dry heat treatment</th>
<th>Wet heat treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Endospore inactivation rate (day(^{-1}))</td>
<td>No. of days to reach LOD</td>
<td>R(^2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>20</td>
<td>5.28±0.07A (A)</td>
<td>0.012±0.012A,a</td>
<td>4.97±0.16A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NA†</td>
<td>-0.281±0.097A,a</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>5.21±0.04A (A)</td>
<td>-0.024±0.054A,a</td>
<td>4.99±0.17A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NA</td>
<td>-0.278±0.092A,a</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.22±0.10A (A)</td>
<td>0.025±0.027A,a</td>
<td>5.23±0.04A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NA</td>
<td>-0.276±0.025A,a</td>
</tr>
<tr>
<td>65</td>
<td>20</td>
<td>5.15±0.22A (A)</td>
<td>-0.012±0.079A,a</td>
<td>5.07±0.18A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NA</td>
<td>-0.309±0.186A,a</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>4.48±0.04B (B)</td>
<td>0.059±0.050A,B,a</td>
<td>4.35±0.28B</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NA</td>
<td>-0.311±0.189A,a</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>5.05±0.10A (A)</td>
<td>-0.176±0.043B,b</td>
<td>5.05±0.21A</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>25.1±5.9</td>
<td>-1.008±0.050B,b</td>
</tr>
</tbody>
</table>

* Values are expressed as log mean ± standard deviation for triplicate trials. Means with the same upper case letter in the same column are not significantly different (p > 0.05) for a selected temperature of composting at different MC levels and the means with the same lower case letter in the same column are not significantly different (P>0.05) for compost with a selected MC level subjected to different temperatures.

†Not applicable due to very low inactivation rates in dry heat treatment and very low R\(^2\) values.
Table 4.4 Ribotyping of *C. difficile* isolates at each temperature-MC combination after the 3-day heat treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dry heat</th>
<th>Wet heat</th>
</tr>
</thead>
<tbody>
<tr>
<td>55°C-20% MC</td>
<td>Ribotype 596 (n=9)</td>
<td>Ribotype 033(n=1), ribotype 596 (n=8)</td>
</tr>
<tr>
<td>55°C-30% MC</td>
<td>Ribotype 033(n=3), ribotype 060 (n=1), ribotype 596 (n=5)</td>
<td>Ribotype 596 (n=9)</td>
</tr>
<tr>
<td>55°C-40% MC</td>
<td>Ribotype 060 (n=1), ribotype 596 (n=8)</td>
<td>Ribotype 596 (n=9)</td>
</tr>
<tr>
<td>65°C-20% MC</td>
<td>Ribotype 596 (n=9)</td>
<td>Ribotype 060 (n=1), ribotype 596 (n=7)</td>
</tr>
<tr>
<td>65°C-30% MC</td>
<td>Ribotype 596 (n=9)</td>
<td>Ribotype 060 (n=3), ribotype 596 (n=6)</td>
</tr>
<tr>
<td>65°C-40% MC</td>
<td>Ribotype 596 (n=9)</td>
<td>Ribotype 060 (n=4), ribotype 596 (n=5)</td>
</tr>
</tbody>
</table>
Figure 4.1 Experimental design for assessing the effect of dry and wet heat treatments on the persistence of *C. difficile* endospores in compost.

Dried (MC <10%) and sieved dairy compost

Mixed and adjusted the MC to 20, 30, & 40% with a final level of $3 \times 10^5$ CFU/g

**DRY** heat treatment in environmental chamber at 55 and 65°C (2-day come-up time and 3-day holding time)

Sampled at 24 h intervals

Plated on BHIA-YE-CYS-CC-T agar and incubated anaerobically for 24 h at 37°C

Selected colonies (n=107) randomly after 3-day holding time and ribotyped

**WET** heat treatment in water bath at 55 and 65°C (come-up time is <2 h and 3-day holding time)

Sampled at come-up time and 24 h intervals

TEM analysis for the ultrastructure
Figure 4.2 Changes of MCs during the dry heat treatments at 55°C (A) and 65°C (B), and wet heat treatment at 55°C (C) and 65°C (D). The dotted lines (A & B) indicate the come-up time of dry heat treatment. The come-up times were approximately 30, 60, and 75 min for compost with 40, 30, and 20% MCs at 55°C, respectively, and approximately 25, 48, 60 min for compost with 40, 30, and 20% MCs at 65°C, respectively, for the wet heat treatment.
Figure 4.3 Changes of indigenous microflora in dairy compost with 20, 30, and 40% MCs, respectively, at 55°C (A) and 65°C (B) during the dry heat treatment and 55°C (C) and 65°C (D) during the wet heat treatment. The dotted line (A & B) represents the come-up time, which was programmed to be 2 days in dry heat treatment. The come-up times were approximately 30, 60, and 75 min for compost with 40, 30, and 20% MCs at 55°C, respectively, and approximately 25, 48, 60 min for compost with 40, 30, and 20% MCs at 65°C, respectively, in wet heat treatment.
Figure 4.4 Ultrastructures of C. difficile endospores of ribotype 033 (A), ribotype 60 (B), and ribotype 596 (C). CR-core; CX-cortex; CT-coat.
CHAPTER 5
SURVIVAL OF CLOSTRIDIUM DIFFICILE IN FINISHED DAIRY COMPOST UNDER CONTROLLED CONDITIONS

ABSTRACT

Survival of Clostridium difficile vegetative cells and endospores was compared in autoclaved and unautoclaved dairy composts with different moisture contents (MCs). Both types of composts adjusted to MCs of 20, 30, and 40% were inoculated with C. difficile at a final concentration of ca. 5-6 log CFU/g of vegetative cells and ca. 5 log CFU/g of endospores. The inoculated composts were then stored at room temperature (22°C) for 1 year in a humidity controlled chamber. The MCs of both types of composts were very stable during the year-long storage and no significant changes (p>0.05) in MC were observed except for the autoclaved compost with 30% MC. The level of indigenous microflora was very stable during the storage after day 7 in both types of compost. The greatest reductions of C. difficile vegetative cell counts were observed during the first 24 h of aerobic storage for all samples, which were 4.7 and 5.51 log CFU/g with 20% MC, 1.85 and 2.13, log CFU/g with 30% MC, and 2.32 and 1.31 log CFU/g with 40% MC, respectively, for autoclaved and unautoclaved compost. For the first 120 days of storage, the level of MC of compost and the duration of storage have significant (p < 0.05) effects on the survival of vegetative cells. The slow inactivation of C. difficile in compost with 30 and 40% MCs during the initial aerobic exposure was supported by the modeling data as well. The reduction of endospore counts during the year-long storage for both types of compost at all MCs was in the range 0.1-0.8 log CFU/g compost. There were no significant changes (p>0.05) in the endospore counts in both types of composts except the autoclaved compost with 30% MC. This study reported the survival of highly resistant C. difficile endospores for more than a year.
while vegetative cells died off exponentially upon the initial aerobic exposure, but leaving relatively more survivors in drier compost in the tailing phase of the survival curve. The longer survival of *C. difficile* in the contaminated biological soil amendments may transmit the pathogen to fresh produce, water or field workers in pre- and post-harvest conditions.

**INTRODUCTION**

*Clostridium difficile* is an anaerobic, Gram-positive and endospore-forming bacillus, which causes enteric diseases in humans and animals. *C. difficile* has been isolated from agricultural environments including soil, water (al Saif and Brazier, 1996; Zidaric et al., 2010), farm animal feces (Thitaram et al., 2011), and produce or produce related products (al Saif and Brazier, 1996; Metcalf et al., 2010; Rodriguez-Palacios et al., 2014), suggesting its probabilities of circulating in the pre-harvest environment. Both infected humans and animals including livestock, pet animals, and wild animals shed *C. difficile*. Human excreta contain more than $10^4$ CFU/g of *C. difficile* vegetative cells and endospores (Weese et al., 2000; Foster and Riley, 2012).

Prevalence of *C. difficile* in dairy cattle in the USA has been estimated to be around 2-2.4% from surveys of 17 states (APHIS, 2011) and 35 states (Thitaram et al., 2011). Poor farm management practices have increased the prevalence of this pathogen among calves (36%) and cattle (10%) in dairy farms (Bandelj et al., 2016). Land application of these livestock manure and pestering operations, which mix the fresh fecal matter to the agricultural environment (Moore et al., 1989), can facilitate the transmission of human pathogens to the agricultural fields. Transferring of human pathogens such as *Listeria monocytogenes*, *Salmonella* spp. and *Escherichia coli* O157:H7 from manure-amended soil or improperly processed compost to fresh
produce (Oliveira et al., 2012; Islam et al., 2005) has been reported. Several studies reported *C. difficile* contaminations in vegetables that grew both above and inside soil (al Saif and Brazier, 1996; Metcalf et al., 2010; Rodriguez-Palacios et al., 2014) suggesting the transferring possibilities from contaminated fields. The potential sources of *C. difficile* in the food chain could be contaminated soil or compost, animal manure, worker, wild life, water, and so on.

Dairy manure, a good source of nutrients for plant growth, is generally composted for agricultural farming (Cooperband, 2002). However, finished compost can be contaminated by *C. difficile* from above sources, which may survive longer during the storage. Chen et al (2018) reported that the composition of compost, inoculum level, and storage temperature affect on the survival of pathogens in manure-based compost. According to their study, *E. coli* O157:H7 survived 154 and 70 days in two types of dairy manure-based composts and *Salmonella enterica* survived for 77 and 14 days in two types of hen manure-based composts during the storage at room temperature. Non-O157:H7 shiga toxin producing *E.coli* survived for >125 days in dairy composts with 30 and 40% moisture contents (MCs) at room temperature (Wang et al., 2017). However, the survival of anaerobes in compost under the storage conditions would be different from Gram-negative enteric pathogens as the major obstacle for anaerobes is oxidative stress in the aerobic conditions.

The vegetative form of *C. difficile* loses viability faster upon aerobic exposure (Kim et al., 1981), but can survive nearly 3 h aerobically in the presence of sufficient moisture (Jump et al., 2007). However, the required moisture level for the survival is uncertain. The vulnerability of *C. difficile* vegetative cells is due to the poor aero-tolerance rather than the competition from indigenous microflora and other factors related to the matrix during the aerobic storage (Weese et al., 2000). On the contrary, the endospores are aero-tolerant and survive for an extended
period of time, e.g. > 5 months aerobically in nosocomial environments (Kim et al., 1981) and at least four years in farming environments (Båverud et al., 2003). These endospores or vegetative cells of *C. difficile* surviving in the environment could infect humans via the consumption of contaminated food. Therefore, in this study we assessed the effect of three moisture content levels (20, 30, and 40%) in dairy compost on the survival of artificially inoculated *C. difficile* vegetative cells and endospores during the room temperature storage. Mathematical modeling was applied to describe the effect of MC of compost on the survival of *C. difficile* vegetative cells.

**MATERIALS AND METHODS**

**Compost preparation.** Commercial dairy manure compost was directly purchased from the farm (Wallace Farm Soil Product Inc., Huntersville, NC). As listed on the label, the compost samples contained 0.5% total nitrogen, 0.5% available phosphate, 0.5% soluble potash, and no more than 1% chlorine. Autoclaved and unautoclaved composts were prepared as described previously (Dharmasena and Jiang, 2018). Briefly, air-dried dairy compost was sieved (sieve screen pore size: 3 × 3 mm) to remove the large particles and used as the unautoclaved compost. Autoclaved compost was prepared by autoclaving a portion of sieved compost at 121°C for 20 min, for three consecutive days, to reduce the initial level of background microflora.

**Inoculum preparation.** The stock culture of *C. difficile* (ATCC 43593) stored at -80°C was grown on pre-reduced brain heart infusion agar (Becton & Dickinson, Sparks, MD) supplemented with 0.5% yeast extract (Hardy Diagnostics, Santa Maria, CA), 0.1% L-cysteine (Alfa Aeser, Ward Hill, MA) (BHIA-YE-CYS agar). During anaerobic incubation at 37°C, the pure culture was transferred on to the same medium at 12 h intervals twice to minimize the
endospore formation. Biomass (12 h old) on BHIA-YE-CYS agar (3-4 plates) was collected with sterile cotton tipped applicators (Puritan, Guilford, ME) and suspended in pre-reduced brain heart infusion broth (Becton & Dickinson) supplemented with 0.5% yeast extract and 0.01% L-cysteine (BHIB-YE-CYS broth). The optical density of the suspension was adjusted approximately 0.2-0.3 at OD_{600nm}. Ten milliliters of this inoculum were added to 750 ml of pre-reduced BHIB-YE-CYS broth, and incubated anaerobically in an anaerobic jar with anaerobic gas packs (Becton & Dickinson) for 12 h at 37°C. Four batches (4 × 750 ml) of pre-reduced BHIB-YE-CYS broth were inoculated per each trial. Followed by centrifugation at 7,000 × g for 15 min at 4°C, the pellet was washed with 0.85% saline twice and the pellet was resuspended in the saline to have approximately 0.5 at OD_{600nm} in each batch. The suspension was further concentrated (10×) by centrifugation. Sufficient amount of inoculum was prepared to yield ca. 10^7-10^8 C. difficile vegetative cells/ml by the time of inoculation.

**Inoculation and storage of dairy compost.** The experimental design is presented in Figure 5.1. C. difficile inoculum was sprayed onto ca. 1,200 g of autoclaved and unautoclaved compost separately using a sterile spray nozzle at a ratio of 1:10 (v/w) (final concentration of vegetative cells ca. 10^6-10^7/g). The MC of compost was adjusted to 20, 30, and 40% with sterile tap water and mixed for 5 min in a pre-autoclaved series stand mixer (Professional 600 series, KitchenAid, Benton Harbor, MI) to distribute the inoculum and MC homogenously. Approximately, 100 g of inoculated dairy compost at each MC were distributed into 24 oz sterile Whirl-pak® stand-up bag (Nasco, USA) and closed by folding the wired top down twice and labeled according to the sampling day. For each trial, 12 bags of compost were prepared to sample on each day and sealed sample bags were then packed tightly in a single layer in a poly vinyl tray (10” by 12”). The tray was placed in a larger poly vinyl container (18” by 36”) with a lid that contained a
saturated solution of KCl \((a_w=0.845)\) and stored at room temperature \((22^\circ C)\) up to 1 year. There were 3 storage conditions: 20% MC-RT, 30% MC-RT, and 40% MC-RT per each type of compost and the experiment was repeated in triplicate.

**Physical and indigenous microflora analysis.** The moisture content of compost was measured on each sampling day using a moisture analyzer (model IR-35, Denver Instrument, Göttingen, Germany).

For assessing the indigenous microflora, the inoculated dairy compost was sampled on pre-selected sampling days \((0, 7, 30, 60, 120, 240, \text{ and } 365)\). On each sampling day, single compost bag \((100 \text{ g of inoculated compost})\) was withdrawn and mixed by hand from outside to homogenize compost inside the bag. Then, 10 g of the sample in duplicate were taken and mixed with 90 ml of sterile saline in a sterile Whirl-Pak sampling bag and homogenized using a Stomacher 400 laboratory blender (Brinkman Instruments, Inc., Westbury, NY) at the medium speed \((230 \text{ rpm})\) for 1 min. The total viable mesophilic bacterial counts were enumerated after plating ten-fold serial dilutions onto trypticase soy agar \((\text{TSA- Becton & Dickinson})\) and incubating at \(37^\circ C\) for 24 h.

**Analysis of vegetative cells and endospores of C. difficile.** To enumerate vegetative cells, the inoculated dairy compost was sampled on pre-selected sampling days \((0, 1, 3, 5, 7, 14, 21, 30, 60, \text{ and } 120)\). Dilutions were prepared as described above and selected serial dilutions in saline were plated on pre-reduced BHIA-YE-CYS agar supplemented with cycloserine-cefoxitin \((\text{SR0096E, Oxoid, Basingstoke) BHIA-YE-CYS-CC agar})\) in duplicate. Compost bags were withdrawn on day 240 and 365 additionally for endospore analysis. Endospores were enumerated by plating the serial dilutions on BHIA-YE-CYS-CC agar supplemented with 0.1% \((\text{w/v})\) sodium taurocholate \((\text{Alfa Aeser, Ward Hill, MA, USA})\) \((\text{BHIA-YE-CYS-CC-T agar})\) after heat-
shocked at 60°C for 25 min. Plates were anaerobically incubated at 37°C for 24 h. The sampled compost bag was returned to the same tray to maintain approximately the same packed status of compost to prevent infiltration of additional air inside the compost.

**Survival kinetics of C. difficile vegetative cells.** The inactivation of vegetative cells of *C. difficile* should be due to the oxidative and desiccation stresses when the MC was not sufficient. However, the natural genetic heterogeneity of bacteria being exposed to stress also could be a reason for the differences in resistance (Coroller et al., 2006). Since the inactivation of *C. difficile* vegetative cells in dairy compost has been shown to be first-order type, exponential model was used to describe the inactivation kinetics of vegetative cells. The exponential decay model used with three parameters (Chen et al., 2013) is as follows.

$$\log_{10}[N_i(t)] = \alpha + \beta \exp^{-\mu \log \text{time}} + \epsilon_i$$

where, $N_i(t)$ is the number of *C. difficile* vegetative cells at time $t$ (time of aerobic storage) for the $i$th observation, $\alpha$ is the long-term ($t \to \infty$) log count of vegetative cells, $\beta$ is the initial log count of the vegetative cells, $\mu$ is the inactivation/decay rate or slope of inactivation/decay curve, and $\epsilon_i$ is the random error for the $i$th observation. Regression coefficient ($R^2$) was calculated for each regression model to assess the goodness-of-fit of the model.

**Statistical analysis.** Plate count data for vegetative cells, endospores of *C. difficile*, and indigenous microflora were converted to log values of CFU/g in dry weight basis. The mean counts on each sampling day were compared among MC and compost types with analysis of variance (ANOVA), followed by Tukey’s honest significant difference (HSD) procedure.

For the inactivation kinetics of vegetative cells, means of the estimated model parameters for each trial were compared among MC and type of compost with an ANOVA, followed by
Tukey’s HSD procedure. All calculations were performed using JMP® Pro 12 (SAS Institute Inc., Cary, NC, 1989-2007). Any \( p \geq 0.05 \) was considered evidence of statistically significant.

RESULTS

Change of moisture content and indigenous microflora of compost during the storage. The saturated KCl bath controlled MC of compost almost constant throughout the entire storage time. Unautoclaved composts retained the MCs without significant changes \( (p>0.05) \) during the storage at all three levels of MC (Table 5.1). For autoclaved composts, the MC changes throughout the year-long storage was significant \( (p<0.05) \) only in compost with 30% MC.

Unautoclaved compost contained ca. 7-8 log CFU/g of indigenous mesophilic microflora immediately after the MC was adjusted while autoclaved compost, which was used as a control had a reduced level of indigenous microflora (ca. 4 log CFU/g) on day 0 (Figure 5.2). However, a rapid increase of the indigenous mesophilic population was observed in autoclaved composts during the first 7 days of storage. In both types of composts with all MC levels, increases of indigenous microflora were observed during the storage compared to the counts on day 0 and both MC and storage time had significant \( (p<0.05) \) effects on those increases. On each sampling day, the indigenous microflora counts were not significantly different \( (p>0.05) \) from each other among the different MC levels except for day 120, for both types of composts with 20% MC, whereas the counts were significantly lower compared to those for the other 2 levels of MC on the same day. Both types of composts with 30% MC had higher counts for indigenous microflora on day 120, but the counts were not significantly \( (p>0.05) \) different from the counts in compost with 40% MC.
**Population changes of *C. difficile* vegetative cells in dairy compost during the storage.**

Finished dairy compost used in this study was confirmed to be free of *C. difficile* by enrichment method. The level of initial inoculum of vegetative cells for each trial was targeted to have ~$10^6$ - $10^7$ CFU/g. However, there were slight deviations from the expected counts for some trials. The survival curves for the vegetative cells of *C. difficile* during storage under aerobic conditions at each MC and compost type is shown in Figure 5.3. Within the first 24 h of storage, there were ca. > 4 log reductions of *C. difficile* in both types of composts with 20% as compared with ca. 1.3-2.3 log CFU/g reductions in both compost types with 30% and 40% MCs. After the first 24 h, the vegetative cell counts in compost with 20% MC had slight fluctuations for the 120 days of the storage for both types of compost, which were not significantly (p>0.05) different from the counts on day 1. However, there were further reductions for *C. difficile* in compost with 30 and 40% MCs during the same duration of storage after 24 h. A 4-log reduction of vegetative cells was observed for both types of composts with 30 and 40% MCs by day 60 and 30 of storage, respectively. *C. difficile* vegetative cell count for composts with 40% reached the detection limit by day 60 and below the detection limit by day 120. Both storage time and the MC had significant (p<0.05) effects on the survival of *C. difficile* vegetative cells. However, at each MC, the compost type did not have a significant (p>0.05) effect on the change of vegetative cell counts over the time of 120 days.

**Population changes of *C. difficile* endospores in dairy compost during the storage:** The survival curves for endospores in compost with selected moisture levels during the storage are presented in Figure 5.4. The counts of endospores over the storage were nearly constant, but had slight reductions for every compost type-MC combination by day 365. For autoclaved composts
with 20, 30, and 40% MCs, the reductions of endospore counts were 0.80±0.79, 0.31±0.32, and 0.27±0.07 log CFU/g, respectively, as compared with the reductions of endospore counts in unautoclaved composts with 20, 30, and 40% MCs, 0.32±0.12, 0.39±0.28, and 0.77±0.17 log CFU/g respectively. The changes of endospore counts were not significantly different for both types of composts with 20% (autoclaved: p=0.786 and unautoclaved: p=0.709), unautoclaved compost with 30% MC (p=0.450), and both types of composts with 40% MC (autoclaved: p=0.985 and unautoclaved: p=0.139). Only autoclaved compost with 30% MC had a significant (p=0.027) change of endospore counts during the storage.

**C. difficile vegetative cells and survival kinetics:** Survival curves for the log vegetative cell counts of *C. difficile* were fit using the exponential decaying model, and all parameter estimates and R² values are presented in Table 5.2. Two phases in the survival curves were observed of vegetative cells at each MC, i.e. the initial exponential decay and the tailing until day 120. The α values for the long term log count of vegetative cells are the baseline value fit by the model, which were not significantly (p>0.05) different on the compost types and MC levels. β values for the initial log count of the vegetative cell counts were not significantly (p>0.05) different at all MCs for both types of composts as well. The fastest decay rates (µ) of vegetative cells were observed for both types of composts with the least MC (20%) used in this experiment, which was significant (p<0.05) from the decaying rates in composts with higher MCs (30 and 40%) for both types of composts. Moreover, the decay rate of *C. difficile* in unautoclaved compost with 20% MC was significantly (p<0.05) higher than that of autoclaved compost with 20% MC. Both types of composts with 30% MC had the slowest decaying rate of *C. difficile* vegetative cells during the storage. However, those decaying rates were not significantly (p>0.05) different from the
decaying rates in both types of composts with 40% MC. For the tailing of the survival curves, the highest MC (40%) had the lowest survival compared to the survival of vegetative cell counts in composts with lower MCs, 20 and 30%.

DISCUSSION

Several studies have reported the survival of non-endospore-forming Gram-negative pathogens in compost as affected by some factors such as compost type, storage condition, and initial inoculum (Chen et al., 2018). Unlike aerobic bacteria, the survival of anaerobic bacteria has an additional stress, i.e. the oxidative stress under the aerobic exposure.

Very limited studies have reported the survival of vegetative *C. difficile* cells in different matrices aerobically, but with controversial observations. When 26 artificially inoculated equine fecal samples with *C. difficile* were stored at 4°C, *C. difficile* was recovered up to 2.5 ± 2.52 days in 2 samples during the aerobic storage, whereas 25 samples were still positive after 30 days of storage in an anaerobic chamber (Weese et al., 2000). Freeman and Wilcox (2003) also assessed the survival of both *C. difficile* vegetative cells and endospores in human fecal suspensions at refrigeration (4°C) and freezing (-20°C) temperatures stored aerobically for 56 days. In their study, both vegetative cells and endospores survived for the entire time of storage (56 days) exhibiting approximately constant counts. Above studies, suggest the differences in survival of the strict anaerobe, *C. difficile*, upon aerobic and anaerobic exposure at low temperatures. Our study revealed that majority of the vegetative *C. difficile* cells lost the viability rapidly, but a small population survived during the aerobic storage of dairy compost at room temperature for 120 days. On the other hand, *C. difficile* endospores are highly resistant to aerobic exposure with slight reductions (0.1-0.8 logs) during a year-long storage.
Following the first order kinetics, the log reductions of *C. difficile* vegetative cells in both types of composts with 20% MC, were ca. >4 as compared with ca. <2.5 log reductions in both compost types with 30 and 40% MCs during the first 24 h of storage. However, the survival patterns of *C. difficile* during the tailing phase were opposite to the survival in exponential decaying phase. In the tailing phase, the compost with the highest MC (40%) had the lowest survival as compared with the composts with lower MCs (20 and 30%). Moisture content is a key factor that determines the survival of enteric pathogens in bio-solid amended soil (Schwarz et al., 2013) and compost (Wang et al., 2017). In the crop fields, most enteric pathogens in soil have increased decaying rates, when the MC of soil decreases (Schwarz et al., 2013). Agreeing to that, our results clearly demonstrated a faster decay rate in compost with the lowest MC during the initial exponential decaying phase of survival. In addition to oxidative stress, low water activity can affect microbial activity significantly. Decline of MC in soil is correlated with increases of soil temperature, which may desiccate bacteria (Schwarz et al., 2013). Desiccation killed 6 logs of vegetative *C. difficile* cells on a dry glass slide in ca.15 min at 37°C exposed to aerobic and anaerobic conditions (anaerobic chamber) (Jump et al., 2007). This emphasizes the quick die-off was due to desiccation even in the absence of oxidative stress. Having provided with sufficient moisture using nutrient free agar, *C. difficile* vegetative cells could survive for 3 h with a sporadic growth up to 12 h aerobically, but up to 24 h without a decline in the initial inoculum level (6 logs) anaerobically (Jump et al., 2007). As our inoculum was not on surface that exposes directly to the aerobic conditions, inside the compost may be more beneficial for the survival of *C. difficile*. Furthermore, the compost with the least MC contained more air exposing the vegetative cells to more oxygen and desiccation. Usually, bacteria get well-adapted to the environmental stresses. For example, the gene responsible for heat shock protein, groEL is up-
regulated under stressed conditions such as heat, sub-inhibitory antibiotic concentrations, and pH changes in *C. difficile* (Hennequin et al., 2001). Hennequin et al (2001) demonstrated that *groEL* gene in *C. difficile* is produced several folds higher under stressed conditions and reach a plateau. Based on that, the analysis of survival curve data for the exponential decay of vegetative *C. difficile* cells, it is clear that *C. difficile* in comports with lower MC had increased decaying rates, reaching a plateau within 24 h of storage. Vegetative cells of bacteria that up-regulate genes to adopt the stress survive longer (Chen et al., 2013). Hence, we hypothesize that *C. difficile* vegetative cells in compost with sub-optimal MC (20%) used in this experiment may be induced with desiccation adaptation and survived longer, which needs to be further confirmed by gene expression experiments. In contrast, the compost with the highest MC (40%) had more water activity and less air providing a beneficial environment for *C. difficile* vegetative cells without inducing stress adaptation. This may lead to a rapid mortality of vegetative cells during the storage as compared with the cells in drier compost.

Regulation of the oxidative stress by vegetative cells is another possible explanation for the tailing phase of survival curve. Obligate anaerobes such as members of clostridia do not possess sufficient levels of enzymes to react with reactive oxygen species (superoxides and hydrogen peroxide) produced upon aerobic exposure (Imlay, 2002). However, recent studies revealed that *C. difficile* genome contains *sodA* gene (Li et al., 2015), a superoxide dismutase (SOD), and 7.6 % of genes encode the proteins involved in electron transport chain, which are up-regulated upon oxidative stress conditions (Emerson et al., 2008). The SOD production rate in anaerobes is usually slower compared to the aerobes. Rolfe et al (1978), explained that bacteria with low SOD activity such as anaerobes, acclimatize to the aerobic conditions and survive longer periods of time by reducing oxygen at a slower rate, than the bacteria with low SOD
activity that can readily reduce oxygen. The longer survival at the lowest MC (20%) therefore, could potentially be due to slower SOD activity or up-regulation of enzymes associated with electron transport chain in C. difficile vegetative cells compared to the survival at 40% MC in the tailing phase of the survival curve.

The indigenous microflora may affect the survival of more aero-tolerant vegetative C. difficile cells in compost indirectly. After 7 days of the storage, the background microflora in each type of compost was 8-9 log CFU/g. When the MC of soil is sufficient, the respiration of aerobic mesophiles reduce the redox potential of surrounding soil generating anaerobic microenvironments, which enhances the survival of anaerobic bacteria (Husson, 2013). The same phenomenon might happen in the storage of compost as well by extending the survival of vegetative cells of C. difficile. The least decaying rate of C. difficile vegetative cells was observed in both types of composts with 30% MC, where the highest level of background flora was observed compared to the composts with 20 and 40% MCs by the day 120 could be a reason. The levels of initial indigenous microflora in autoclaved composts with 30 and 40% MCs on day 0 were higher, but were not significant (p>0.05), than that of autoclaved compost with 20% MC. In the presence of sufficient moisture (30 and 40% MC) the indigenous microflora may metabolically be more active and their respiration create potentially more anaerobic micro-environments facilitating the survival of C. difficile vegetative cells in compost. However, an opposite trend was observed in composts with 20% MC, the driest compost used in this study. The vegetative cells in autoclaved compost with 20% MC had a slower decaying rate as compared with those in the compost with the same MC level of unautoclaved compost. The level of initial inoculum in composts with 20% MC was ca. 6 logs, which was ca. 2 logs higher and ca. 1 log lower than the level of indigenous microflora, respectively, in autoclaved and unautoclaved
compost. Lack of moisture in compost slows down the metabolic rate of indigenous microflora as well and therefore, a less initial interactions may have resulted in a lower inactivation rate in autoclaved compost with 20% MC.

Usually, the indigenous microflora of compost suppresses the survival of most enteric pathogens (Pietronave et al., 2004). According to Pietronave et al (2004), artificially inoculated *Salmonella arizonae* and *E. coli* counts (ca. 5 log CFU/g) had a regrowth of ca. 1 log in autoclaved compost while counts of both pathogens had ca. 1 log reduction in non-sterile compost after 30 day storage. The mesophilic counts in their study ranged 9-7 log CFU/g in non-sterile compost with 40 and 80% MCs, probably contributed to the suppressing the growth of inoculated pathogens during storage. The indigenous microflora in both types of composts used in our study had a growth, with <1 log in unautoclaved compost during the storage and >4 logs in autoclaved compost by day 7 of the storage time. There were slight changes in the indigenous microflora in both types of composts after day 7. However, the counts for the survival of *C. difficile* vegetative cells on each day were not affected significantly (p>0.05) by the type of compost suggesting that oxidative stress has a stronger impact on the survival of *C. difficile* vegetative cells in compost. However, the vegetative cells in autoclaved compost with 20% MC had a slower decaying rate as compared with the same MC level of unautoclaved compost. Autoclaved composts with higher MCs (30 and 40%) had higher initial indigenous microflora levels on day 0, but were not significant (p>0.05), than that of autoclaved compost with 20% MC. In the presence of sufficient moisture (30 and 40% MC) the indigenous microflora may metabolically be more active and their respiration create potentially more anaerobic micro-environments facilitating the survival of *C. difficile* vegetative cells in unautoclaved compost. Therefore, acclimatization by a limited population of vegetative cells to lower oxygen
environments and the occurrence of anaerobic pockets due to the respiration of mesophiles in the matrix may cumulatively aid the survival of vegetative *C. difficile* cells in compost.

Endospores are the resistant form of some Gram-positive bacteria, which maintain the viability upon the exposure of various environmental stresses. In this study, during the year-long storage, endospore counts remained almost unchanged regardless of MC levels in compost, except the significant (p>0.05) fluctuations in autoclaved compost with 30% MC. The constant counts prove again the resistance of endospores in the aerobic conditions. The MC also had a significant (p>0.05) change in autoclaved compost with 30% MC. However, it is not certain of the relationship between endospore survival and MC of compost. In our study, the MC of autoclaved compost with 30% MC dropped significantly (p<0.05) from day 240 to 365, but, which can not be sufficient for drying off the endospores. Probably, this could be due to the heterogenous distribution of inoculum on compost particles. Soil type that accompanies with its water holding capacity, temperature or season of the year, and the ribotype of *C. difficile* affect on the survival of endospores in biosolid waste amended soil (Xu et al., 2016). Though the composition of the matrices used was not the same, the log changes of *C. difficile* endospores counts in our study agree with Xu et al. (2016), who reported 0.49-0.63 log reduction/g by day 350 in the land applied with the inoculated bio-solid amended soil. A recent study reported the survival of artificially inoculated *C. difficile* endospores in to swine manure-based compost at different temperatures (Usui et al., 2017). *C. difficile* was found to regrow during the storage at 25 and 37°C by day 5, but no significant changes in endospore counts were observed for the entire storage time of 30 days. Overall, all studies reported the persistence of endospores of different *C. difficile* ribotypes at different temperatures, soil amendments, MCs, and soil type conditions without losing viability for a year at least.
LIMITATIONS

The resistance to the oxidative and desiccation stresses upon exposure to the aerobic environment and the capability of sporulation in adverse environments could be variable among different strains of *C. difficile*. Therefore using a single strain of *C. difficile* is a limitation of this study. Moreover, the inactivation kinetics could be varied according to the compositions of different compost matrices and strains of *C. difficile* used in experiments.

CONCLUSION

Our study clearly demonstrated that moisture content of compost has a significant (p<0.05) effect on the survival of *C. difficile* vegetative cells in compost stored under aerobic conditions. A trend of slower decaying of *C. difficile* vegetative cells was observed at the early stage of aerobic exposure when the moisture content of compost was increased. However, *C. difficile* in compost with both 30, and 40% MCs had non-significant (p>0.05) differences in the decaying rates fit by the exponential decay model. The $R^2$ values support that using three-parameter exponential model was appropriate in fitting the model to the changes in vegetative cell counts at all MCs for both types of compost. Furthermore, the survival of *C. difficile* vegetative cells persisted well in drier compost (20 and 30% MC) than wet compost (40% MC). The growth of indigenous microflora in compost may affect indirectly on the extended survival of *C. difficile* vegetative cells by creating anaerobic pockets inside the compost matrix. Endospores of *C. difficile* maintain the viability for an extended length of time in dairy compost at all MCs tested in this study, despite the slight reductions observed by the end of the year-long storage. Therefore, using improperly composted animal manure, manure amended soil, and land application of unprocessed manure may contaminate agricultural fields with *C. difficile*. 

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endospores and vegetative cells. Long-term survival of C. difficile endospores in compost environment allows the transmission of the pathogen into fresh produce at pre- and post-harvest conditions and increase the risk of community associated C. difficile infections.

REFERENCES


Table 5.1: Fluctuations of moisture contents of compost during the storage for 1 year under the controlled conditions

<table>
<thead>
<tr>
<th>Time (Day)</th>
<th>Moisture contents (%)</th>
<th>Autoclaved compost</th>
<th>Un autoclaved compost</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20</td>
<td>30</td>
<td>40</td>
</tr>
<tr>
<td>0</td>
<td>19.30±0.82A</td>
<td>29.16±0.62A,B</td>
<td>39.04±0.41A</td>
</tr>
<tr>
<td>1</td>
<td>19.84±0.75A</td>
<td>28.88±0.07A,B,C</td>
<td>39.16±0.72A</td>
</tr>
<tr>
<td>7</td>
<td>19.21±0.85A</td>
<td>29.16±0.50A,B</td>
<td>39.12±0.70A</td>
</tr>
<tr>
<td>14</td>
<td>19.52±1.02A</td>
<td>29.58±1.00A,B</td>
<td>39.36±0.43A</td>
</tr>
<tr>
<td>30</td>
<td>19.18±0.44A</td>
<td>28.95±0.57A,B,C</td>
<td>39.39±0.94A</td>
</tr>
<tr>
<td>60</td>
<td>19.24±1.34A</td>
<td>29.31±0.29A,B</td>
<td>39.72±0.34A</td>
</tr>
<tr>
<td>120</td>
<td>18.87±1.12A</td>
<td>29.96±0.11A</td>
<td>39.69±0.28A</td>
</tr>
<tr>
<td>240</td>
<td>17.84±0.55A</td>
<td>28.43±0.22B,C</td>
<td>38.77±0.41A</td>
</tr>
<tr>
<td>365</td>
<td>18.00±0.48A</td>
<td>27.51±0.50C</td>
<td>38.04±0.81A</td>
</tr>
</tbody>
</table>

*MC readings are expressed as means ± standard deviation for three trials. Means with the same letter in the same column are not significantly different (p > 0.05).
Table 5.2 Parameter estimates of the inactivation model for *C. difficile* vegetative cells stored at room temperature under controlled conditions*

<table>
<thead>
<tr>
<th>Compost Type</th>
<th>Moisture content (%)</th>
<th>Long-term log count (α-log CFU/g)</th>
<th>Initial log count of vegetative cells (β-log CFU/g)</th>
<th>Inactivation rate (μ-log CFU/g/day)†</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclaved</td>
<td>20</td>
<td>1.24±0.45A</td>
<td>4.97±0.34A</td>
<td>-3.17±0.95B</td>
<td>0.93</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.80±0.89A</td>
<td>4.48±0.57A</td>
<td>-0.56±0.29A</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>1.41±0.48A</td>
<td>4.21±1.22A</td>
<td>-1.65±0.90A,B</td>
<td>0.85</td>
</tr>
<tr>
<td>Unautoclaved</td>
<td>20</td>
<td>1.44±0.34A</td>
<td>4.98±0.52A</td>
<td>-5.33±0.95B</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.64±0.82A</td>
<td>4.31±0.37A</td>
<td>-0.41±0.21A</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>-0.01±1.32A</td>
<td>5.36±1.99A</td>
<td>-0.56±0.42A</td>
<td>0.91</td>
</tr>
</tbody>
</table>

*Data are expressed as means ± standard deviations and means with different letters in the same column for each compost type are not significantly different (P >0.05).
†The inactivation rates were assessed for the exponential phase of the survival curves.
Figure 5.1 Experimental design for the analysis of survival of *C. difficile* vegetative cells and endospores in dairy compost.

Air dried (MC <10%) and sieved dairy compost

Mixed and compost was adjusted with MCs to 20, 30, & 40% using sterile tap water and to a final level of $10^5-10^6$ vegetative cells and ca. $10^4$ endospores/g.

Mixed in a food processor for 5 min

Placed ca. 100 g of inoculated compost in Stand-up bags and stored in a humidity controlled chamber

Sampled at selected time intervals and tested for MC, background flora, *C. difficile* vegetative cells, and endospores
Figure 5.2 Changes of indigenous microflora in autoclaved (O) and unautoclaved (Δ) dairy compost with 20% (A), 30% (B), and 40% (C) during the storage at room temperature. Bars represent the standard deviations on each sampling day.
Figure 5.3 Changes of *C. difficile* vegetative cells in autoclaved (O) and unautoclaved (Δ) dairy compost with 20% (A), 30% (B), and 40% (C) during the storage at room temperature. The dotted lines indicate the detection limits by direct plating, ca. 0.8, 0.85, and 0.9 log CFU/g in dry weight respectively for composts with 20, 30, and 40% of MCs. Bars represent the standard deviations on each sampling day.
Figure 5.4 Changes of *C. difficile* endospores in autoclaved (O) and unautoclaved (Δ) dairy compost with 20 (A), 30 (B), and 40% (C) MCs during the storage at room temperature. Bars represent the standard deviations on each sampling day.
CONCLUSIONS

Toxigenic *C. difficile* is an opportunistic pathogen with a fecal-oral transmission route. Acquisition of the pathogen occurs frequently from healthcare environments, but during the last two decades, *C. difficile* has been isolated from various environmental sources such as water, soil, livestock animals and their manure, fresh produce, and ready-to-eat produce salads. The pathogenesis by *C. difficile* requires the disturbing the equilibrium of intestinal microflora. Therefore, determining the exact source of pathogen is difficult in patients. As *C. difficile* contaminations have already been identified in fresh produce, testing manure-based compost as a vector for this pathogen is appropriate. Due to lack of standard isolation protocol for *C. difficile* from environmental samples, improving culture media to have reproducible results is important.

Although *C. difficile* is an anaerobic bacterium, which is difficult to grow, our results demonstrated that satisfying its growth requirements provides a reproducible growth within overnight incubation even without manipulating this bacterium inside an anaerobic chamber. Increased counts after a thermal treatment implies the induction of dormant endospores, however, choosing the temperature-time combination that provides the best yield is recommended. Although, enrichment of environmental samples to isolate *C. difficile* is a common practice, the composition and the selective supplements used in an enrichment broth are more important. We found that BHIB-YE-CYS-MN-T broth is a more sensitive enrichment broth to isolate *C. difficile* from compost and it would be effective for environmental samples with a high level of background flora. The efficacy of both media and enrichment method developed in this study was confirmed by analyzing more than 100 manure/compost samples. Our results demonstrate that commercially available compost is a potential vector for pathogenic *C. difficile* in fresh produce with a prevalence of 36%. To investigate the fate of *C. difficile*
endospores in composting, minimal recommended composting conditions were compared with a higher temperature that may be reached during composting at different moisture levels. We found that minimal treatment was not effective despite the moisture level, whereas maintaining higher temperatures (65°C) with ≥ 40% moisture level in composting materials for more than 4 days is more effective with turnings to prevent occurrence of dry spots. This information would be helpful for compost producers to improve the microbial safety of their products from pathogenic C. difficile. Preventing the routes of contaminations of finished compost/agricultural fields by C. difficile is required as endospores can persist well in compost longer and sub-optimal moisture contents of compost may induce the stress adaptation of C. difficile vegetative cells allowing an extended survival. Further studies related to gene up-regulation of C. difficile under stressed conditions are needed to confirm the adoptability of these strict anaerobes for the aerobic survival.