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Hongye Wang
Clemson University, hongyew@g.clemson.edu

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DETERMINING THE FATE OF NON-O157 SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN DAIRY COMPOST DURING STORAGE USING THE OPTIMIZED DETECTION METHOD

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Food, Nutrition and Culinary Sciences

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by
Hongye Wang
December 2014

Accepted by:
Dr. Xiuping Jiang, , Committee Chair
Dr. T.R. Jeremy Tzeng
Dr. Paul Dawson

ABSTRACT

Shiga toxin-producing *Escherichia coli* (STEC), a member of *Enterobacteriaceae* family, has been recognized as emerging pathogens. Dairy compost is commonly applied to farmland as a soil amendment. Despite the agricultural benefit of manure-based soil amendment, the inadequately treated compost can contribute to fresh produce contamination on the farm. Moreover, the epidemiological results showed that the non-O157 STEC cases have surpassed those of *E. coli* O157. Therefore, it is critical to evaluate the behavior of non-O157 STEC strains in the dairy manure-based compost. The objectives of this study were to: 1) optimize a culturing method for detecting non-O157 STEC from dairy compost, 2) determine the growth potential of top six non-O157 STEC serovars in dairy compost, and 3) conduct a persistence study of non-O157 STEC in dairy compost being held at room temperature.

First, we optimized a culturing method for detecting STEC during enrichment. Cefixime-tellurite Sorbitol MacConkey Agar supplemented with 5 mg/l novobiocin (CTN-SMAC) was chosen for enumerating non-O157 STEC cells before or after enrichment, as CTN-SMAC is more cost effective than Modified Rainbow Agar (mRBA) and both agar plates enumerated the same level of STEC. The single step selective enrichment recovered ca. 0.54 log CFU/g more cells as compared to the two-step enrichment. In addition, without enrichment step, the detection limit of individual STEC serovar ranged from 250 to 2,500 CFU/g in dairy compost. For STEC O26 and O145, the detection limit by IMS was 2,500 CFU/g, but for other STEC serotypes (O45, O103, O111, and O121), the detection limit was 250 CFU/g. Our results demonstrated that a low

level of STEC (ca. 100 CFU/g) could be detected within one day from dairy compost by culturing method through optimized enrichment procedure followed by immunomagnetic beads separation (IMS).

Next, we investigated the survival potential of non-O157 STEC in dairy compost during storage at room temperature. A mixture of six non-O157 STEC serovars was inoculated into commercially available dairy compost with 30% moisture content at a final concentration of ca. 5.5 log CFU/g. During storage at room temperature for up to 42 days, STEC counts and other factors such as indigenous microorganism population, moisture contents and pH were analyzed at selected sampling intervals. Both moisture contents and pH values in dairy compost remained unchanged (p>0.05) during the entire duration of trials, and so did the background bacterial level. As for the STEC population, a growth of ca. 0.5 log CFU/g was recorded within the first day post inoculation, followed by a rapid decrease of ca. 1.5 log CFU/g during 14 days of storage. By the end of the experiment, the population level of non-O157 STEC reduced ca. 1.7 logs, and the survival curve displayed an extensive tailing. Randomly selected colonies from the last 3 sampling times were confirmed as STEC by PCR.

Our results demonstrated that low-level of STEC could be detected within one day from the finished dairy compost by culturing method through optimized enrichment procedure followed by IMS, and non-O157 STEC persisted in dairy compost for at least 42 days, indicating the long-term survival of non-O157 STEC in the finished dairy compost. Therefore, proper handling and testing of the finished dairy compost as soil

amendment is critical for ensuring the microbiological safety of fresh produce and the farm environment.

DEDICATION

I would like to dedicate this work to my grandparents and my parents for their love and support. And I would like to dedicate this work special to my dearest grandmother for her love.

ACKNOWLEDGMENTS

I would like to sincerely thank my advisor, Dr. Xiuping Jiang, for her guidance, encouragement, and patience. I would like to thank Dr. Paul Dawson and Dr. T.R. Jeremy Tzeng for serving on my thesis committee. I would also like to thank all past and current lab members I have worked with while completing my Master's for their friendship and assistance.

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CHAPTER ONE

LITERATURE REVIEW

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) have been recognized as emerging pathogens (Gyles *et al*., 2007). Although many severe cases of hemorrhagic diseases and deaths are frequently associated with *E. coli* O157:H7, foodborne illnesses linked to non-O157 STEC, such as serotypes O26, O45, O111, O103, O145 and O121, have been increasing according to the data reported by Centers for Disease Control and Prevention (CDC)'s FoodNet (Hoefer *et al*., 2011). STEC outbreaks were mostly linked to dairy products, ground beef and fresh produce. Due to inadequate epidemiological and laboratory investigation, non-O157 STEC infections are often under-reported. In the United States, unlike *E. coli* O157:H7 which became nationally emerging pathogen in 1994, non-O157 STEC infections were reportable in 2000. In the Federal Register Notice published on Sept. 20, 2011, Food Safety and Inspection Service (FSIS) declared the top six STEC serotypes as adulterants on raw and/or beef-related products, in the same status as *E. coli* O157:H7 (Johnson *et al*., 2011).

Ruminants, like cattle, sheep and goat, are recognized as important reservoirs for STEC, whereas animal wastes also appear to be the potential sources for the transmission of these pathogens to crops that are then consumed by humans. Composting of animal waste is an effective way to kill pathogens prior to the on-farm use. The composting process is driven by microbial activities under aerobic condition by metabolizing and converting the organic material into a stable soil amendment (Larney *et al.*, 2003).

Nevertheless, these nutrient-rich environments may allow the persistence of pathogenic cells in the finished compost and facilitate the transfer of these cells to the compost-amended soil (Berry *et al.*, 2013).

As the transmission of STEC in animal waste to fresh produce is a valid hypothesis, the factors contributing to the survival and growth of STEC in animal waste and to the contamination of farm land should be thoroughly investigated in order to develop the practical strategies for STEC control in pre-harvest phase.

Data gathering

In order to review all the related information on the research topic, a literature review was conducted (Fig1.1). The literature review was carried out by searching EBSCO and Google Scholar databases. The scientific papers available in full text and published in English between January 1990 to 2014 were searched, using the following keywords: (Detection "of" *E. coli* Non O157*) OR (Detection "of" *E. coli* O157*) OR (*E. coli* Non O157); (*E. coli* Serogroups*) AND (Immunomagnetic separation*) OR (Modified Rainbow Agar*) OR (Modified TSB*); (*E. coli* Serogroups*) OR (Immunomagnetic separation*) OR (sorbitol MacConkey agar*) OR (Modified TSB*) OR (CHROMagar Non O157*); (*E. coli* Non O157*) OR (*E. coli* O157:H7*) OR (Shiga toxin producing*) AND (Manure*) OR (Compost*) OR (Soil*) OR (Biological soil amendment*) OR(Ground Beef*) OR (Fresh produce*).

A total of 812 articles were selected, and 188 duplicate records were removed. After initial screening by titles and abstracts, 407 irrelevant papers were deleted. Fifty records

were excluded because they are short reports without conclusive results. Thus, a total of 167 articles were analyzed and classified into three groups. Briefly, 1) Detection and isolation methods of STEC from different matrices; 2) Worldwide outbreaks related to STEC and its causative serotypes; 3) Microbiological safety of manure or manure-amended soil, including the pathogen survival during composting process.

From above 3 groups, this review was performed as following:

1. Introduction of bacteriological characteristics of STEC
2. Identification of pathogen survival in manure or manure-amended soil
3. Evaluation of survival and regrowth of pathogens especially STEC during composting process and in the finished compost
4. Outbreaks and causative STEC serotypes
5. Current detection and isolation methods for STEC

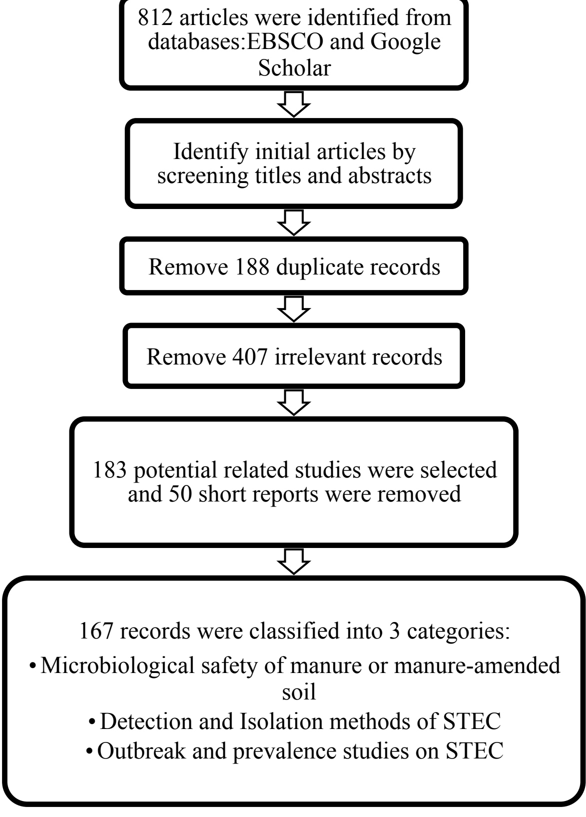

Figure 1.1 Flow chart of literature review.

Non-O157 Shiga toxin-producing *E. coli*

STEC belong to the *Enterobacteriaceae* family and the *Escherichia* genus. They are Gram-negative bacillus and characterized by their serogroups and virulence genes. Shiga toxins encoded by *stx* genes carried by lysogenic phages are the main virulence factors for STEC (Sandvig *et al*., 2001). Shiga toxins are capable of binding the cellular receptors and inhibiting the protein synthesis in several organs such as kidney, brain and liver causing severe diseases (Smith *et al.*, 2013). Based on current studies, STEC strains expressing *stx2* genes are more likely to cause Hemolytic Uremic Syndrome (HUS) as compared to strains that express *stx1* alone (Ethelberg *et al.*, 2004; Lindgren *et al.*, 1994).

E. coli O157:H7 is the most common STEC serotype involved in human gastrointestinal infections around the world. Nonetheless, non-O157 STEC has recently caused growing concern over their ability to cause of human gastrointestinal disease (Mainil *et al.*, 2005). Besides *stx1* and *stx2*, most non-O157 STEC strains frequently associated with severe outbreaks carry a 43-kb pathogenicity island called locus of enterocyte effacement (LEE). LEE contains *eaeA* gene that encodes intimin outer membrane protein, which is required for intestinal colonization of STEC (Boerlin *et al.*, 1999; Barrett *et al.,* 1992).

Non-O157 STEC is a zoonotic pathogen that can infect both human and animals. Several studies reported that the *stx* genes could be isolated from human sewage and animal fecal sources (Garćıa-Aljaro *et al.*, 2005; Jenkins *et al*., 2003). A prevalence study throughout New Zealand showed a 3.8% incidence of STEC in bovine fecal samples (Moriarty *et al.*, 2011). Similar prevalence studies reported that the fecal prevalence of non-O157 STEC in France ranged from 7.9 to 34% (Pradel *et al.*, 2000; Rogerie *et al*., 2001). As a result, the fecal contamination can take place from animal hides to the carcass during the slaughtering process, although the level of non-O157 STEC was reduced after the process (Rogerie *et al.*, 2001; Leung *et al*., 2001). Arthur *et al*. (2002) found STEC proportion was 58.3% of pre-evisceration samples and 8.3% of post-processing samples. As those meat products destined for human consumption, the sporadic cases of STEC due to contaminated meat product were also reported (Bosilevac *et al.*, 2007; Arthur *et al.*, 2002).

Some of the STEC strains are stress-resistance to the environment and other chemical-physical treatments. For example, Duffy *et al.* (2006) found that the STEC

O157 and O26 survived for 18 days in yogurt and 30 days in orange juice at 4°C, respectively. Hiramatsu *et al.* (2005) also claimed that STEC O157, O26 and O111 showed high resistance to desiccation under refrigerated storage conditions. *E. coli* O157:H7 had been considered as the representative serotype for evaluating the thermal tolerance of STEC in the food samples (Juneja *et al.*, 1997; Smith *et al.*, 2001.). Recently, several studies claimed that cooking times and temperatures that adequate to inactivate *E. coli* O157 could also be effective against non-O157 STEC strains (Luckansky *et al.*, 2013; Enache *et al.*, 2011). Luchansky *et al.* (2013) compared the D-values of *E. coli* O157:H7 strain versus seven strains of non-O157 STEC, and conducted that non-O157 STEC was within the thermal tolerance range of *E. coli* O157. Moreover, Enache *et al.* (2011) found that *E. coli* O157:H7 had D-values similar to or higher than the individual six non-O157 STEC serotypes (O26, O45, O103, O111, O121, O145) in apple juice.

As most foodborne pathogens are transmitted through fecal-oral route, STEC can survive extremely or moderately acidic environments (Lin *et al.*, 1996). Studies have shown that the acid resistance of STEC strains is an additional phenotype that may be related to the genotype (Lee *et al.*, 2012). Barua *et al.* (2002) in his study created three *E. coli* O157 mutants by inserting mini-Tn5 to the *fcl, wecA (rfe)* and *wecB (rffE)* genes, and concluded that the surface polysaccharides is indispensable to the organic acid resistance of *E. coli* O157 (Wang *et al.*, 1998; Samuel *et al.*, 2004; Barua *et al.*, 2002). As observed by Bergholz *et al.* (2007), stationary-phase O26 and O111 strains were less acid resistant than *E. coli* O157 in a model stomach system. The better adaptation to gastric acidity of *E. coli* O157 than the non-O157 STEC serotypes tested may result from the higher

activity of the glutamate decarboxylase system in *E. coli* O157. On the other hand, some studies showed that there was a great variability in survival of STEC strains (O26, O88, O91, O111, O113, O116, O117, O157, O171, OX3, O113, O121 and O157) when inoculated into acidified broth (Lino *et al.*, 2011; Molina *et al.*, 2003). In agreement with Bergholz's study, Molina *et al.* (2003) also reported that STEC O26 had the least acid resistance in Luria-Bertani broth with pH value of 3.0. Additionally, the non-O157 STEC isolates showed a greater acid tolerance response at 25°C as compared to 32°C (Brudzinski *et al.*, 1998).

Understanding that STEC including *E. coli* O157 and non-O157 strains could survive in a wide range of the animal host, animal feces and fresh produce is important as these pathogens may cause life-threatening human infection. Furthermore, the easy transmission and low infective dose of STEC, ca. 10 cells (Etcheverría *et al.,* 2013), pose great challenges for preventing fecal-oral contamination of this group of microorganisms.

Pathogen survival in manure and manure-amended soil

The ability of a pathogen to survive in manure and manure-amended soil is a major factor for on-farm contamination and fecal-oral transmission to food supplies (Larrie-Bagha *et al.*, 2013). Over 335 million tons of dry matters are produced annually in the United States at concentrated animal feeding operations (CAFOs) and animal-feeding operations (AFOs) (USDA, 2006) containing about 8.3 million tons of nitrogen (N) and 2.5 million tons of phosphorus (P). The proper application of animal manure in different forms provides essential nutrients for crop growth, and also can improve soil quality by

increasing soil organic matter reserves, improving water-holding capacity and enhancing water infiltration rates (USDA, 2008).

As proposed by the Food and Drug Administration (FDA) Food Safety Modernization Act (FSMA), the presence of human pathogen in biological soil amendment of animal manure could lead to the amendment acting as an inoculum that results in replication of pathogen in the produce growing field, which can lead to the contamination of fresh produce (FSMA 2012).

Animal manure contains a wide variety of microorganisms, including human pathogens, such as *E. coli*, *Salmonella*, *Listeria monocytogenes*, *Clostridium*, *Campylobacter*, and *Yersinia enterocolitica* and so on (Table1.1). The colonized cows may shed 2 to 7 log CFU/g of *Salmonella* spp. and 2 to 5 log CFU/g of *E. coli* O157:H7 in animal feces (Himathongkham *et al.,* 1999). Microbiological surveys have shown that the prevalence of microorganisms in fecal sample depends on microbial species, pathogen serotypes, animal species, geographic areas and so on (Table 1.1). Noted that even in the same sampling field, the prevalence of pathogen varied throughout different sampling sites (Simango *et al.*, 2006; Bagge *et al.*, 2009; Hancock *et al.*, 1997). For example, the shedding level of *E. coli* O157 was higher in herds samples (75%) compared with fecal samples (1.41%) based on analysis of 12,664 fecal samples and 37 herds samples collected in 100 feedlots in 13 states in USA (Hancock *et al.*, 1997).

There were several studies documenting the population level of STEC or Shiga toxin genes in animal farms worldwide. Fecal testing of dairy cattle around the world showed that the prevalence of *E. coli* O157 ranged from 0.2 to 48.8% whereas non-O157 STEC

were in the range of 0.4 to 74% (Hussein *et al*., 2005). Cobbold *et al*. (2004) isolated STEC strains from 7.4% of fecal samples collected from 22 farms in Washington State, USA. Ennis *et al*. (2012) determined the prevalence of STEC by collecting 650 fecal samples from 12 beef farms in the Ireland, and reported that 13.7% were *stx* positive including serotypes *E. coli* O157 and non-O157 STEC. The shedding level of STEC was serotype-dependent, which was also significantly associated with the origin of animal source, environmental source and seasons. For examples, Jeon *et al*. (2006) investigated the prevalence of non-O157 STEC in South Korea, and reported that 6.67% fecal samples were serotyped as STEC O26 compared with 4.57% for STEC O111. Irino *et al.* (2005) found that the STEC isolation rate ranged from 3.8 to 84.6% depending on the dairy farm investigated. Franz *et al.* (2007) observed difference in the prevalence of STEC between organic dairy farm (61%) and low-input conventional dairy farm (36%). There was also a seasonal factor as the lower STEC prevalence was always found in winter (Cobbold *et al*., 2004; Hancock *et al*., 2001; Van *et al*., 1999; Barkocy-Gallagher *et al.*, 2003).

STEC can be sporadically carried by healthy ruminants and present in feces for a long time (Kearney *et al.*, 1993; Wang *et al.*, 1996). Several studies have shown that the pathogens can persist in the animal manure for weeks or months, and this survival was influenced by a variety of factors, i.e. animal species diets, handling and treatment of manure, location of manure pile, moisture content, sampling seasons and other organic residues present in the soil (Himathongkham *et al*., 1999; Fukushima *et al.*, 1999b). Kudva *et al*. (1998) reported that *E. coli* O157:H7 survived for 47 days in moist middle layers of aerated bovine manure piles whereas 21 months in non-aerated bovine manure

piles under different environmental conditions with a bacterial concentration ranging from 2 to 6 log CFU/g.

STEC can survive for long period after manure being incorporated into the cropland (Lynn *et al.*, 1998; Fenlon *et al.*, 2000). Artificially inoculated non-O157 STEC strains persisted in the manure-amended soil with survival time ranging from 16.5 to 98.2 days depending on the soil types and serotypes (Bolton *et al*., 2011; Fremaux *et al*., 2008; Ma *et al*., 2014). A greenhouse study conducted under different seasons reported a survival time of 5 to more than 28 days, suggesting that, the survival potential of pathogens was influenced by bacterial species, temperature, light intensity and moisture content (Kim *et al.*, 2010). Cross-contamination occurs when the contaminated agricultural water or soil amendment is applied to the field. Islam *et al.* (2005) conducted a field study and showed that *E. coli* O157:H7 survived in soil samples for 154–196 days, and was detected for 74 and 168 days on onions and carrots, respectively, suggesting that on-farm contamination through the animal wastes might result in the contamination of fresh produce.

In summary, animal waste is routinely applied to agricultural land as fertilizer or soil amendment, which may contain pathogenic microorganisms. As a result, pathogens become potential microbial hazards contaminating fresh produce. Thus, it should be emphasized that reducing pathogens in livestock manure is critical to curtail contamination of environment and our food supplies.

Table 1.1 Summary of studies on foodborne pathogens in fecal or environmental samples

Pathogen	Year/Location	Sample source	Sample type	Sample size	Prevalence	Reference
Arcobacter	2000/Minnesota US	Health cattle and Hogs	Fecal samples	N.A[a]	11% for cattle and 46% for hogs	Wesley *et al.,* 2000
Campylobacter	2002/Ohio US	Livestock	Fecal samples	686	7%	Dodson *et al.,* 2005
	2003/Wisconsin US	Organic and conventional farms	Fecal samples	1191	27.9%	Sato *et al.,* 2004
	2002/Texas US	Cattle	Fecal samples	100	64-68%	Beach *et al.,* 2002
	2000/Minnesota US	Health cattle and Hogs	Fecal samples	N.A	24.57% for cattle and 69.28% for hogs	Wesley *et al.,* 2000
	2008/Canada	Manure storage tanks & fresh pooled feces	Fecal samples	359	36.5 %	Farzan *et al.,* 2010
	N.A/ Netherlands	Pigs	Fecal samples	N.A	85%	Weljtens *et al.,* 1993
	2003/UK	3–17 months young stock	Fecal samples	N.A.	62.5%	Ellis-Iversen *et al.,* 2009
	2001-2003/ US	Mature cattle	Fecal samples	610	23.4%	Gharst 2004
Clostridium	N.A./Canada	Horses	N.A.	135	10.3%	Schoster *et al.,* 2012
	2008/Ohio US	Cattles	Fecal samples	944	1.8%	Rodriguez-Palacios *et al.,* 2011
	N.A./Germany	Diseased cow	Fecal samples	196	22.5%	Krüger *et al.,* 2012

Table 1.1 *Cont.*

Pathogen	Year/Location	Sample source	Sample type	Sample size	Prevalence	Reference
Clostridium	2005-2007/Sweden	Soil, manure and substrate from biogas plants	Muscle, blood, manure and soil samples	254	Muscle samples (32%), biogas process (10%), absent in soil samples	Bagge *et al.,* 2009
	N.A./Zimbabwe	Homestead	Environmental samples	656	64.7%	Simango *et al.,* 2006
E. coli O157	2002/Ohio US	Livestock	Fecal samples	1026	*E. coli* O157 2.1%	Dodson *et al.,* 2005
	N.A/France	Manure & sewage sludge	Fecal samples	752	*E. coli* O157 21%	Vernozy *et al.,* 2002
	1994/ Wisconsin US	Dairy farm	Environmental samples	560	1.8%	Faith *et al.,* 1996
	N.A./France	Manure, slurry and sewage sludge	Environmental samples	752	24%	Vernozy-Rozand *et al.,* 2002
	2008/Canada	Swine manure storage tanks & pooled faces	Fecal samples	359	*E. coli* O157 3.3 %	Farzan *et al.,* 2010
	N.A./Australia	Diagnostic bovine	Fecal samples	191	47.1% yielded STEC or EPEC	Hornitzky *et al.,* 2005
	N.A./Virginia US	Ruminant	Fecal samples	287	N.A.	Pao *et al.,* 2005
	N.A./Belgium	Farms	Fecal samples	59	*E. coli* O157 100%	Verstraete *et al.,* 2014

Table 1.1 *Cont.*

Pathogen	Year/Location	Sample source	Sample type	Sample size	Prevalence	Reference
E. coli O157	N.A./Washington US	Cattle	Fecal samples and herds samples	3410	8.58%	Hancock *et al.,* 1994
	2011/Switzerland	Slaughtered cattle	Fecal samples	563	7.8%	Hofer *et al.,* 2012
	2008-2010/ California US	Leafy green production region	Environmental samples	13650	2.6%	Cooley *et al.,* 2013
	2004/Netherland	Organic and conventional farms	Manure sample	N.A.	61% for ORG and 36% for LIC	Franz *et al.,* 2007
	1994/Oregon US	Cattle house	Fecal herds samples	12701	76.41%	Hancock *et al.,* 1997
	1995-1996/Canada	Cattle	Fecal samples	247	2.6-7.5%	Van *et al.,* 1999
	1994/US	Cattle	Fecal samples	11881	*E. coli* O157 1.8%	Hancock *et al.,* 1997
		2002/Brazil	Animal	Fecal samples	454	30.4%
	N.A./Kansas US	Cattle	Fecal samples	3152	*E. coli* O157 1.3%	Sargeant *et al.,* 2000
	2004-2005/Kansas US	Cattle	Fecal samples	891	*E. coli* O157 9.2%	Alam *et al.,* 2006
	N.A./Oregon&Washington US	Cattle	Environmental samples	735	11.1%	Hancock *et al.,* 1998

Table 1.1 *Cont.*

Pathogen	Year/Location	Sample source	Sample type	Sample size	Prevalence	Reference
Non-O157 STEC	1997-1999/Switzerland	Plants	Stool samples	5590	47 strains	Stephan *et al.*, 2000
	2006/Bangladesh	Plants	Fecal samples	423	71 strains	Isam *et al.*, 2008
	2001/Brazil	Dairy farm	Feces	153	3.8 to 84.6%	Irino *et al.*, 2005
	2002-2004/Scotland	Cattle farm	Fecal pats	6086	O26: 23% O103: 22% O145: 10%	Pearce *et al.*, 2006
	1997/Spain	Lambs flocks	Fecal swabs	1300	36%	Blanco *et al.*, 2003
	2002-2004/South Korea	Beef and dairy cattle farm	Fecal samples	809	O26: 6.67%, O111: 4.57% and 1.98% for both	Jeon *et al.*, 2006
	2008-2010/ California US	Leafy green production region	Environmental samples	13650	14%	Cooley *et al.*, 2013
	Germany	Cattle farm	Fecal samples	-	29-82%	Geue *et al.*, 2002
	2002/Washington US	Farm	Fecal samples	1440	STEC 7.4%	Cobbold *et al.*, 2004
	2004/UK	Sheep	Fecal samples	1082	*E. coli* O26 4%	Evans *et al.*, 2008
	2001/UK	Bovine feces	Fecal samples	745	O26: 85% & O103: 37%	Jenkin *et al.*, 2003
	2002/Brazil	Animal	Fecal samples	454	O111: 18.9% & O113: 3.3%	Vicente *et al.*, 2005
	N.A./US	Feedlot cattle	Fecal samples	1897	1.8%	Kalchayanand *et al.*, 2013

Table 1.1 *Cont.*

Pathogen	Year/Location	Sample source	Sample type	Sample size	Prevalence	Reference
Non-O157 STEC	2011/Switzerland	Slaughtered cattle aged between three and 24 months	Fecal samples	563	O145: 41.9%, O103: 25.9%, O26:0.8%, and O111: 23.9%	Hofer *et al.,* 2012
	2002-2004/South Korea	Beef and dairy cattle farm	Fecal samples	809	6.67% for *E. coli* O26, 4.57% for *E. coli* O111 and 1.98% for both	Jeon *et al*., 2006
	2002/Brazil	Animal	Fecal samples	454	*E. coli* O111 18.9% & *E. coli* O113 3.3%	Vicente *et al.,* 2005
Listeria	2008/Canada	Manure storage tanks & fresh pooled faces	Fecal samples	359	3.3 %	Farzan *et al.,* 2010
Salmonella	2008/Canada	Manure storage tanks & fresh pooled faces	Fecal samples	359	31.5 %	Farzan *et al.,* 2010
	1995-1996/Canada	Cattle at processing	Fecal samples	247	0.08%	Van *et al.,* 1999
	2002/Texas US	Cattle	Fecal samples	100	3-5%	Beach *et al.,* 2002
	2002/Ohio US	Livestock	Fecal samples	585	6.7%	Dodson *et al.,* 2005

Table 1.1 *Cont.*

Pathogen	Year/Location	Sample source	Sample type	Sample size	Prevalence	Reference
Yersinia enterocolitica	2008/Canada	Manure storage tanks & fresh pooled faces	Fecal samples	359	5.8 %	Farzan *et al.,* 2010

[a] N. A. not applicable
[b] –, negative for STEC

Composting and fate of pathogen during composting process

Composting is a controlled biological process undergoing a thermophilic decomposition of organic residues such as manure, animal carcasses, straw, and yard trimmings by aerobic microorganisms (SSSA, 1997). The process of composting broadly consists of four typical phases based on the temperature generated and active microbial community regardless of the materials used (USEPA 1999): mesophilic, thermophilic, cooling and maturation phases (Haug *et al.*, 1993). Due to the temperature fluctuations, the activity and diversity of microbial community change during the composting process (Hassen *et al.*, 2001). This microbial metabolism also generates heat that can inactivate pathogens and convert the organic matter to a more uniform, stable and nutrient-rich soil amendment (Larney *et al.*, 2003). In general, the heat generated during composting is the leading factor for pathogen reduction. However, there are other factors that should be well-controlled in order to ensure the microbial safety of composting process. These key factors include balanced nutrient level from raw ingredients (carbon to nitrogen ratio, C: N), proper moisture content, pH and O_2 level of the compost mix, the heterogenetic nature due to heap turning and the outdoor environment of composting process (Sherman

et al., 2005; Erickson *et al*., 2009; Shepherd *et al*., 2010; Ceustermans *et al.,* 2007; Sundberg *et al.*, 2004; Green *et al*., 2004).

Composting is an environmental friendly way to degrade organic waste. It is relatively easy to handle and effective to kill pathogens along with a reduction of the manure volume approximately fifty to sixty-five percent (Flynn *et al.*, 1996). The finished compost is an excellent humus-like soil amendment, and can be applied to agricultural fields as a soil amendment to improve soil structure and increase microbial and enzymatic activities (Spiehs *et al.*, 2007). The quality compost should be thoroughly decomposed and pathogen-free, as the composting process is considered as a "process to further reduce pathogens" (PRFP). The composting process is regulated by the US Environmental Protection Agency under the Code of Federal Regulations Title 40 Part 503 Biosolids rule (USEPA, 2008). Based on this guideline (Table 1.2), in order to obtain a Class A soil amendment, the static aerated composting or in-vessel composting should be maintained at 55°C for 3 days, whereas windrows should reach 55°C for 15 days with a minimum of 5 turnings. Additionally, according to the USDA-National Organic Program standards, the growers should ensure that the composts maintain temperatures in the range of 55-77°C for a minimum of 3-15 days depending on type of composting system (NOSB 2002).

Table 1.2 USEPA temperature-time requirements for biosolid

Composting method	Time/Temperature guidelines	Microbial level
Static aerated heap	>55°C for consecutive 3 days	<1000 MPN/g *E. coli* in 1 g dry weight and <3 MPN *Salmonella* in 4 g dry weight
Window	>55°C for 15 days with a minimum of 5 turnings	

There were several laboratory-based studies providing scientific data to predict the fate of pathogens in composting process. Lung *et al*. (2001) reported that with an initial level of ca. 7 log CFU/g, *E. coli* O157:H7 and *Salmonella* could not be detected after 3 days when held at 45-48°C. However, Jiang *et al*. (2003) found that *E. coli* O157: H7 survived for 14 days in all the locations of the bioreactor holding at 50°C.

The fate of pathogen during composting may be overestimated under the controlled lab condition. In the real outdoor composting environment, the dynamics of pathogen inactivation may be influenced by different sampling locations of the compost heaps, compost ingredients, composting method and environmental variations. Therefore, some studies were conducted to investigate the inactivation of pathogens by composting in a field setting (Shepherd *et al.*, 2007; Larney *et al.*, 2003). As was observed by Himathongkham *et al.* (1999), *E. coli* O157 and *Salmonella* spp. survived longer in the top layer of stacked manure piles compared to the middle or toe part. In another field study, *E. coli* O157:H7 was not recovered from top layer sample after 28 days in the turned manure-based piles whereas for the unturned stockpiles, *E. coli* O157:H7 was tested positive up to 42-52 days at the top layer, and 84 days from the toe samples. *Salmonella*, *Campylobacter* spp., and *L. monocytogenes* were not detected in either top or

toe samples at the end of the composting period (Berry *et al*., 2013). As concluded from these mentioned studies, the variability in the pathogen inactivation in different heap locations may result from the highly heterogeneous nature of compost ingredients and the stratification of temperature that occurs throughout the heaps.

In conclusion, animal manure-based composting may achieve the adequate level of pathogen reduction by following the standards established by federal and state agencies. However, conditions such as improper composting process and inadequate treated compost ingredients may contribute to the survival of pathogen during composting.

Pathogen regrowth and persistence in the finished compost

The high quality finished compost should be stabilized and sanitized. However, a few pathogenic cells may be reintroduced or survived after composting, and these pathogenic cells could grow or persist under certain conditions. This regrowth and survival of pathogen in the finished compost is an increasing microbial safety concern, since the potential regrowth of pathogen may take place due to insufficient treatment from the active composting process or the environmental contamination.

It is well-known that moisture content (MC) of the compost mixture can influence the growth of microorganisms by upholding the microbial activity (Zaleski *et al.*, 2005). As observed by Kim *et al*. (2009), the regrowth of *E. coli* O157 was found in dairy compost with at least 20% moisture content. And in another study of Kim *et al.* (2010), with an initial inoculation level of ca. 1 log CFU/g, *E. coli* O157:H7 was detected over 28

days by enrichment in compost with a 30% moisture content regardless of season under greenhouse condition.

Furthermore, finished compost containing certain populations of indigenous microflora plays an important role in pathogen suppression and regrowth (Kim *et al*., 2011; Zaleski *et al.*, 2005; Wolna-Maruwka *et al.*, 2012). Kim *et al.* (2011) showed a negative correlation between the growth of *E. coli* O157:H7 and the population of background microorganism. *Actinomycetes* and fungi were found as critical indigenous microorganisms for the suppression of *E. coli* O157: H7. In another study, Kim *et al.* (2009) reported that regrowth of *E. coli* O157: H7 was suppressed by ca. 6.5 log CFU/g of background microflora in compost. Apparently, the competition for nutrients and available water between pathogenic bacteria and background microbes leads to a reduction of pathogen.

According to the Leafy Greens Management Association (LGMA) and Food Safety Leadership Council On-Farm Produce Standards (FSLC) standards for soil amendments, the finished compost should be tested and free of human pathogens, and applied to the field for at least 45 days before harvest (LGMA, 2008).

As the animal waste-based soil amendment is one of the major risk factors in the contamination of fresh produce, controlling pathogen regrowth in compost is critical to ensure microbial safety of these crops.

Outbreaks associated with causative STEC serotypes

Sporadic cases of illnesses and outbreaks linked to non-O157 STEC serotypes other than *E. coli* O157 appear to be on the rise worldwide. From 1984 to 2009, the epidemiological results showed that the most common non-O157 STEC serotypes reported worldwide were O26 (37%), O111 (31%), O103 (6%), O121 (5%), O145 (5%) and O45 (1%) (Kalchayanand *et al*., 2012).

An epidemiological study throughout Minnesota area between 2000-2006 reported that urban site human outbreaks, patients with non-O157 STEC infection were more likely to be related to the consumption of water. Conversely, a significantly higher proportion of patients with *E. coli* O157 was linked to ground beef (Hedican *et al*., 2009). Another prevalence study conducted in Nebraska revealed that non-O157 STEC were as prevalent as *E. coli* O157. From 335 stool samples, 4.2% were positive for STEC with five non-O157 STEC serotypes being isolated from those positive samples, i.e. O111: NM, O26:H11, O145: NM, O103:H2, and Orough: H2. Four of them (O111, O26, O145 and O103) belong to the top six STEC as designated by USDA (Fey *et al.,* 2000).

From the FoodNet 2012 surveillance report, the number and incidence of laboratory-confirmed non-O157 STEC infections were listed in Table 1.3, indicating that 6 STEC serogroups (O26, O45, O103, O111, O121, and O145) account for ca. 75% of the reported non-O157 STEC illnesses in the United States (Johnson *et al*., 2006; Robbins *et al*., 2014; USDA, 2012a). By taking consideration of the growing public concern on non-O157 STEC, Food Safety and Inspection Service (FSIS) proposed the zero-tolerance policy for top six non-O157 STEC (O26, O45, O103, O111, O145 and O121) as the same way to *E. coli* O157:H7 in beef product (USDA 2012b).

Table 1.3 Number and incidence of laboratory-confirmed non-O157 STEC infections

Rank	STEC serogroup	Cases confirmed	Prevalence for per 100,000 persons
1	O26	120	0.26
2	O103	77	0.16
3	O111	56	0.12
4	O145	22	0.05
5	O121	16	0.03
6	O45	15	0.01

[*]Adapted from Foodborne Diseases Active Surveillance Network (FoodNet) 2012 annual report, United States

Current detection and isolation methods for non-O157 STEC

As discussed above, the cross-contamination between live healthy animals and the finished meat products may occur during slaughtering processing. Moreover, the on-farm fecal contamination is also a leading factor for the outbreaks related to STEC. Since the non-O157 STEC was regarded as emerging foodborne pathogens more recently, a reliable, accurate and rapid detection method is needed to detect non-O157 STEC in a variety of high-risk matrices.

Current detection and isolation methods include culture-based, PCR-based and immunoassays for Shiga toxin or major STEC strains. Prior to the actual detection procedure, sampling and sample preparation are critical steps, since the pathogen always present in the complex matrix (Stevens *et al.*, 2004). Both FDA Bacteriological Analytical Manure (BAM) Chapter 4A (FDA, 2012) and FSIS Microbiology Laboratory Guidebook 5B have described methods for non-O157 STEC detection (USDA 2014). These methods combine traditional, immunological, and molecular biological

approaches. With the enrichment step involved, the detection duration is always 3-4 days. Many test kits are available in recent years for the rapid detection of non-O157 STEC, and more novel protocols are being tested (Wang *et al*., 2013). For example, the BAX® System real-time PCR assay suite for detecting STEC was approved by USDA most recently (USDA, 2014). The following sections briefly review the current detection methods for STEC from different matrices.

Culture-based methods consist of two basic steps, i.e. enrichment and plating onto selective agar, followed by biological or serological tests for further confirmation (Ge *et al.*, 2009). Due to the presence of interfering background microflora, the proper selection of selective supplements is critical. The selective reagents for non-O157 STEC are sometimes developed from *E. coli* O157 detection (Wang *et al*., 2013). As listed in Table 1.4, bile salts, novobiocin, tellurite and cefixime (CT supplement) are commonly used as selective reagents for STEC detection used in the enrichment broth or selective agar (Cooley *et al*., 2013; Hara-Kudo *et al*., 2000; Fukushima *et al*., 1999b; Varela-Hernández *et al*., 2007; Fratamico *et al*., 2014; Lionberg e*t al*., 2003 *etc*.). Bile salts and novobiocin are capable of inhibiting gram-positive bacteria (MCDonough *et al*., 2000; Lionberg *et al.*, 2003), whereas cefixime and tellurite can inhibit the growth of most non-verocytotoxigenic *E. coli* (Association of Public Health Laboratories, 2012). Hofer *et al.* (2012) applied 16 µg/l novobiocin in modified TSB for selecting STEC O26, O103 and O145. In contrast, even the higher concentration of novobiocin (16-20 µg/l) was widely used for *E. coli* O157 isolation from different matrices (Himathongkham *et al*., 1999; Almeid *et al*., 2013). USDA FSIS procedure suggests a lower concentration of

novobiocin (8 μg/l) for isolating non-O157 STEC (USDA 2014; Tillman *et al.*, 2012), which was also in agreement with other published studies (Cooley *et al*., 2013; Fratamico *et al*., 2014; Auvary *et al.*, 2007; Kanki *et al*., 2011).

When the target microorganism is very low, the enrichment step is essential to allow the target cell to grow to a detectable level. Table 1.4 listed the most common enrichment broths being used for enriching STEC from different matrices. USDA FSIS protocol included mTSB supplemented with novobiocin is recommended for enriching STEC from meat product. Similar to USDA FSIS protocol, Kanki *et al*. (2011) compared four enrichment broths for detecting STEC O91, O103 O111, O119, O121, O145 and O165 from food samples and pure culture, and concluded that enrichment in mTSB containing bile salts was useful for detecting non-O157 STEC cells from food samples. Moreover, mTSB enrichment broth was also applicable to fecal or fresh produce samples (Tutenel *et al*., 2003; Hofer *et al*., 2012; Hara-Kudo *et al.*, 2000).

As for the enrichment conditions, incubation at 42°C is often preferred, because it improved suppression of competing microflora and gave better recovery of STEC in fresh produce (Drysdale *et al.*, 2004; Hara-Kudo *et al.*, 2000; Gonthier *et al.*, 2001). The temperature-duration condition (42°C for 6 h) showed relatively higher sensitivity for recovering STEC from fecal samples and ground beef samples (Lionberg e*t al*., 2003; Tutenel *et al*., 2003).

There are several commercial available selective agars such as CHROMagar®STEC, Rainbow® agar O157 and R&F® STEC chromogenic agar for STEC enumeration or isolation (Table 1.4). Normally, there was no requirement of supplement for these

chromogenic agars for selecting STEC; however, several studies modified Rainbow agar by adding novobiocin and CT supplement for selecting non-O157 STEC (Cooley *et al*., 2013; Hara-Kudo *et al*., 2000; Tillman *et al*., 2012). Due to the cost of above chromogenic agar, some selective agars were developed on the basis of some cost effective agar bases, such as MacConkey agar (Tang *et al*., 2014; Fukushima *et al*., 1999a; Varela-Hernández *et al*., 2007; Novicki *et al*., 2000). Bibbal *et al*. (2014) showed that on the basis of MacConkey agar, sorbitol and raffinose could serve as the primary carbohydrate sources for isolating STEC. Posse *et al*. (2007) developed a novel medium using a mixture of carbohydrate sources, which was capable of differentiating STEC O26, O103, O111 and O145. Moreover, as most pathogenic *E. coli* strains are acid tolerant, the post acid treatment was also applied in some protocols in order to isolate *E. coli* from food and environmental samples (Waterman *et al.*, 1996; Tillman *et al*., 2012).

Table 1.4 A list of selected current selective agars media and enrichment broths for detecting STEC from different sample matrices

Agar or Broth	Media	Target strains	Matrix	Supplement	References
Selective agar	CHROMagar ®STEC	Top six and O91, O104, O128	Fresh produce	N.A[b]	Kase *et al*., 2014
		STEC	Fecal samples	N.A	Zhao *et al*., 2013
		Top six	Beef and cattle feces	N.A	Kalchayanand *et al*, 2013
		STEC	Sheep	N.A	Asakura *et al*., 1998
		STEC	Fecal, plant, soil and water sample	N.A	Cooley *et al*., 2013
		STEC	Beef carcasses	N.A	Varela-Hernández *et al*., 2007
		STEC O26, O103, O111, O118, O121, O145 and O157	Fresh produce	N.A	Tzschoppe *et al*., 2012
		STEC O26, O45, O103, O111, O121, O145, O157	Pure culture	N.A	Tang *et al*., 2014
		STEC O157, O26, O103, O111, and O145	Food	N.A	Gill *et al*., 2012
	RB® O157[a]	STEC	Fecal, plant, soil and water sample	Novobiocin and tellurite	Cooley *et al*., 2013
		Top six and O91, O104, O128	Fresh produce	N.A	Kase *et al*., 2014
		STEC non-O157	Fresh produce	N.A	Kase *et al*., 2012

Table 1.4 *Cont.*

Agar or Broth	Media	Target strains	Matrix	Supplement	References
Selective agar	RB® O157[a]	Top six	Beef	N.A	Fratanico *et al*., 2014
		Top six	Pure culture	N.A	Windham *et al*., 2013
		STEC O26, O157, O103, O121 and O111	Stool samples	N.A	Novicki *et al*., 2000
		STEC O26, O45, O103, O111, O121, O145, O157	Pure culture	N.A	Tang *et al*., 2014
		STEC O26	Ground beef and radish sprout	Novobiocin	Hara-Kudo *et al*., 2000
		STEC O26, O45, O103, O111, O121, and O145	Ground beef	Novobiocin, Tellurite and cefixime	Tillman *et al*., 2012
	R&F® STEC	Top six and O91, O104, O128	Fresh produce	N.A	Kase *et al*., 2014
		Top six	Beef and cattle feces	N.A	Kalchayanand *et al*., 2013
		STEC O157	Fresh produce	N.A	Kase *et al*., 2012
		STEC O26, O45, O103, O111, O121, O145, O157	Pure culture	N.A	Tang *et al*., 2014
	SHIBAM	Top six and O91, O104, O128	Fresh produce	N.A	Kase *et al*., 2014
		STEC	Fresh produce	N.A	Lin *et al*., 2012
	L-EMB	Top six and O91, O104, O128	Fresh produce	N.A	Kase *et al*., 2014

Table 1.4 *Cont.*

Agar or Broth	Media	Target strains	Matrix	Supplement	References
Selective agar	L-EMB	STEC	Fresh produce	N.A	Lin *et al*., 2012
		STEC O157	Fresh produce	N.A	Kase *et al*., 2012
	SMAC	STEC	Beef carcasses	Tellurite and cefixime	Varela-Hernández *et al*., 2007
		STEC O157	Fresh produce	Tellurite and cefixime	Kase *et al*., 2012
		STEC O26, O111 and O157	Feces	Tellurite and cefixime	Fukushima *et al*., 1999a
		STEC	Sheep	N.A	Asakura *et al*., 1998
		STEC O26, O45, O103, O111, O121, O145, O157	Pure culture	N.A	Tang *et al*., 2014
		STEC	Fecal, plant, soil and water sample	Tellurite and cefixime	Cooley *et al*., 2013
	RMAC	STEC O26 and O111	Minced beef	Tellurite and cefixime	Catarame *et al*., 2003
Enrichment broth	mTSB	STEC	Beef carcasses	Cefixime, cefsulodin and vancomycin	Varela-Hernández *et al*., 2007
		STEC O26 and O111	Minced beef	Cefixime, vancomycin and potassium tellurite	Catarame *et al*., 2003
		Top six	Beef	Novobiocin	Fratamico *et al*., 2014
		STEC O157	Feces	Novobiocin	Tutenel *et al*., 2003
		E. coli O26, O103,O111,O145 and O157	Fecal samples	Novobiocin	Hofer *et al*., 2012

Table 1.4 *Cont.*

Agar or Broth	Media	Target strains	Matrix	Supplement	References
Enrichment broth	mTSB	*E. coli* O26, O103, O111, O145 and O157	Beef	Novobiocin	Auvary *et al.*, 2005
		STEC O26	Ground beef and radish sprout	Vancomycin, cefixime and cefsulodin	Hara-Kudo *et al.*, 2000
		STEC O91, O103, O111, O119, O121, O145 and O165	Beef	Novobiocin	Kanki *et al.*, 2011
		STEC O157, O26, O103, O111, and O145	Food	Vancomycin	Gill *et al.*, 2012
	BPW	STEC O157	Feces		Tutenel *et al.*, 2003
		STEC O157	Ground beef	Cefixime+cefsulodin	Lionberg *et al.*, 2003
	mBPWp	STEC	Fresh produce		Kase *et al.*, 2012
	mEC	STEC O157	Groun d beef	Bile salt +novobiocin	Lionberg *et al.*, 2003
	R&F EB	STEC O91, O103, O111, O119, O121, O145 and O165	Beef	Novobiocin	Kanki *et al.*, 2011
		STEC O157	Ground beef	N.A	Lionberg *et al.*, 2003
		Non-O157 STEC	Food	N.A	Restaino *et al.*, 2012
		STEC O157	Feces		Tutenel *et al.*, 2003
		STEC O157	Feces	Bile salt +novobiocin	MCDonough *et al.*, 2000
		STEC	Fecal samples	N.A	Hornitzky *et al.*, 2005

[a] Agar abbreviations: RB® O157= Rainbow agar O157; R&F® STEC=R&F STEC chromogenic agar (O157 & non-O157); SHIBAM= STEC heart infusion washed blood agar with mitomycin-C; L-EMB=Levine's

eosin–methylene blue agar; SMAC= Sorbitol-MacConkey agar RMAC= rhamnose MacConkey agar; mTSB=modified tryptone soy broth; BPW=buffered peptone water; mBPWp=modified BPW with pyruvate; mEC= Modified EC broth; R&F® EB= R&F® STEC (O157 & non-O157) enrichment broth.
[b] N.A – not applicable.

The immunological assays include enzyme-linked immunosorbent assay, immunomagnetic separation (IMS), latex agglutination, *etc*. Most of them are commercial available as test kits (Wang *et al.*, 2013). Immunomagnetic beads coated with serotype-specific antibodies have been applied to capture STEC cells from naturally or artificially contaminated samples, thereby concentrating the target pathogens by removing inhibitors from samples simultaneously (Ge *et al*., 2009). As the antibodies recognize the specific antigens, the STEC cells can be isolated directly from matrices through IMS. The current USDA FSIS protocol uses RapidChekCONFIRM®non-O157 STEC IMS beads to detect non-O157 STEC from ground beef (USDA 2014; Tillman *et al.*, 2012). Additionally, IMS normally applies after overnight enrichment prior to some further tests such as PCR or real-time PCR as a concentration step (Hoffer *et al.*, 2011; Bibbal *et al*., 2014). However, there were some published studies claimed that the sensitivity of IMS depended on the formation of complex between antibodies and antigens, the IMS procedure, pathogen serotype and the matrix properties. Willford *et al*. (2011) reported that the detection sensitivity of IMS for non-O157 STEC ranged from 75.9 to 93% with the detection limit yielded from 5 to 6 log CFU/ml in pure culture.

Molecular-based methods such as PCR or real-time PCR are also used for screening *stx* or other virulence factors in non-O157 STEC. Conventionally, most PCR assays targeted *stx1*/*stx2* and *eae* genes that encode for Shiga toxins and intimin, respectively. In addition, multiplex PCR or real-time PCR assays target *stx* and *eae* genes along with

other virulence factors. Table 1.5 listed some selected PCR or real-time PCR assays for targeting virulence genes of STEC from different matrices. The time duration for the assay is normally shorter with a high sensitivity (1-10^3 CFU/reaction) (Sharma *et al*., 2002; Perelle *et al*., 2005; Verstraete *et al*., 2014; Kisler *et al*., 2011). Nonetheless, the assay performance also depends on the sample types and whether enrichment step is involved (Fratamico *et al*., 2011; Anklam *et al*., 2012). Additionally, *stx* and *eae* genes are regarded as the most frequently targeted genes for detecting STEC, however, other virulence factors such as *hlyA*, *wzx*, *wzy* are also included for developing the multiplex PCR assay (Paton *et al*., 1998; Madic *et al*., 2011; Kumar *et al*., 2012; Anklam *et al*., 2012).

Moreover, PCR assay has been combined with other methods such as immunological assays to enhance the detection sensitivity. For example, Auvray *et al.* (2007) identified 5 STEC serogroups by using real-time PCR combined with culture-IMS method. Luo *et al*. (2002) reported that PCR combined with immuno-capture method could enhance the detection sensitivity and allow identifying the pathogen serotype from non-pathogenic cells. Besides, the loop-mediated isothermal amplification (LAMP) methods showed as a specific and cost-effective pathogen detection method in food testing. Wang *et al.* (2012) used LAMP assay to detect top six non-O157 STEC from beef and fresh produce with a lower detection limit of 1-2 CFU/g of target cells from these samples after enrichment.

Table 1.5 A list of selected PCR and real-time PCR assay for STEC detection

Platform	Serogroup	Target genes	Matrix	Sensitivity	Reference
PCR	O111	*stx1, stx2, eae,* rfb_{O111}	Clinical	10^3 CFU/reaction	Paton *et al*., 1998
Real-time PCR	O26, O111	*stx1, stx2,* eae_{O26}, eae_{O111}	Beef and bovine feces	1-10 CFU/g	Sharma *et al*., 2002
PCR	O26, O103, O111, O145	wzx_{O26}, eae_{O103}, $wbdI_{O111}$,$ihp1_{O145}$	DNA	5-25 copies/ reaction	Perelle *et al*., 2004
PCR, Real-time PCR	O145	*stx1, stx2, wzx, wzy*	Food sample	2 CFU/25g	Fratamico *et al*., 2011
Real-time PCR	O26, O103, O111, O145	*stx, eae,* wzx_{O26}, wzx_{O103}, $wbdI_{O111}$, $ihp1_{O145}$	Cheese	> 5 CFU/25g	Madic *et al*., 2011
Real-time PCR	Top six	*stx1, stx2, eae, wzx*	Ground beef	50 CFU/reaction, 1–2 CFU/25 g after enrichment	Fratamico *et al*., 2014
Real-time PCR	STEC	*stx1, stx2, eae*	Feces	<2.7~3.7 copies/g	Verstraete *et al*., 2014
Real-time PCR	O26, O103, O145, O111 and O157	*stx1, stx2,* wzx_{O26}, eae_{O103}, $wbdI_{O111}$,$ihp1_{O145}$,$rfbE_{O157}$	Cattle	N.A	Hofer *et al*., 2012
PCR	O26	*stx1, stx2, eae, hlyA*	Feces	10^3 CFU/reaction	Evans *et al*., 2008
Real-time PCR	STEC	N.A	Fecal samples	10^3copies/g	Zhao *et al*., 2012
PCR	O103, O26, O111 and O145	*stx1*and *stx2*	Fecal samples	N.A	Jenkins *et al*., 2003
PCR	STEC	*stx, hlyA* rfb_{O157}	Fresh seafood and meat	N.A	Sanath *et al*., 2001

Table 1.5 *Cont.*

Platform	Serogroup	Target genes	Matrix	Sensitivity	Reference
PCR	STEC	*stx1*and *stx2*	Stool samples	N.A	Kumar *et al*., 2012
Real time PCR	STEC	*stx1, stx2, eae*	Fecal samples	9 copies/g	Kisler *et al*.,2011
Real time PCR	Top six and O157	*stx1, stx2, eae,* wzx_{O26}, wzx_{O103}, wzy_{O145}, $manC_{O111}$,wzx_{O121},$rfbE_{O157}$	Pure culture and fecal sample	10^3~10^4 CFU/ml w/o enrichment	Anklam *et al*., 2012

[a]*Partial adapted from text of Wang et al., 2013*

As listed in Table 1.6, for different study matrices, instead of using only one detection method, some studies included all three mentioned methods. According to the current USDA guide, detection of non-O157 STEC from ground beef consisted of culture, immunoassay, and molecular-based assay (USDA, 2014). This STEC detection guideline was in agreement with a large number of published studies (Evans *et al*., 2008; Kanki *et al*., 2011; Pao *et al*., 2005; Cooley *et al*., 2013; Berry *et al*., 2013 *etc*.). As some food samples may contain injured cells, pre-enrichment step in the universal enrichment broth is preferred prior to the selective enrichment (Kanki *et al.*, 2011). Nevertheless, for the high-background microflora matrices, such as fecal samples, the pre-enrichment step could be skipped (Hofer *et al.*, 2012).

With the increased demand for rapid and sensitive detection methods for STEC, biosensor as another detection system has been increasingly researched. Anderson *et al.* (2013) applied one biosensor (particle DNA recognition system) system to achieve the detection limit as 10^5 CFU/ml and 5 CFU/ml at the cost of \$2 and \$1.8 per sample, respectively. In addition, Subramanian *et al.* (2012) used carbon nanotube biosensor to

detect STEC O157 and O145, and found a higher detection sensitivity without specific requirement for DNA preparation.

There are obstacles for STEC detection in food and environmental samples. First, non-O157 STEC is lack of distinguished phenotypic characteristics for routine culturing in lab. Secondly, no typical virulence marker has been found for all O-serogroup genes. As a result, some non- pathogenic STEC strains may also reveal a false positive result when screening (DebRoy *et al.*, 2011). Thirdly, in addition to the target bacteria, the background microflora present in the matrix is also a critical factor affecting detection sensitivity and specificity, especially in high background matrices, such as soil or other environmental samples. Unlike food matrices, there are even more challenges associated with non-O157 STEC detection in animal waste-based compost, due to complex composting materials involved, high-level of microflora background as well as the difficulty in getting consistent samples (Fairbrother *et al.*, 2006).

Table 1.6 A list of selected of methods for STEC detection from different matrices

Pathogen Detected	Matrix Studied	Detection and Isolation Method				Reference
		Culture-Based		Molecular-Based	Immunoassay-Based	
		Medium name	Selective protocol			
E. coli O26, O103,O111,O145 and O157	573 Fecal samples	Sheep blood agar	mTSB+16 mg/l novobiocin	Real-time PCR	Dynabeads EPEC/VTEC	Hofer *et al.,* 2012
E. coli O26	Animal feces	CT-RMAC ;TBX	Rhamnose fermatation was tested	PCR	IMS was carried out without washing steps	Evans *et al.,* 2008
Non-O157 STEC (O91, O103, O111, O119, O121, O145 and O165) strains	Pure culture and beef samples	CT-SBMAC	UPB, mEC with novobiocin, mTSB and mTSB with novobiocin	LAMP assay	IMS assay with immunomagnetic beads targeting serotypes O157, O26 and O111	Kanki *et al.,* 2011
E. coli O157	Bovine feces	CT-SMAC	N.A[b]	PFGE	IMS	Vali *et al.,* 2007
E. coli O157	Ground beef and unpasteurized milk	CT-SMAC and CHROMagar® O157	mTSB+n combined with IMS	PNA-FISH	IMS-Dynabeads MAX *E. coli* O157 kit	Almeida *et al.,* 2013
E. coli O157	Ruminant feces	CT-SMAC	N.A.	PCR	Enzyme Immunoassay	Pao *et al.,* 2005
E. coli O157:H7	Bovine feedlot manure compost	CHROMagar® O157	N.A	Multiplex PCR	*E. coli* O157 latex agglutination reagents	Berry *et al.,* 2013
E. coli O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28	Cattle feces	CT-SMAC: *E. coli* O157, O103, and O111, CT-RMAC: *E. coli* O26 and *E. coli* O145	N.A	PCR and PFGE	Immunomagnetic separation (IMS)-based isolations were performed using Dynabeads	Bibbal *et al.,* 2013

Table 1.6 *Cont.*

Pathogen Detected	Matrix Studied	Detection and Isolation Method				Reference
		Culture-Based		Molecular-Based	Immunoassay-Based	
		Medium name	Selective protocol			
STEC	Fecal, plant , soil and water sample	CT-SMAC, NT-RA, modified sheep blood agar and CHROMagar®	N.A	Quadraplex PCR	IMS with anti-O157 beads	Cooley *et al.,* 2013
Top six STEC	Beef	mRBA	mTSB+N, mTSB w/o novobiocin enrichment for O111	BAX System real-time PCR assay for STEC screening	IMS	Fratanico *et al.,* 2014
E .coli O157	Bovine feces	CT-SMAC	N.A	N.A.	Standard IMS	Fox *et al.,* 2007
E. coli O157:H7, O26:H11, O103:H2, O111:H8, and O145:H28	Cattle feces	CT-SMAC: E. coli O157, O103, and O111, CT-RMAC: *E. coli* O26 and CT-RMAC for *E. coli* O145	N.A	PCR and PFGE	Immunomagnetic separation (IMS)-based isolations were performed using Dynabeads	Bibbal *et al.,* 2013
Top six STEC	Ground beef	mRBA, sheep blood agar	mTSB+N and acid treatment	PCR	Post IMS	Tillman *et al.,* 2012
STEC	Mincemeat	EC+N	N.A.	Real time PCR	Enzyme-linked fluorescent assay (ELFA)	Stefan *et al.,* 2007
E. coli O26, O103, O111, O145 and O157	164 minced beef samples	mTSB+N and CT-MCB	N.A.	Real-time PCR	Dynabeads IMS	Auvray *et al.,* 2007
E. coli O26, O103, O111, O145 and O 157	Dairy product, meat and feces	Pre-enrichment /selective enrichment	N.A.	PCR	IMS after enrichment	Posse´ *et al.,* 2007

[a] Agar abbreviations: CT-RMAC=rhamnose MacConkey agar supplemented with cefixime and tellurite; TBX=tryptone bile X-glucuronide agars; CT-SBMAC=Sorbose MacConkey Agar supplemented with cefixime; CT-SMAC= Sorbitol-MacConkey agar supplemented with cefixime and tellurite; CT-MCB= cefixime tellurite MacConkey broth; NT-RA=RMBA with novobiocin (20 mg/ml) and tellurite (0.8 mg/ml); RB® O157= Rainbow agar O157;SMAC= Sorbitol-MacConkey agar RMAC= rhamnose MacConkey agar; mTSB=modified tryptone soy broth; mEC= Modified EC broth; UPB= Universal Pre-enrichment broth.
[b] N.A: not applicable.

Conclusions

Animal manure is rich in nutrients for supporting crop growth. Through the composting treatment, the animal and agricultural wastes are converted into a humus-like product through microbial activities. Generally, the thermophilic stage during the composting processes is considered to be effective for the inactivation of human pathogens (Brito *et al.*, 2008; Jiang *et al*., 2003). Although this process has been regarded as an environment-friendly way to handle the on-farm wastes and animal manure, the inadequately processed compost appear to be the potential source for foodborne pathogens such as *E. coli* O157:H7 and non-O157 STEC. Furthermore, in order to effectively control STEC in biological soil amendment, sensitive and easy detection methods are needed. The knowledge on growth and survival of non-O157 STEC will provide valid scientific data to make guidelines for agricultural applications of animal manure-based soil amendment.

The objectives of this study were:

1. To optimize a culturing method for detecting non-O157 STEC from dairy compost.

2. To determine the growth potential of top six non-O157 STEC serovars inoculated

in dairy compost.

3. To conduct a persistence study of non-O157 STEC in dairy compost being held at room temperature.

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CHAPTER TWO

OPTIMIZING A CULTURING METHOD FOR DETECTING NON-O157 SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* FROM DAIRY COMPOST

Abstract

An optimized culturing method for detecting non-O157 Shiga toxin-producing *Escherichia coli* (STEC) after enrichment was investigated in this study. The finished dairy compost with 30% moisture content was inoculated with a cocktail of six non-O157 STEC serovars at a concentration of ca. 100 CFU/g. Afterwards, STEC cells in the inoculated dairy compost were enriched by four methods, followed by plating onto CTN-SMAC and mRBA plates. Immunomagnetic bead separation (IMS) was used to enumerate individual non-O157 STEC serotype after enrichment to determine the growth rates of each serotype. There was no significant difference ($p>0.05$) between CTN-SMAC and mRBA for non-O157 STEC enumeration. The single step selective enrichment recovered ca. 0.54 log CFU/g more cells ($p<0.05$) as compared to the two-step enrichment. Furthermore, the detection duration of non-O157 STEC from dairy compost was optimized by selective enrichment, followed by IMS. Among six non-O157 STEC serotypes, serotypes O45 and O145 grew faster in dairy compost, and the cell populations reached up to 7.4 and 7.8 log CFU/g within 16 h of incubation, respectively. In addition, without enrichment step, the IMS detection limit of individual non-O157 STEC serovar ranged from 250 to 2,500 CFU/g in dairy compost. These results demonstrated that low level of non-O157 STEC (ca. 100 CFU/g) could be detected within

one day from dairy compost by culturing method through the optimized enrichment procedure followed by IMS.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) has recently been recognized as emerging pathogens. STEC is capable of causing watery diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) in humans (Karch *et al*., 1999). Although severe hemorrhagic diseases and deaths were frequently associated with *Escherichia coli* O157:H7, the number of non-O157 STEC infections due to the contaminated food has risen every year since it was documented as a nation-wide infection in 2000 based on the Centers for Disease Control and Prevention (CDC)'s FoodNet (Hoefer *et al*., 2011; Karch *et al*., 1999). According to the epidemiological results summarized by Scallan *et al.* (2011), the non-O157 STEC cases have surpassed those of *E. coli* O157. The most common non-O157 STEC serogroups: O26, O111, O103, O121, O45, and O145 accounted for 75% of reported foodborne illnesses in the United States (Gould *et al*., 2009; CDC, 2012; USDA/FSIS, 2012). The Federal Register Notice published on Sept. 20, 2011, Food Safety and Inspection Service (FSIS) (Johnson *et al*., 2011) declared these top six STEC serotypes as adulterants on raw and/or beef-related products in the same status as *E. coli* O157:H7.

Over the last decades, there was an increased demand for organic products. The most common soil amendments applied to the farmland for organic production are animal

manures or composted animal wastes (Kuepper *et al.*, 2000). However, if the animal waste is not adequately treated, there is the possibility of contamination of agricultural crops by manure-born foodborne pathogens (Fairbrother *et al.*, 2006). Multi-state epidemiological studies around United State have revealed a high herd prevalence and environmental contamination of STEC from pre-harvest phase (Cho *et al*., 2006; Cobbold *et al*., 2004; Hussein *et al*., 2007; Hancock *et al.*, 1998). Although the thermophilic stage during the composting process is considered to be effective for the inactivation of pathogens in animal wastes (Brito *et al*., 2008; Jiang *et al*., 2003), the regrowth of pathogens in compost has also been reported (Elving *et al*., 2010; Kim *et al*., 2009). Hence, in order to control STEC in biological soil amendments, sensitive and easy detection methods are needed.

There are methods for detecting STEC including *E. coli* O157 and non-O157 STEC from different matrices (He *et al.*, 2011; Verstraete *et al.*, 2010; Cooley *et al.*, 2013; Catarame *et al.*, 2003), but no study has been done on detecting non-O157 STEC from dairy compost. Culture-based methods are widely used and considered as the first step for pathogen detection. For example, in order to isolate and detect STEC from meat products, current USDA-FSIS methods entailed enrichment and culturing steps for preliminary identification of STEC prior to biochemical and real-time PCR confirmations (USDA 2014). Unlike food matrix, there are more challenges associated with non-O157 STEC detection in animal waste-based compost due to high level of indigenous microflora and heterogeneous nature of compost samples (Fairbrother *et al.*, 2006). As a result, it is critical to use proper selective supplements for culturing methods. Media supplemented

with selective reagents such as bile salts and novobiocin developed from *E. coli* O157 detection have been applied for the detections of other STEC (Evans *et al.*, 2008; Wang *et al.*, 2013; Vimont *et al.*, 2007; Fukushima *et al.*, 1999). Additionally, immunomagnetic bead separation (IMS) was also proven as an effective isolation method. Willford *et al.* (2011) reported that in combination with serotype-specific IMS, the detection sensitivity of enzyme-labeled phage assay for non-O157 STEC (O111, O145, O26 and O123) ranged from 75.9 to 93% depending on the serotype. Studies also confirmed that IMS was effective in concentrating STEC O26 and O111 from ground and minced beef after enrichment (Catarame *et al*., 2003; Hara-Kudo *et al*., 2000).

In this study, a culturing method based on the USDA-FSIS protocol was optimized for detecting STEC in dairy compost, and the culturing method combined with IMS was also applied to compare growth rates of different STEC serotypes during enrichment in dairy compost.

Materials and Methods

Sample preparation. Three commercially available dairy composts were used in this study. Compost #1 (Wallace Farm Soil Product Inc., Huntersville, NC, USA) was directly purchased from Wallace farm in Huntersville, North Carolina, whereas compost #2 (Jolly Gardener Products, Inc., Poland Spring, Maine, USA) and compost #3 (Black Gold Compost Company, Oxford, FL, USA) were purchased from a local supermarket. As listed on the labels, the compost samples contain 0.5% total nitrogen, 0.5% available phosphate, 0.5% soluble potash, and no more than 1% chlorine. All the compost samples

were dried under the fume hood until moisture content was reduced to less than 10%; afterwards compost samples were screened to the particle size of less than 3 mm using a sieve. Initial moisture content of the finished dairy compost samples was measured with a moisture analyzer (Model IR-35 Infrared analyzer; Denver Instrument, Denver, CO, USA). Sufficient samples were collected for the entire study and stored in a sealed container at 4°C until use.

Bacterial strains and inoculum preparation. The bacterial strains and species used in this study are listed in Table 2.1. All bacterial strains were obtained from STEC Center at Michigan State University (East Lansing, MI, USA). Bacterial cultures were stored at -80°C in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD, USA) with 20% glycerol.

To prepare for the inoculation, the frozen stock cultures of these six STEC serovars were streaked twice on tryptic soy agar (TSA; Becton Dickinson, Sparks, MD, USA), and incubated at 37°C for 24 h. Single colony of each STEC strain was transferred into 30 ml TSB and grown overnight at 37°C. The overnight cultures were harvested by centrifugation (5,000 g for 15 min at 4°C), and individual cultures were washed 3 times with sterile saline (0.85% NaCl). The optical density (OD_{600}) of washed cultures was adjusted to ca. 0.5 (ca. 10^8 CFU/ml) and serially diluted to the desired concentration (ca. 10^4 CFU/ml), and then equal volumes of the six STEC serovars were combined to provide the inocula for following experiments.

Growth rate of STEC in TSB. The growth curve of individual STEC strain was determined in the TSB. The OD_{600} of each culture in 96-microwell plate (Corning,

Corning, NY, USA) was measured every hour up to 14 h using µQuant Microplate Spectrophotometer (BioTek Instrument, Inc. Winooski, Vermont, USA).

Moisture content, pH, and electrical conductivity. Moisture content was determined using a moisture analyzer (model IR-35, Denver, CO, USA). The pH value and electrical conductivity in dairy compost were measured according to the methods described by U.S. Composting Council (2002). Briefly, the compost sample was mixed with water at a ratio of 1:5, w/v equivalent basis, and shaken at room temperature for 20 min. Electrical conductivity and pH value of above compost slurry were measured using a multiparameter benchtop meter (Orion VERSA Star meter, Thermo Fisher Scientific Inc., Fort Collins, CO, USA).

Selection of dairy compost matrix for detecting non-O157 STEC and microbiological analysis. To select the compost matrix for recovering non-O157 STEC, compost #1, compost #2 and compost #3 with 30% moisture content were compared by enumerating detectable STEC-like indigenous microflora before or after enrichment (Table 2.2). Ten grams of each dairy compost were added to 90 ml of sterile saline in a Whirl-Pak® sampling bag and homogenized using a stomacher (Brinkman Instruments, Inc., Westbury, NY, USA) at medium speed (230 rpm) for 1 min. Serial dilutions were made from each suspension and spiral-plated onto Cefixime-Tellurite Sorbitol MacConkey Agar (Becton Dickinson, Sparks, MD, USA) supplemented with 5 mg/l novobiocin (CTN-SMAC) and Modified Rainbow Agar (Rainbow® Agar O157 Biolog Inc., Hayward, CA, USA) containing 5.0 mg/l novobiocin, 0.05 mg/l cefixime trihydrate and 0.15 mg/l potassium tellurite (mRBA) (USDA,2014) for enumerating STEC-like

bacteria before enrichment. In order to check for STEC-like background microflora after enrichment, all samples were enriched by enrichment methods described later, followed by plating onto the selective agar as mentioned above. These experiments were performed in two separate trials.

For enumeration of total background bacteria, serial dilutions of chosen compost sample were spiral-plated on TSA, and incubated overnight at 37 and 55°C for enumerating mesophiles and thermophiles, respectively. Actinomycetes Isolation Agar (AIA; Becton Dickinson, Sparks, MD, USA) and Rose Bengal Agar (RBA; Becton Dickinson, Sparks, MD, USA) were used for enumeration of *Actinomycetes* and fungi, respectively.

Selection of culturing media. Universal Pre-enrichment Broth (UPB; Neogen, Lansing, MI, USA) was used as non-selective enrichment broth. R&F non-O157 STEC Enrichment Broth (STEC-EB; R&F Laboratories, Downers Grove, IL, USA) and modified TSB (Becton Dickinson, Sparks, MD) with novobiocin (8 mg/l) plus casamino acids (mTSB+n) were used as selective enrichment broths.

The following enrichment methods were used for recovering non-O157 STEC cells in dairy compost samples (Fig 2.1):

1) Two-step enrichment (**A**): Twenty five grams of compost sample was incubated in UPB at 42°C for 20 h, and then 25 ml enrichment broth was transferred to 75 ml mTSB+n and inoculated for another 16-22 h at 42°C ;

2) Two-step enrichment (**B**): Twenty five grams of compost sample was incubated in

UPB at 42°C for 20 h, and then 25 ml enrichment broth was transferred to 225 ml STEC-EB and inoculated for another 16-22 h at 42°C；

3) One-step enrichment (**C**): Twenty five grams of compost sample was incubated in 75 ml mTSB+n at 42°C for 16-22 h；

4) One-step enrichment (**D**): Twenty five grams of compost sample was incubated in 225 ml STEC-EB at 42°C for 16-22 h;

The following selective agars were used for STEC enumeration:

1) Cefixime-tellurite Sorbitol MacConkey Agar (Becton Dickinson, Sparks, MD, USA) supplemented with 5 mg/l novobiocin (CTN-SMAC);

2) Modified Rainbow Agar (Rainbow® Agar O157 Biolog Inc., Hayward, CA, USA) containing 5.0mg/l novobiocin, 0.05 mg/l cefixime trihydrate and 0.15 mg/l potassium tellurite (mRBA) (USDA, 2014).

Evaluation of enrichment procedure and selective agar. Compost #1 was chosen to evaluate the enrichment procedures and selective agars for non-O157 STEC detection. The above six-strain STEC cocktail was inoculated into the compost sample with 30% moisture content. Approximately 100 g of dairy compost were placed in a sterile tray covered with alumina foil. STEC cultures were inoculated at a ratio of 1:100 vol/wt into the compost sample surface using a pipette to yield a ca. 10^2 CFU/g inoculation level, followed by mixing bacterial mixtures into the compost wearing sterile gloves. Immediately, 1 ml of sterile saline was inoculated into 100 g dairy compost (1:100, v/w) as the control samples.

Both inoculated and control samples were enriched in enrichment broth as mentioned above. Samples taken at the beginning of inoculation (0 h) were used to determine the initial populations of STEC. Enriched cultures sampled at 22 h were plated onto mRBA and CTN-SMAC after 10-fold serial dilutions, and then incubated for 20 h at 37°C. These experiments were performed in two separate trials.

Optimization of enrichment time. Based on preliminary results, CTN-SMAC was chosen as the selective agar to optimize enrichment time. One-step selective enrichment of 16 h was conducted to compare the growth of background microorganisms and STEC cocktail in dairy compost during enrichment (Fig 2.2). Briefly, the STEC cocktail inoculated into dairy compost was enriched in mTSB+n and STEC-EB for 16 h, and sampled every 2 h. Serial dilutions were made from each sample, and then spiral-plated onto CTN-SMAC. At the meantime, control compost without inoculation was enriched and sampled by the same procedure. TSA and CTN-SMAC were used to enumerate the background microorganisms in the control samples and STEC population in inoculated compost during enrichment, respectively. The detection limit of plate count was 1.7 log CFU/ml. These experiments were performed in two separate trials.

Immunomagnetic Separation. Following enrichment for 16 h (Fig 2.2), 1 ml of the enrichment mixture was pipetted into a 1.5 ml microcentrifuge tube (Fisher Scientific, USA) containing 50 µl of immunomagnetic beads coated with serotype-specific antibodies, separately [SDIX RapidChek® CONFIRM STEC Immunomagnetic Separation (IMS) Kit, Newark, DE, USA]. This mixture was rotated at room temperature for 15 min. Afterwards, serial dilutions were made from the IMS mixture, followed by

plating onto selective agar to determine the growth rate of STEC serovars enriched in dairy compost. These experiments were performed in two separate trials. The losses after IMS were calculated as (Total log CFU/ml before IMS-Total log CFU/ml after IMS) /Total log CFU/ml before IMS (%).

Furthermore, the detection limit for STEC serovars directly from dairy compost using IMS was determined as well. Briefly, STEC cocktail was prepared as described above and then inoculated into dairy compost using a sterile spray nozzle and thoroughly mixed to a final concentration of ca. 10^2, 10^3, and 10^4 CFU/g. Twenty five grams of inoculated sample was transferred into three sampling bags containing 75 ml mTSB+n enrichment broth to achieve target detection limit of ca. 25, 250 and 2,500 CFU/g, respectively. After IMS, the beads were directly streaked onto two selective agars to determine the detection limit of IMS. These experiments were performed in two separate trials.

Statistical analysis. Plate count data were converted to log CFU/g in dry weight. Data analysis was performed by SigmaPlot 12.3 (Systat Software Inc., San Jose, CA, USA). Analysis of variance (ANOVA), followed by the least significant differences (LSD) test, was carried out to determine whether significant differences (p<0.05) existed among different treatments.

Results

Compost matrix selection and microbiological analysis

The pH values of all three tested dairy compost samples (#1, #2 and #3) were 6.66±0.01, 6.82±0.03 and 7.72±0.01, respectively, and electrical conductivity values were 4.32±0.02, 3.46±0.01 and 2.33±0.01ms/cm, respectively. Due to the lack of STEC-like colonies, compost #1 was chosen for following experiments (Table 2.2).

For the dairy compost sample used in this study (compost #1), the initial population of mesophilic microflora, actinomycetes, and fungi was 8.19±0.13, 7.50±0.18 and 3.03±0.45 log CFU/g, respectively, and the populations of interfering colonies observed on CTN-SMAC ranged from 3.27 to 5.56 log CFU/g in different batches of compost sample #1 whereas only a few non-STEC colonies were observed on mRBA. However, those interfering colonies on CTN-SMAC plate presented with different color and morphology that can be easily differentiated from those of STEC.

Growth of single STEC serovars

Table 2.3 presented the OD_{600} value of STEC serotypes during 14 h of growth in the nutritional broth. After ca. 6 h of lag phase, all six STEC serotypes grew at the similar rate ($p>0.05$).

Evaluation of enrichment procedures and selective agars

Among four enrichment methods tested for enriching low level of STEC cocktail in dairy compost, the higher levels of STEC cells were obtained from one-step selective enrichment methods (STEC-EB and mTSB+n) ($p<0.05$) (Table 2.4). At the end of 22 h enrichment, an average of 0.54 log more of STEC cells was enumerated by the single step enrichment as compared to the two-step enrichment methods (UPB-STEC-EB and UPB-

mTSB+n). As compared with mTSB+n enrichment, the STEC-EB recovered slightly more STEC cells, which were 9.61±0.01 log CFU/g on mRBA, and 9.49±0.06 log CFU/g on CTN-SMAC at 22 h. However, according to the LSD test, there was no significant difference ($p>0.05$) between these two one-step methods after 22 h enrichment.

For all enrichment methods, there was no significant difference ($p>0.05$) observed between CTN-SMAC and mRBA for STEC enumeration. Therefore, CTN-SMAC could be used for enumeration of STEC after enrichment as considering the cost in using mRBA. Nevertheless, all STEC serovars showed the same morphology when plated onto CTN-SMAC.

Next, we evaluated the optimal time for STEC enrichment from dairy compost. Due to the presence of background microorganisms, overgrowth of those microorganisms can affect STEC growth adversely. The growth of background microorganisms and STEC in the same enrichment broth during 16 h enrichment were summarized and compared in Fig 2.3 and Fig 2.4. The growth of background microflora was inhibited significantly in STEC-EB ($p<0.05$), as population of background microorganisms increased 3.51 and 2.14 log CFU/ml in mTSB+n and STEC-EB, respectively, at the end of 16 h enrichment. For STEC serovars, an exponential growth of STEC serovars in dairy compost was observed from 2 to 8 h, followed by the stationary phase from 8 to 16 h. However, after 6 h enrichment, STEC outgrew background microorganisms, suggested that 6 h enrichment in both mTSB+n and STEC-EB was sufficient for detecting STEC from dairy compost.

Growth rate and detection limit among STEC serovars in dairy compost using IMS

In the dairy compost investigated in this study, serotypes O45 and O145 were

identified as two fast growing serotypes after enrichment in mTSB+n ($p < 0.05$) for 16 h as cell populations reached up to 7.4 and 7.8 log CFU/g on both selective agars, respectively (Table 2.5). A similar result was also observed when compost was enriched in STEC-EB (data not shown). For each serovar, the populations of STEC enumerated by CTN-SMAC and mRBA were not significant different (p>0.05). The after IMS lost rate for all serovars combined was (10.94±0.57)% on CTN-SMAC and (12.40±0.79)% on mRBA (Table 2.5).

Detection limit of STEC without enrichment step was studied by using dairy compost sample #1 artificially inoculated with STEC cocktail at three target levels (25, 250, and 2,500 CFU/g). For O26 and O145, the detection limit by IMS was 2,500 CFU/g, but for other serotypes (O45, O103, O111, and O121), the detection limit was 250 CFU/g (Table 2.6).

Discussion

Dairy compost is an excellent soil amendment for growing agricultural crops, but it is also known as a potential source of harboring foodborne pathogens such as *E. coli* O157:H7, non-O157 STEC, *Salmonella*, and *L. monocytogenes* (Beuchat *et al.*, 1996; Berger *et al.*, 2010; Stine *et al.*, 2005). Previous studies showed that non-O157 STEC is present in animal feces, pasture land and manure-amendment soils (Bolton *et al*., 2011; Fremanux *et al*., 2007), suggesting that a reliable, accurate and rapid detection method is needed to detect low level of non-O157 STEC from animal waste and compost.

USDA-FSIS has published laboratory procedures on detection of top six non-O157 STEC from meat products (USDA, 2014). In USDA-FSIS protocol, meat products were preliminarily identified for STEC by culturing method prior to other confirmations. In order to detect STEC from dairy compost, our first approach was to choose the appropriate selective agars. Our results showed that CTN-SMAC could be used for enumerating non-O157 STEC cells before or after enrichment as CTN-SMAC is more cost effective than mRBA and both agars enumerated the same level of STEC. SMAC contains sorbitol that serves as primary carbon source to support growth of non-O157 STEC (Association of Public Health Laboratories, 2012). The selective agents, cefixime and tellurite, inhibit the growth of most non-verocytotoxigenic *E. coli*. Fukushima *et al*. (1999, 2004) also confirmed that CT-SMAC could isolate presumptive STEC from fecal samples.

USDA-FSIS procedures recommend adding a lower concentration of novobiocin to both mTSB (8 mg/l) and mRBA (5 mg/l) media. In our study, novobiocin added into mTSB (8 mg/l) and CTN-SMAC (5 mg/l) as a selective supplement was adequate to inhibit the interfering background microflora present in dairy compost. It was documented in a number of studies that novobiocin functions as selective agent for STEC by inhibiting gram-positive bacteria such as some butyrate-producing bacteria, an inhibitor of STEC growth (Brinton *et al*., 2009). Cooley *et al.* (2013) used RBA containing 20 µg/ml novobiocin and 8 µg/ml tellurite for culturing STEC from wild animal and livestock fecal samples. Similarly, Hara-Kudo *et al.* (2000) reported that after a secondary selective enrichment in modified *E. coli* broth containing novobiocin (25

mg/l), the inoculated *E. coli* O26 was successfully isolated from food sample by plating onto mRBA. However, Vimont *et al*. (2007) did not add novobiocin to the enrichment broths for the detection of STEC from food, and argued that the addition of novobiocin could inhibit the recovery of injured cells. The difference in selecting the type and concentration of selective agents among studies is attributed to the difference in microflora of sample matrix.

In general, pathogens are present in animal wastes at low levels along with large numbers of indigenous microorganisms (Catarame *et al.*, 2003). In order to detect a few pathogenic cells, enrichment culturing is routinely used but it should strike a balance between providing conditions that are optimal for growth of the pathogens but inhibiting the growth of background microflora (Baylis *et al.,* 2001; Catarame *et al.*, 2003). Pre-enrichment step is commonly used to recover injured cells (Reinders, *et al.*, 2002; Possé *et al.,* 2008). For example, Kanki *et al.* (2011) claimed that mTSB+n was found less effective for enriching freeze-injured STEC cells from beef sample as compared with UPB followed by mTSB enrichment. In the present study, the combination of pre-enrichment broth with selective enrichment broth and application of selective enrichment broth alone was evaluated for their ability to selectively enrich STEC serovars in dairy compost. Our results showed that the single-step selective enrichment method by STEC-EB or mTSB+ n recovered ca. 0.54 log CFU/g more ($p<0.05$) cells of STEC as compared to the two-step enrichment methods, suggesting that pre-enrichment in non-selective broth might allow background microflora to overgrow and suppress STEC growth in the compost. These findings are in agreement with other published research on the

enrichment of STEC from different matrices. Reinders *et al.* (2002) proposed that direct enrichment in mTSB+n followed by automated IMS with integrated ELISA detection was a sensitive and efficient procedure for detecting injured or uninjured *E. coli* O157 in raw or pasteurized milk. Kanki *et al.* (2011) compared four enrichment broths for detecting non-O157 STEC O91, O103, O111, O119, O121, O145 and O165 from food samples and pure culture, and concluded that enrichment in mTSB containing bile salts was effective for detecting non-O157 STEC cells from food samples.

Both the non-O157 detection procedures of USDA-FSIS and the instruction of R&F® STEC-EB used in this study recommended a 20-22 h enrichment for STEC recovery from meat products (USDA, 2014). However, it is well-known that *E. coli* has a short generation time under optimal growth conditions (Bachmann *et al.*, 1996). We, therefore, hypothesized that the one-step enrichment time could be shortened in order to detect STEC within one day. In this study, the optimized condition for enriching non-O157 STEC from dairy compost was determined as 42°C for 6 h (Fig 2.3 and 2.4). This finding was in agreement with several previously published studies (Turenel *et al*., 2003; Lionberg *et al*., 2003). Those studies evaluated the selective enrichment broths, including TSB+n, TSB, BPW+cefixime+cefsulodium and R&F® STEC-EB, and reported the relatively higher sensitivity (more than 60%) for recovering STEC from fecal sample and ground beef under this temperature-duration condition (42°C for 6 h) (Vimont *et al*., 2007; Turenel *et al*., 2003; Lionberg *et al*., 2003). For cattle feces, there was an increased isolation efficiency for *E. coli* O157 when the samples were enriched for a short period of time (6 h) as compared with 24 h of enrichment (Tutenel *et al*., 2003). Apparently, with

prolonged enrichment time, detection sensitivity decreased due to the fast growth of indigenous microflora present in the matrix. Verstraete *et al*. (2010) claimed that there was no significant effect between 6 and 24 h enrichment on the recovery rate of 3 non-O157 STEC serotypes (O26, O103 and O111) from cattle feces. Furthermore, incubation at 42°C is often preferred because it can suppress competitive microflora and give better recovery of STEC in fresh produce (Drysdale *et al.* 2004; Hara-Kudo *et al*. 2000; Gonthier *et al.*, 2001).

Some studies have documented that IMS can enhance the detection sensitivity of STEC from environmental and food samples in concentrating pathogenic cells in sample matrices (Wasilenko *et al.*, 2014; Drysdale *et al.*, 2004; Hara-Kudo *et al.*, 2000; Cooley *et al.*, 2013). Nonetheless, there was no study on combining IMS with culturing method for detecting STEC cells from dairy compost. In this study, we used IMS to study the growth rate of STEC serovars (O26, O45, O103, O111, O145 and O121) during enrichment of dairy compost. We observed some differences in growth rate, overall losses after IMS and different detection limit among STEC serovars. As a result, these mentioned factors might contribute to the different detection sensitivity of individual serovar in dairy compost. In supporting of our finding, Conrad *et al.* (2014) reported that the effectiveness of IMS kit ranged from ca. 20 to 100% depending on the STEC serotypes, enrichment broth and antibody binding capacity. In order to avoid the losses by over-washing step, Evans *et al.* (2008) washed IMS beads once instead of 3 times as recommended by manufacturer. Additionally, Verstraete *et al*. (2010) claimed that these non-defined losses could be explained by the fact that the antibody–antigen complexes

were too weak to be washed off by PBS-T for 3 times, thus the antibody may be detached from the antigen after the IMS procedure. Clearly, experiment conditions should be optimized to remove the inhibitors and non-target microbial cells but maintain the binding of target cells to IMS beads.

Conclusions

In summary, this study has optimized the enrichment and plating methods for rapid detection of six non-O157 STEC serovars from dairy compost. Our results indicate that a single-step selective enrichment followed by plating onto CTN-SMAC is capable of detecting STEC with low concentration (ca. 100 CFU /g) within one day. Without the enrichment step, the detection limit of individual non-O157 STEC serovar ranged from 250 to 2,500 CFU/g. Apparently, the differences in growth rate during enrichment and the detection limit for non-O157 STEC serovars by IMS may affect the detection sensitivity of individual serovar in dairy compost.

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Figure Legends

Figure 2.1 Flow chart I of experimental design.

Figure 2.2 Flow chart II of experimental design.

Figure 2.3 Growth of background and STEC serovars in dairy compost during 16 h enrichment in mTSB+n ◆ Background, □STEC serovars.

Figure 2.4 Growth of background microorganism and STEC serovars in dairy compost during 16 h enrichment in STEC-EB. ◆ Background, □STEC serovars.

Tables and Figures

Table 2.1 A list of STEC strains used in this study

STEC serovar	Accession number[*]	Strain name	Isolation origin	*Stx* production
O26:H11	TW07814	97-3250	human	*stx1,2*
O45:H2	TW14003	MI05-14	human (M, 12 y)	*stx1*
O103:H2	TW08101	MT#80	human	*stx1*
O111:H11	TW14960	02019611	human	*stx1*
O145: NM	TW07596	GS G5578620	human	*stx1*

[*] Strains were acquired from the STEC Center at Michigan State University, East Lansing, MI, USA.

Table 2.2 Detection of background STEC in three commercial dairy compost samples

Matrix	Selective agar	One-Step Enrichment		Two-Step Enrichment	
		mTSB+n	STEC-EB	UPB-mTSB+n	UPB-STEC-EB
Compost #1	CTN-SMAC	-[a]	-	-	-
	mRBA	-	-	-	-
Compost #2	CTN-SMAC	-	-	-	-
	mRBA	-	-	-	-
Compost #3	CTN-SMAC	+[b]	+	+	+
	mRBA	+	+	+	+

[a] -, negative for STEC.

[b] +, presumptive STEC as confirmed by growth on selective agar.

Table 2.3 Growth rates of six STEC strains in TSB

STEC serovar	Incubation time (h)								
	6	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
O26	0.007±0.002 a[a]	0.037±0.033a	0.188±0.017a	0.441±0.017a	0.588±0.003a	0.608±0.004a	0.648±0.022a	0.655±0.024a	0.649±0.004a
O45	0.020±0.016 a	0.062±0.005a	0.221±0.015a	0.524±0.009a	0.600±0.011a	0.613±0.030a	0.615±0.021a	0.588±0.003a	0.615±0.020a
O103	0.008±0.003 a	0.050±0.005a	0.228±0.021a	0.479±0.017a	0.584±0.031a	0.611±0.005a	0.608±0.022a	0.610±0.030a	0.608±0.033a
O111	0.009±0.000 a	0.041±0.004a	0.182±0.016a	0.450±0.002a	0.603±0.006a	0.627±0.034a	0.646±0.021a	0.630±0.023a	0.636±0.021a
O145	0.012±0.005 a	0.049±0.007a	0.235±0.014a	0.494±0.003a	0.609±0.042a	0.643±0.030a	0.647±0.005a	0.650±0.007a	0.660±0.006a
O121	0.007±0.001 a	0.054±0.045a	0.196±0.009a	0.461±0.008a	0.588±0.036a	0.605±0.038a	0.609±0.004a	0.608±0.005a	0.615±0.006a

[a] Data are expressed as means±SD of two trials. Means with same lowercase letters in the same column at each incubation time are not significantly different ($p>0.05$) according to the LSD test.

Table 2.4 Comparison of different enrichment methods and selective agars for STEC detection

Enrichment Method	Plating agar	Enrichment time (h)	
		0	22
STEC-EB	CTN-SMAC	2.43±0.17A[a]	9.49±0.06Aa
	mRBA	2.55±0.12A	9.61±0.01Aa
mTSB+n	CTN-SMAC	2.43±0.17A	9.34±0.22Aa
	mRBA	2.55±0.12A	9.40±0.07Aa
UPB-STEC-EB	CTN-SMAC	2.43±0.17A	9.05±0.40Ab
	mRBA	2.55±0.12A	9.04±0.12Ab
UPB-mTSB+n	CTN-SMAC	2.43±0.17A	8.84±0.13Ab
	mRBA	2.55±0.12A	8.82±0.08Ab

[a] Plate count data (log CFU/g) were expressed as means±SD of two trials. Means with the same uppercase letters in the same column for plating agars are not significantly different (P >0.05). Means with different lowercase letters in the same column among different enrichment methods for the same selective agar are significantly different ($p < 0.05$).

Table 2.5 Immunomagnetic separation of top six STEC serovars after enrichment in mTSB+n for 16 h

STEC serovar	CTN-SMAC	mRBA
O26	6.27±0.08A[a]c[b]	6.29±0.05Ad
O45	7.42±0.02Ab	7.47±0.06Ab
O103	5.90±0.03Ad	5.79±0.05Ae
O111	7.39±0.09Ab	7.27±0.08Ac
O145	7.88±0.14Aa	7.85±0.07Aa
O121	5.43±0.16Ae	5.56±0.13Af
Total STEC counts after IMS	8.10±0.04A	8.09±0.06A
Total STEC counts before IMS	9.10±0.10A	9.23±0.16A
Overall lost rate after IMS (%)	10.94±0.57A	12.40±0.79A

*Data are expressed as means±SD of two trials. Means with different lowercase letters in the same column are significantly different ($p< 0.05$) for all serovars. Means with the same uppercase letters in the same row are not significantly different ($p > 0.05$).

Table 2.6 Detection limits of six STEC strains from dairy compost using direct plating methods

STEC serovars	Culture Media	STEC detection limit (CFU/g)		
		2,500	250	25
O26	CTN-SMAC	+[a]	-[b]	-
	mRBA	+	-	-
O45	CTN-SMAC	+	+	-
	mRBA	+	+	-
O103	CTN-SMAC	+	+	-
	mRBA	+	+	-
O111	CTN-SMAC	+	+	-
	mRBA	+	+	-
O145	CTN-SMAC	+	-	-
	mRBA	+	-	-
O121	CTN-SMAC	+	+	-
	mRBA	+	+	-

[a] +, positive by directly streaking the IMS beads on selective agar.

[b] -, negative by directly streaking the IMS beads on selective agar.

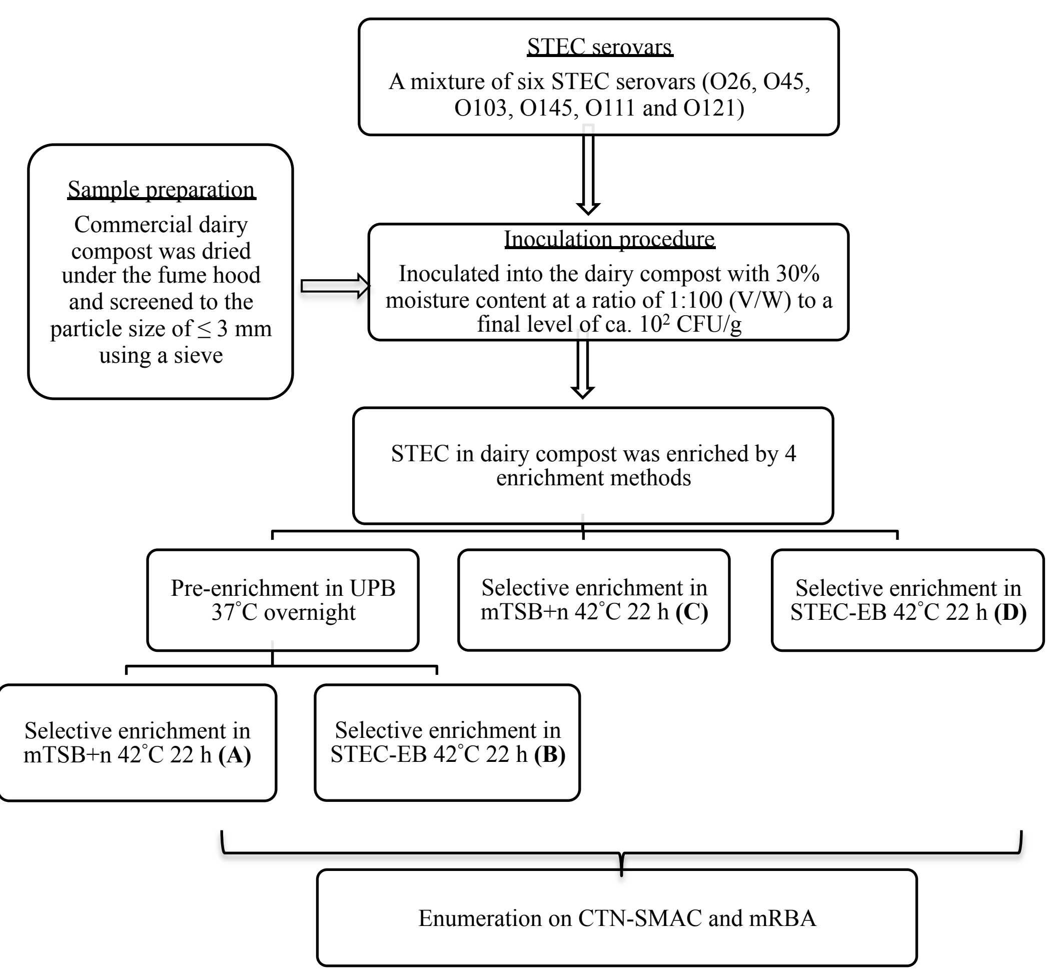

Figure 2.1

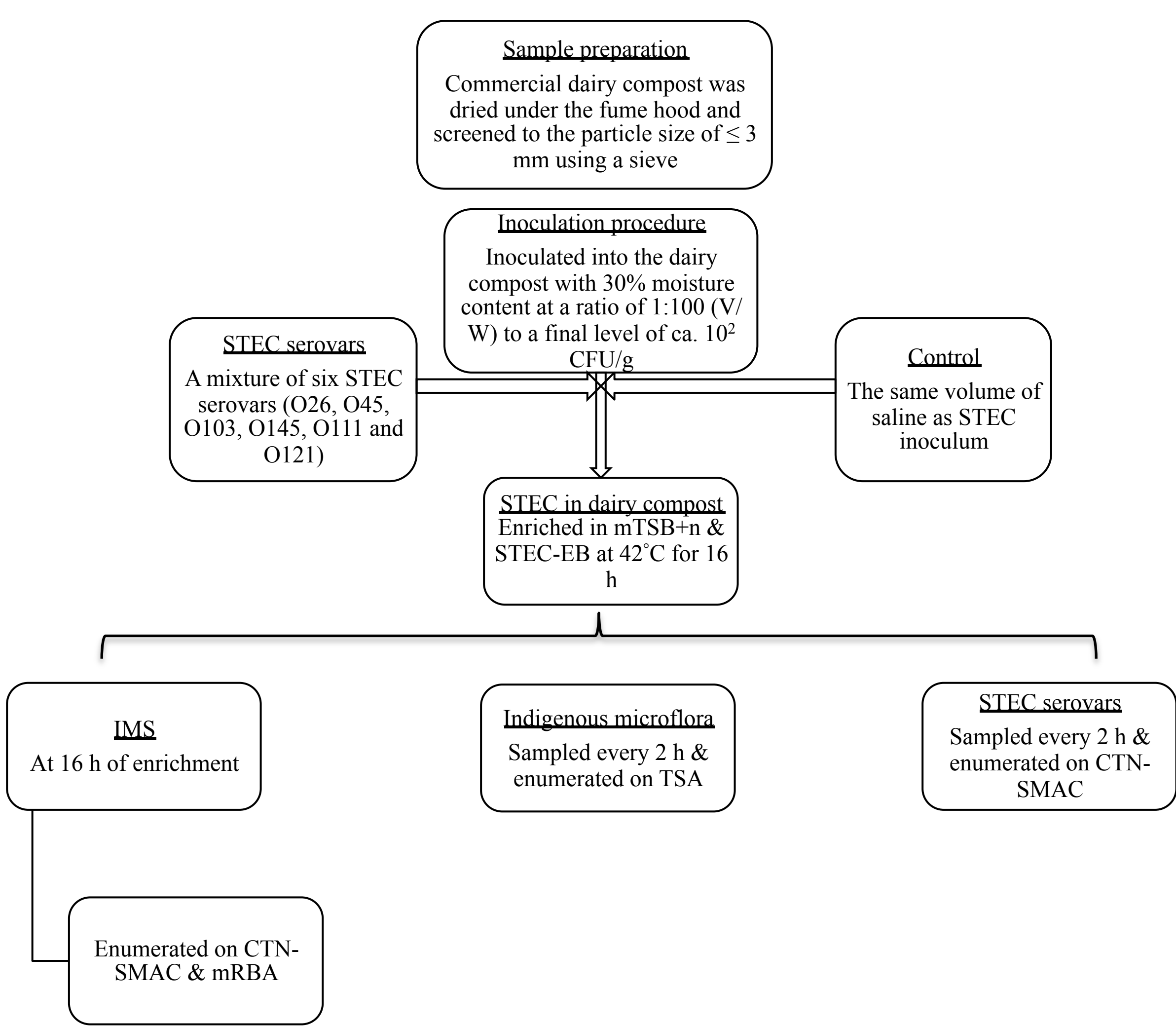

Figure 2.2

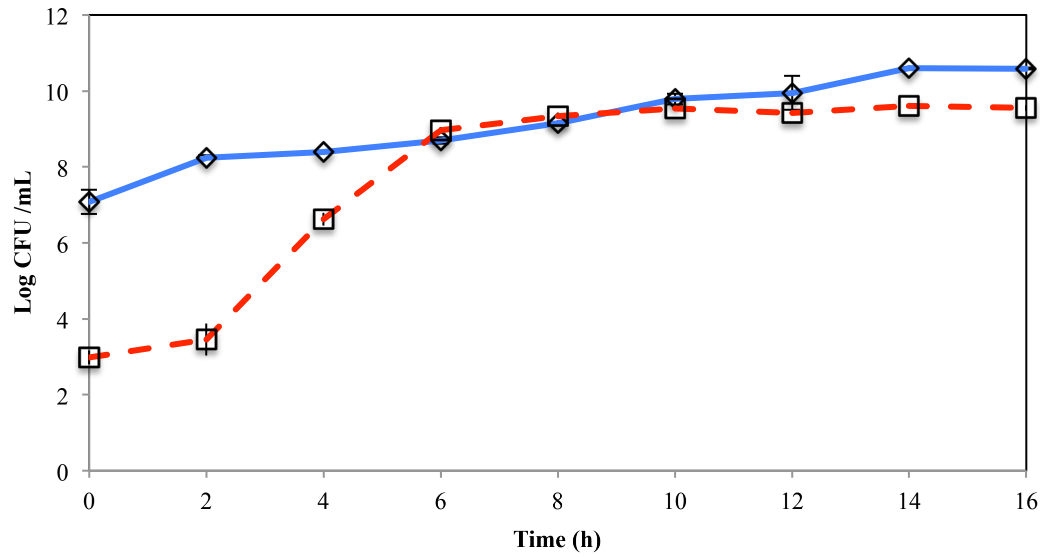

Figure 2.3

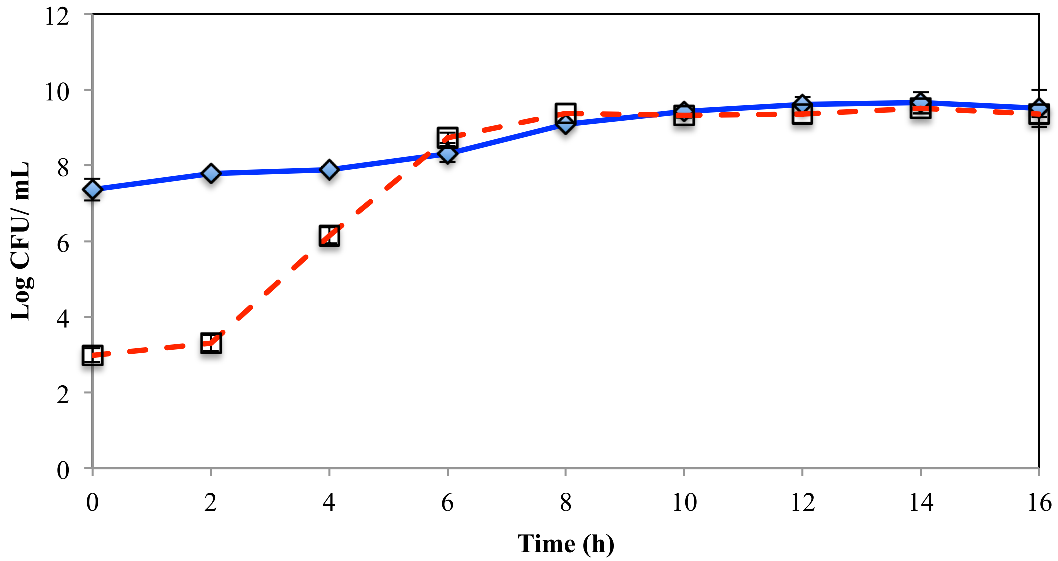

Figure 2.4

CHAPTER THREE

PERSISTENCE OF NON-O157 SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* IN DAIRY COMPOST DURING STORAGE

Abstract

Dairy compost as treated animal wastes is commonly applied to farmland as soil amendment. Despite the agricultural benefit, the finished compost may support the growth and survival of the human pathogens such as non-O157 Shiga toxin-producing *Escherichia coli* (STEC), and become one of the sources of fresh produce contamination on the farm. As a result, it is critical to evaluate the behavior of non-O157 STEC strains in dairy compost. In this study, a mixture of six non-O157 STEC serovars was inoculated into commercially available dairy compost with 30% moisture content at a final concentration of ca. 5.5 log CFU/g and then stored at room temperature for up to 42 days. STEC counts and other factors such as indigenous microbial population, moisture content and pH were analyzed at selected sampling intervals. During entire storage, both moisture content and pH values were remained unchanged, and so did the background bacterial level. As for the STEC population, a regrowth of ca. 0.5 log CFU/g was recorded in the first day post inoculation followed by a rapid decrease of ca. 1.5 log CFU/g during 14 days of storage. By the end of the experiment, ca. 1.7 log of STEC was reduced, and the STEC survival curve displayed an extensive tailing. The randomly selected colonies from the last 3 sampling times were confirmed as STEC by PCR and CHROMagar® STEC.

These results revealed that the STEC persisted in dairy compost for at least 42 days, indicating the long-term survival of non-O157 STEC in the finished dairy compost.

Introduction

Animal waste-based composts are commonly used as biological soil amendments in the fresh produce industry to improve soil quality and fertility (Harris *et al*., 2013). Most pathogens and viruses such as Shiga toxin-producing *Escherichia coli* (STEC) can be killed via proper composting process (Berge *et al.*, 2009). Nonetheless, there is a potential risk that a few pathogenic cells can be reintroduced into the finished product, may proliferate, and persist in the finished compost prior to land application. According to the Leafy Greens Management Association (LGMA) and Food Safety Leadership Council On-Farm Produce Standards (FSLC), the finished compost as soil amendments should be tested and free of human pathogens, and applied to the field for at least 45 days before harvest (LGMA, 2008).

The regrowth or survival of foodborne pathogens in the animal waste or animal manure-amended soil is possible depending on the factors such as moisture content, temperature and indigenous microorganisms (Islam *et al.*, 2004; Kim *et al.*, 2009a; Kim *et al.*, 2009b; Kim *et al.*, 2010). Fremaux *et al.* (2008) reported that at 4 and 20°C, STEC O26 with an initial inoculation level of ca. 6 log CFU/g could be detected in various manure-amended soil for 196 to 365 days depending on different soil type. Similarly, Fukushima *et al.* (1999) also confirmed the long period survival (12 weeks) of STEC O26 in bovine feces with an inoculation level of 5 log CFU/g.

Previous studies have confirmed the transferability of pathogens from soil fertilized with contaminated compost to the fresh produce (Johannessen *et al.*, 2005; Islam *et al.,* 2005; Oliveira *et al.*, 2012). For example, Islam *et al.* (2005) applied the compost contaminated with *E. coli* O157:H7 into produce field, and reported that the pathogen survived in soil samples for 154-196 days, and was detected for 74 and 168 days on onions and carrots, respectively, under field condition. Additionally, improper storage of dairy-based compost prior to use could also result in the regrowth or transmission of pathogens to other fresh produce (Toth *et al.*, 2012)

Above persistence studies were limited to the STEC O157:H7 serotype or generally performed on single non-O157 STEC strain in the animal waste or manure-amended soil, but the fate of non-O157 STEC especially top six non-O157 STEC serovars in dairy compost remains unclear. Therefore, the objective of current study was to investigate the growth and survival of top six non-O157 STEC serovars, O26, O145, O103, O111, O45 and O121 in dairy compost at room temperature.

Materials and Methods

Compost sample. Wallace Farm dairy compost (Wallace Farm Soil Product Inc., Huntersville, NC, USA), directly purchased from Wallace farm in Huntersville, North Carolina, was used in this study. As listed on the label, the compost samples contain 0.5% total nitrogen, 0.5% available phosphate, 0.5% soluble potash, and no more than 1% chlorine. All the compost samples were dried under the fume hood until moisture contents were reduced to less than 10%; afterwards compost samples were screened to

the particle size of less than 3 mm using a sieve. Initial moisture content of the finished compost sample was measured with a moisture analyzer (Model IR-35 Infrared analyzer; Denver Instrument, Denver, CO, USA). Sufficient samples were collected for the entire study and stored in a sealed container at 4°C until use.

Moisture content and pH value. Moisture content checked at each sampling time was determined using a moisture analyzer (model IR-35). The pH value in dairy compost was measured periodically according to the method as described by U.S. Composting Council (U.S. Composting Council, 2002). Briefly, the compost sample was blended with water at a ratio of 1:5, dw/v equivalent basis, and shaken for 20 min at room temperature. The pH value was measured in this 1:5 compost slurry using a multiparameter benchtop meter (Orion VERSA Star meter, Thermo Fisher Scientific Inc., Fort Collins, CO, USA).

Bacterial strains and inoculum preparation. The bacterial strains and species used in this study are listed in Table 2.1. All bacterial strains were obtained from STEC center at Michigan State University (East Lansing, MI, USA). As provided by STEC center, four STEC strains (O45, O103, O111 and O145) harbor *stx1* gene, one (O121) only harbors *stx2* and the O26 strain harbors both *stx1* and *stx2* genes. Bacterial cultures were stored at -80°C in tryptic soy broth (TSB; Becton Dickinson, Sparks, MD, USA) with 20% glycerol.

To prepare for the inoculation, the frozen stock cultures of these six STEC serovars were streaked twice on tryptic soy agar (TSA; Becton Dickinson, Sparks, MD, USA), and incubated at 37°C for 24 h. Single colony of each STEC strain was transferred into 30 ml TSB and grown overnight at 37°C. The overnight cultures were harvested by

centrifugation (5,000 g for 15 min at $4^{\circ}C$), and individual cultures were washed 3 times with sterile saline (0.85% NaCl). The optical density (OD_{600}) of washed cultures was adjusted to ca. 0.5 (ca. 10^8 CFU/ml) and serially diluted to the desired concentration (ca. 10^4 CFU/ml), and then equal volumes of the six STEC serovars were combined to provide the inocula for following experiments.

Persistence of non-O157 STEC in dairy compost: The above six-strain STEC cocktail was inoculated into the compost samples (Fig 3.1). Approximately 2,000 g of the compost with 30% moisture content were placed in a KitchenAid stainless steel bowl that was previously sterilized by autoclaving. STEC mixture was inoculated into the compost sample surface at a ratio of 1:100 vol/wt to yield a final concentration of approximately 10^5CFU/g using a sterile spray nozzle. The compost was then immediately mixed in a KitchenAid Professional 600 series stand mixer (KitchenAid. USA). At meantime, another 2,000 g of compost sample with 30% moisture content were inoculated with the same volume of sterile 0.85% saline served as control. One hundred grams of compost sample were distributed into Whirl-Pak® sampling bags (710 ml) and closed by folding the wired top down twice, and then placed in an aluminum foil covered tray at room temperature.

At selective sampling intervals (0, 1, 2, 3, 5, 7, 14, 21, 28, 35, and 42 days), duplicate samples were taken from one sampling bag to determine pathogen population. Ten g of sample were combined with 90 ml of sterile 0.85% saline in a Whirl-Pak® sampling bag (710 ml) and homogenized using a stomacher (Brinkman Instruments, Inc., Westbury, NY, USA) at medium speed (230 rpm) for 1 min. For enumeration of total

STEC serovars, serial dilutions were made from each suspension, spiral-plated on CTN-SMAC, and incubated overnight at 37°C for 24 h. At the same sampling intervals, TSA was used for enumerating indigenous bacteria in control compost samples. In addition, 0.1 ml aliquot of the control sample dilutions (10^{-1}) was plated onto one CTN-SMAC for monitoring the interfering microorganism. This experiment was conducted for two separate trials.

Detection of virulence genes *stx1* and *stx2* by polymerase chain reaction (PCR). In order to avoid the false positive results, bacterial colonies were randomly selected from the CTN-SMAC with the last 3 sampling times (28th, 35th and 42nd day). Meanwhile, 2 colonies from the control group grew on the CTN-SMAC were also picked. The selected colonies (inoculated sample, n=15; control sample, n=2) were transferred to TSA twice. DNA of each colony was extracted by a boiling method, and stored at -20°C prior to use.

Both *stx1* and *stx2* genes were detected by a PCR method as described previously with some modifications (Lang *et al.*, 1994). The primers listed in Table 3.1 were synthesized by Invitrogen Co. (Carlsbad, CA, USA). For *stx1*, the PCR reaction was carried out in a total volume of 20 µl containing 10 µl of the bacterial DNA preparation, 2 µl PCR buffer (10X), 1.6 µl dNTPs, 0.2 µl of Taq DNA polymerase (5 unite/µl), 2.8 µl $MgCl_2$ (TaKaRa Bio, Inc., Tokyo, Japan), 0.6 µl of each primer and 2.2 µl nuclease-free water. For *stx2*, the PCR reaction was carried out in a total volume of 25 µl containing 10 µl of the bacterial DNA preparation, 2 µl PCR buffer (10X), 1.6 µl dNTPs, 0.2 µl of Taq DNA polymerase (5 unite/µl), 2.8 µl $MgCl_2$ (TaKaRa Bio, Inc., Tokyo, Japan), 3 µl of

each primer and 2.4 µl nuclease-free water. The amplification conditions were 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 58.5°C for 1 min, and 72°C for 1.5 min, and a final extension at 72°C for 10 min (Heringa *et al.*, 2010). The PCR reactions were performed using the Bio-Rad iCycler™ system (Bio-Rad, Inc., Hercules, CA, USA).

The PCR products were detected by gel electrophoresis. Six-µl of the amplification mixture supplemented with 1 µl 6 X loading dye was loaded onto 1.5% agarose gels (Certified PCR Agarose, Bio-Rad, Hercules. CA, USA) in 1 X Tris-acetate-EDTA (TAE) buffer (Bio-Rad, Hercules. CA, USA). After electrophoresis at 70 V for 50 min, the gel was stained by ethidium bromide (10 mg/ml Bio-Rad, Hercules CA, USA), and visualized and photographed by GelDoc (Bio-Rad, Inc., Hercules, CA, USA).

Culture confirmation by CHROMagar® STEC. The colonies confirmed by PCR were streaked onto CHROMagar® STEC (CHROMagar® Paris. France) to check the colony morphology.

Statistical analysis. Plate count data were converted to log CFU/g in dry weight. Data analysis was performed by SigmaPlot 12.3 (Systat Software Inc., San Jose, CA, USA). Analysis of variance (ANOVA), followed by the least significant differences (LSD) test, was carried out to determine whether significant differences ($p<0.05$) existed among different treatments.

Results

Physical parameters

The dairy compost was adjusted to 30% moisture content prior to pathogen inoculation, and moisture content was maintained well under our experimental setup. As shown in Fig 3.2, no significant difference on the moisture level ranging from 28.71 to 30% was observed during the 42 days of storage ($p>0.05$). The pH remained unchanged for selective time intervals with an average value of ca. 6.8 (data not shown).

Indigenous microorganisms in control compost sample

The initial background bacterial count in dairy compost with 30% moisture content used in this study was ca. 7.10 log CFU/g. During entire storage, indigenous populations remained relatively stable in both two trials ($p>0.05$), ranging from 6.87 to 7.30 log CFU/g (Fig 3.3).

STEC survival in dairy compost for 42 days

The non-O157 STEC cocktail was tested for survival in the finished dairy compost at room temperature for 42 days (Fig 3.4). With an initial inoculum level of 5.66 log CFU/g, the STEC population had significant growth ($p<0.05$) of ca. 0.5 log CFU/g within 1 day in the compost for both two trials, whereas an exponential reduction of ca. 1.5 log CFU/g of non-O157 STEC serovars was observed during the first two weeks with die-off rate of 0.113 log CFU/g per day. After this spiked growth, the population level of non-O157 STEC serovars then stayed at a steady state and the survival curve showed an extensive tailing (Fig 3.4). By the end of the experiment, a total of ca. 1.7 logs of STEC were reduced in dairy compost.

STEC confirmation by CHROMagar® STEC and PCR assays

All the suspect colonies (n=15) that randomly selected from CTN-SMAC were confirmed as STEC by screening via PCR assay followed by streaking onto CHROMagar® STEC. As obtained by PCR assays (Fig 3.5, Fig 3.6), 11 of 15 colonies were tested positive for *stx1*, 3 positive for *stx2* (STEC O121) and only one positive for *stx1* + *stx2* (STEC O26). Two colonies from the control group were negative for *stx* genes. All the suspected colonies were mauve (typical color of STEC) on CHROMagar® STEC (Fig 3.7).

Discussion

Generally, the presence of pathogens in finished compost is very low; however a few cells survived through the composting process or be reintroduced to the finished compost may multiply during storage and serve as an inoculum, resulting in the contamination of crops when compost is used as a soil amendment. Previous publications have studied the survival and regrowth potential of *E. coli* O157 in cow manure or manure-amended soil (Kudva *et al.*, 1998; Himathongkham *et al*., 1999; Jiang *et al*., 2002). However, there is a lack of study on if non-O157 STEC can grow or persist in the finished dairy compost during storage. In the present study, we found that dairy compost can support early-stage proliferation and long-term persistence (at least for 42 days) of non-O157 STEC during room temperature storage.

The animal waste-based compost as an excellent soil amendment is rich in nutrients. Thus, the pathogen may multiply under some favorable conditions. There were several studies documenting that animal waste-based compost could support the growth of *E. coli*

O157:H7. This growth potential depends on background and inoculum population, moisture content and compost types (Kim *et al*., 2009a; Miller *et al*., 2013; Sidhu *et al*., 1999). Kim *et al.* (2009a) found that with ca. 3 log CFU/g inoculum level at the presence of ca. 6.48 log CFU/g background microorganisms, *E. coli* O157:H7 increased from ca. 0.39 to 1.23 log CFU/g within 1 day in dairy compost with different moisture contents. The authors stated the minimum moisture content requirement for the growth of *E. coli* O157 in dairy compost with high level background was 20%. Similarly, in the present study, the background microorganism was about 100 times more than the inoculated non-O157 STEC, and the population level of non-O157 STEC increased ca. 0.5 log CFU/g in the dairy compost with 30% moisture content within first day of post inoculation. As compared to the results reported by Kim *et al*. (2009a), non-O157 STEC (ca. 0.5 log CFU/g) might have a relatively lower growth potential than *E. coli* O157 (ca. 0.8 log CFU/g) in the dairy compost with 30% moisture content. In contrast, Miller *et al.* (2013) found that at initial inoculation level of ca. 5 log CFU/g, *E. coli* O157 could not grow in dairy compost but grow approximately 1 log within one day in the fish emulsion compost at the presence of ca. 7 log CFU/g indigenous microflora. And, there were studies arguing that high level of indigenous microflora in compost may suppress the growth of pathogens due to the competition (Kim *et al.*, 2010; Pietronave *et al.*, 2004). This suppression difference may be attributed to the diversity of microorganisms present in different compost type or compost ingredients. Furthermore, all the mentioned studies showed that the population of STEC increased in animal waste-based compost only

occurred in the first day followed by rapid decline of STEC but still remained detectable during storage at room temperature with proper moisture content (>20%).

In the present experiment, an exponential reduction in non-O157 STEC population level with a 0.113 log CFU/g per day die-off rate was observed during the first 2 weeks of storage. Although there were no studies reporting on the fate of non-O157 STEC survival in dairy compost under room temperature storage, our results were very similar with those of Fremaux *et al.* (2007), who found with an initial inoculation level ranging from 5.45 to 6.81 log CFU/ml, the STEC O26 counts in manure slurry decreased within 68 days to a low population level with a daily die-off rate of 0.075 log CFU/ml. Similar with STEC, Erickson *et al.* (2014) reported that at 30°C, *L. monocytogenes* reduced ca. 2 log CFU/g during 4 weeks of storage in dairy compost. However, even for the same pathogen, the different survival potential may result from the different serotype. As for the extended survival among STEC serotypes, a number of studies showed a relatively shorter survival of *E. coli* O157 in animal waste under different conditions compared with non-O157 STEC (Mukherjee *et al.,* 2006; Ogden *et al.*, 2002; Fukushima *et al.,* 1999). For example, Fukushima *et al.* (1999) found that under the same experimental condition and inoculation level, STEC O26 and O111 survived longer in bovine feces for 8 weeks as compared to the 6 weeks of survival of *E. coli* O157. In this study, after 42 days of storage, there were more than 4 logs of STEC remaining culturable, suggesting better adaptation of non-O157 STEC in dairy compost.

Besides, it should be noted that an extensive tailing was observed in the survival curve of non-O157 STEC serovars in dairy compost during storage at room temperature.

This finding is consistent with the data published by Fremaux *et al.* (2008), who suggested the persistent behavior of STEC O26 in manure-amended soil was correctly fitted by the log-linear model curve with tailing. There were studies documenting the similar survival pattern of pathogens, especially for the pathogen survival under stress conditions (Fukushima *et al.,* 1999; Bolton *et al.,* 2011; Jiang *et al.*, 2002). A possible explanation for this tailing is that more environment sensitive cells died off at a relatively faster rate, whereas the more persistent bacteria declined at a slower rate resulting in tailing. This extensive tailing effect should be considered fully when investigating the fate of pathogen survival or developing the strategies for pathogen inactivation. Otherwise, the stress-adapted cells in compost could be a risk factor for contaminating the field and fresh produce. Moreover, the PCR results in this study implied STEC O121 might predominate over other serovars used in this study; however, further study needs to be conducted to verify the most persistent STEC serotype in dairy compost.

Due to the controlled experimental conditions in the lab, the persistence of STEC may be overestimated; further field studies should be conducted to investigate the fate of non-O157 STEC survival under environmental conditions. Moreover, the analysis of the indigenous microbial communities in the inoculated samples is also needed for the further studies.

Nevertheless, these results could also provide some scientific evidences on the growth potential of non-O157 STEC in the finished dairy compost during storage at room temperature, suggesting that the practical strategies for non-O157 STEC control during subsequent storage of dairy compost should be addressed.

Conclusions

In conclusion, the non-O157 STEC strains studied in this research were found to grow initially but survive in the finished dairy compost for at least 42 days at room temperature with the survival curve showing an extensive tailing. In order to ensure the microbiological safety of dairy compost before their application to the field, the regular testing for non-O157 STEC should be performed. Despite the possible over-estimation of the survival time of non-O157 STEC in dairy compost under laboratory conditions, the non-O157 STEC strains were capable of persisting for a long period in the farm conditions. Therefore, proper handling and storage of dairy compost is critical and required to reduce the risk of transmission of non-O157 STEC to fresh produce and farm environment.

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Figure Legends

Figure 3.1 Flow chart of experimental design.

Figure 3.2 Change of moisture content during storage.

Figure 3.3 Change of indigenous microflora in dairy compost during storage.

Figure 3.4 Persistence of STEC serovars in dairy compost during storage.

Figure 3.5 Detection of STEC by polymerase chain reaction (PCR) with the specific primer *stx1*. Lane 1, 100 bp DNA ladder; Lane 2, positive control; Lane 3, negative control; Lane 4-18, DNA extracts of randomly selected suspected colonies; Lane 19-20, DNA extracts of colonies from control group.

Figure 3.6 Detection of STEC by polymerase chain reaction (PCR) with the specific primer *stx2*. Lane 1, 100 bp DNA ladder; Lane 2, positive control; Lane 3, negative control; Lane 4-18, DNA extracts of randomly selected suspected colonies; Lane 19-20, DNA extracts of colonies from control group.

Figure 3.7 CHROMagar®STEC plate.

Tables and Figures

Table 3.1 Polymerase chain reaction primers and control strains

Gene	Primer	Primer sequence (5'-3')	PCR product size (bp)	Positive control strains	Reference
stx1	STX1F	GACTGCAAAGACGTATGTAGATTCG	150	*E. coli* O157:H7 C7927	Lang *et al.*, 1994
	STX1R	ATCTATCCCTCTGACATCAACTGC			
stx2	STX2F	ATTAACCACACCCCACCG	210	*E. coli* O157:H7 C7927	Lang *et al.*, 1994
	STX2R	GTCATGGAAACCGGTTGTCAC			

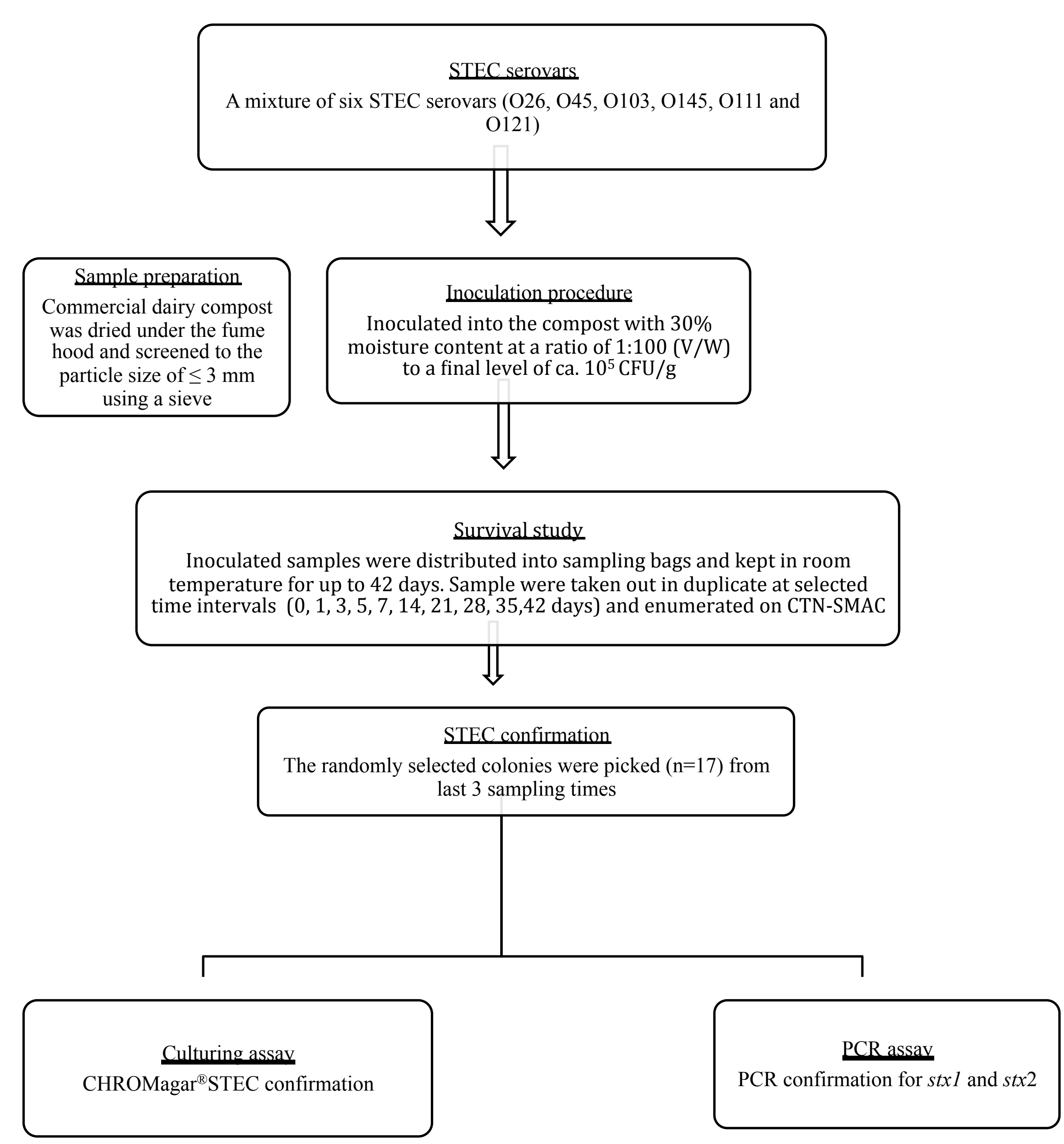


Figure 3.1

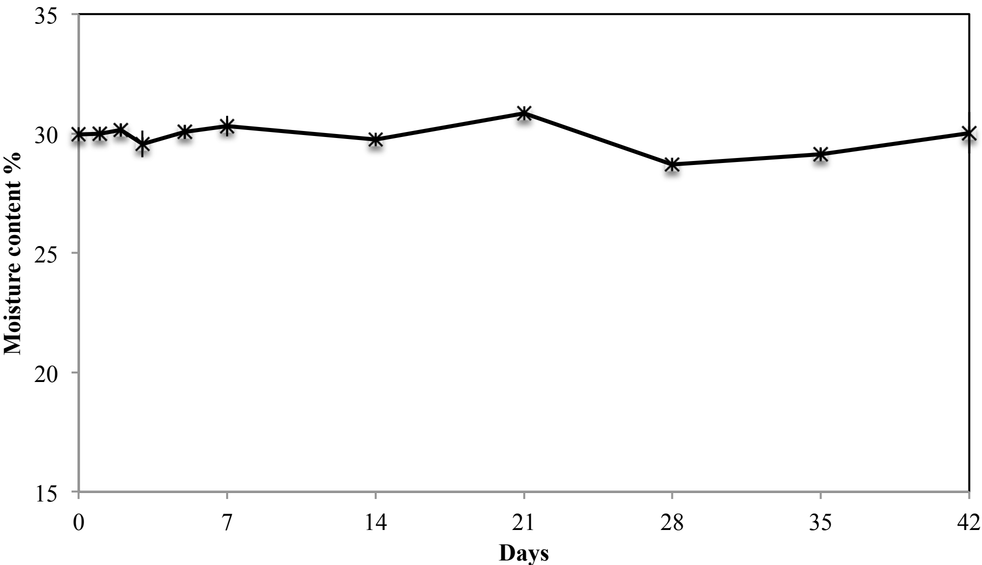
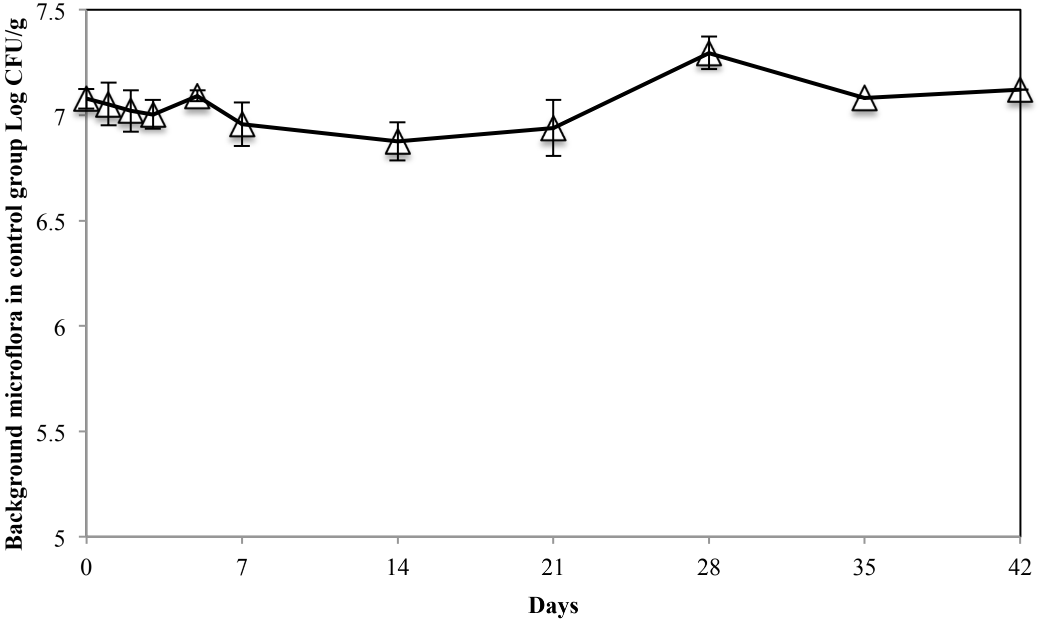


Figure 3.2



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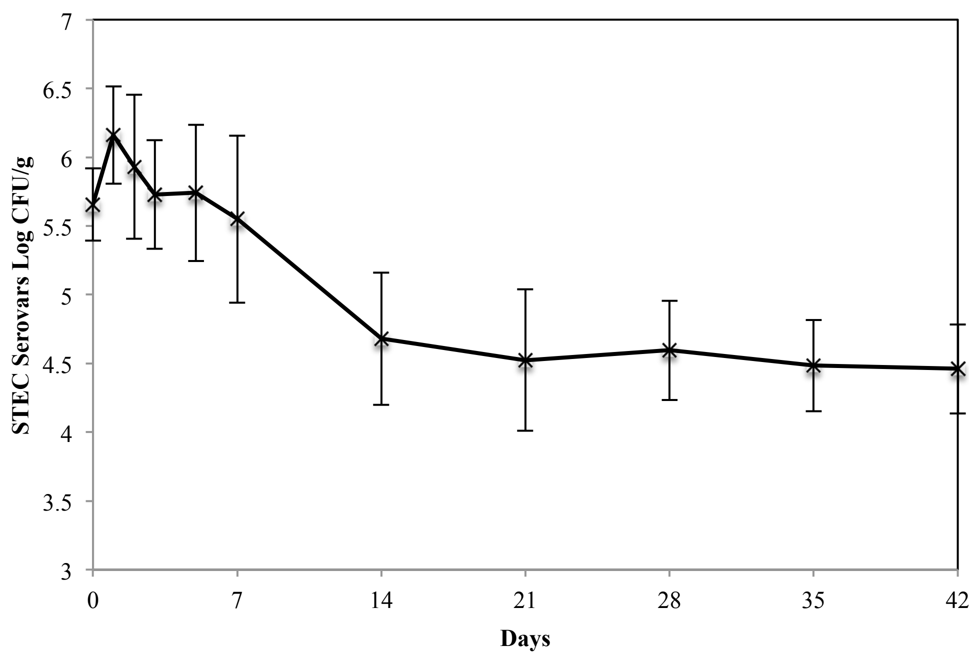

Figure 3.4

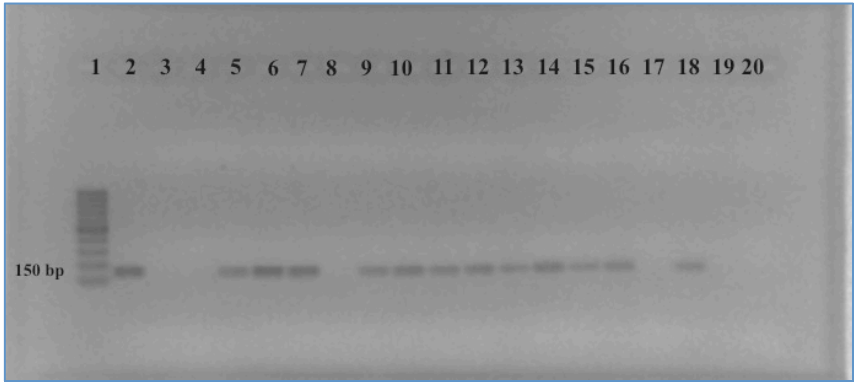

Figure 3.5

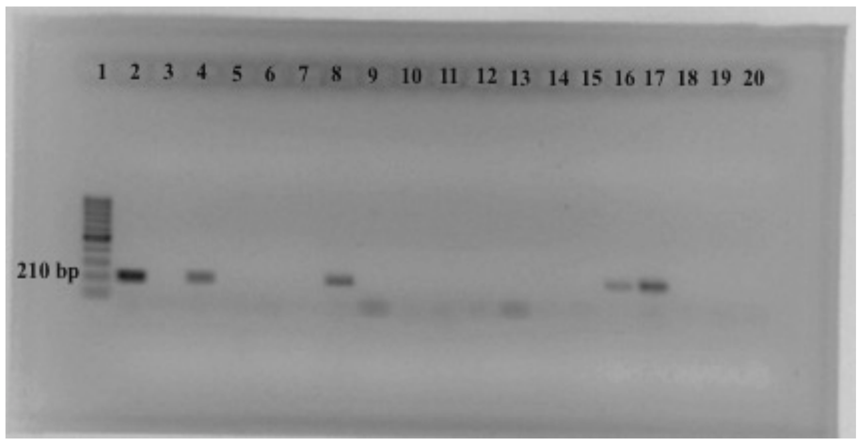

Figure 3.6

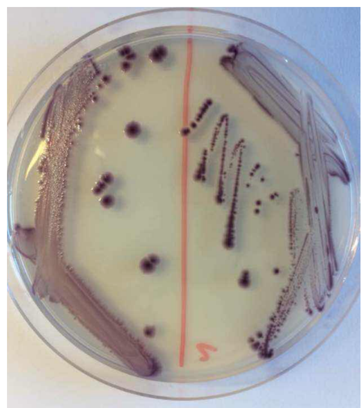

Figure 3.7

CHAPTER FOUR

CONCLUSIONS

In this study, our first approach was to optimize the enrichment and plating methods for rapid detection of STEC from dairy compost. Our results indicated that a single-step selective enrichment followed by plating onto CTN-SMAC is capable of detecting STEC with low concentration (ca. 100 CFU/g) within one day. Without the enrichment step, the detection limit of individual STEC serovar ranged from 250 to 2,500 CFU/g. Apparently, the differences in growth rate during enrichment and detection limit for STEC serovars by IMS may affect the detection sensitivity of individual serovar in dairy compost. In our survival study, non-O157 STEC was found to survive in the finished dairy compost for at least 42 days at room temperature, with survival curve showing extensive tailing. Results from our study indicated that in order to ensure the microbiological safety of the dairy compost before their application to the land, the regular testing for non-O157 STEC should be performed. Despite the possible overestimation of the survival time of STEC in dairy compost under laboratory conditions, the non-O157 STEC was nevertheless to persist for long period in the farm conditions. Therefore, proper handling and storage of animal waste-based compost is critical and required to reduce the risk of transmission of STEC to fresh produce and the farm environment.