Clemson University
TigerPrints

All Dissertations Dissertations

12-2011

THERMAL INACTIVATION OF STRESS ADAPTED PATHOGENS IN COMPOST

Randhir Singh
Clemson University, sainirandhir74@gmail.com

Follow this and additional works at: https://tigerprints.clemson.edu/all_dissertations

Part of the Microbiology Commons

Recommended Citation
Singh, Randhir, "THERMAL INACTIVATION OF STRESS ADAPTED PATHOGENS IN COMPOST" (2011). *All Dissertations*. 873.
https://tigerprints.clemson.edu/all_dissertations/873

This Dissertation is brought to you for free and open access by the Dissertations at TigerPrints. It has been accepted for inclusion in All Dissertations by an authorized administrator of TigerPrints. For more information, please contact kokeefe@clemson.edu.

THERMAL INACTIVATION OF STRESS ADAPTED PATHOGENS IN COMPOST

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Microbiology

---

by
Randhir Singh
December 2011

Accepted by:
Dr. Xiuping Jiang, Committee Chair
Dr. Paul Dawson
Dr. Annel K. Greene
Dr. J. Michael Henson

ABSTRACT

In the United States, 1.3 billion tons of animal wastes are produced annually. Disposal of this huge amount of waste on agricultural land without proper treatment is a public health safety issue as animal waste is a potential source of several human pathogens. Therefore, composting of animal wastes is an economical solution to this problem. The high temperature reached during this process also brings about inactivation of pathogens in the waste. However, survival of pathogens has still been reported from different composting studies, indicating the complex nature of this process. The objectives of this study were to: 1) study thermal inactivation of heat-shocked *Escherichia coli* O157:H7, *Salmonella* spp. and *Listeria monocytogenes* in dairy compost under isothermal conditions, 2) evaluate thermal inactivation of *E. coli* O157:H7 and *Salmonella* spp. in different types of compost at elevated composting temperatures by simulating early stage of on-farm composting, 3) determine thermal inactivation of acid-adapted *E. coli* O157:H7 in fresh dairy compost by simulating early phase of composting process, 4) develop predictive mathematical model for thermal inactivation of stress-adapted *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* in compost, and 5) study stress and virulence gene expression of *E. coli* O157:H7 in fresh dairy compost using real-time PCR.

Thermal inactivation of *E. coli* O157, *Salmonella*, and *L. monocytogenes* was studied in different types of compost in an environmental chamber under either isothermal conditions or by simulating early phase of the composting process. A three strain mixture of pathogen (except gene expression study) was used in every study.

Pathogens were inoculated at a final conc. of ca. 10^7 CFU/g for compost or liquid medium (10^7 CFU/ml) (broth or saline) study. The inoculated compost packed in Tyvek pouches were used for thermal inactivation study. An inactivation temperature of 50, 55, and 60°C was used for most of the study.

Thermal resistance of heat-shocked (at 47.5°C for 1 h) *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* was compared to non-heat-shocked (control) cultures in finished dairy compost under isothermal conditions. The heat-shocked *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* survived longer ($P < 0.05$) at 50°C with reductions of 2.7, 3.2 and 3.9 log CFU/g, respectively, within 4 h of heat exposure as compared with reductions of 3.6, 4.5, and 5.1 log CFU/g, respectively, in control cultures. The heat-shocked cultures of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* had 1.2, 1.9 and 2.3 log reductions, respectively, within 1 h at 55°C, whereas the corresponding control cultures had 4, 5.6 and 4.8 log reductions, respectively. At 60°C, a rapid population reduction was observed during the come-up time of 14 min in control cultures of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* with 4.9, 4.8 and 2.3 log reductions, respectively, as compared with 2.6, 2.4 and 1.7 log reduction, respectively, in heat-shocked cultures. *L. monocytogenes* survival curves for all three temperatures had extensive tailing. The mixed Weibulll distribution model was a good fit for the survival curves of pathogens, with difference in the shape parameter of heat-shocked and control cultures.

Thermal inactivation of *E. coli* O157:H7 was studied in fresh dairy compost by simulating the early phase of composting process (2 and 5 days of come-up time).

Compost mixture with 40 or 50% initial moisture content was used. Optimal and suboptimal compost mixes, with carbon to nitrogen (C/N) ratio of 25:1 and 16:1, respectively, were also compared in this study. In the optimal compost mix *E. coli* O157:H7 survived for 72, 48, and 24 h in compost with 40% moisture and for 72, 24, and 24 h with 50% moisture at 50, 55, and 60°C, respectively, following 2 days of come-up time (rate of heating up). However, in the sub-optimal compost mix (C:N as 16:1), the pathogen survived for 288, 72, and 48 h in compost with 40% moisture and 240, 72, and 24 h in compost with 50% moisture at the same temperatures, respectively. Pathogen survival was longer with 5 days of come-up time compared with 2 days of come-up time.

In the study on thermal inactivation of *Salmonella* in fresh poultry compost, a compost mixture with 40 or 50% initial moisture was used. In poultry compost with optimal moisture content (50%), *Salmonella* spp. survived for 96, 72, and 24 h at 50, 55, and 60°C, respectively, as compared with 264, 144, and 72 h at 50, 55, and 60°C, respectively, in compost with suboptimal moisture (40%). Pathogen decline was faster during the come-up time probably due to higher ammonia volatilization.

In the investigation on cross-protection of acid-adaptation and non-adaptation (control) on thermal inactivation of *E. coli* O157 in compost, *E. coli* O157:H7 acid-adapted in tryptic soy broth without dextrose (TSB w/o D) (pH 5.0) was inoculated into fresh dairy compost. *E. coli* O157 grown in TSB w/o D with pH 7.0 served as control. In fresh dairy compost with 2 days of come-up time, acid-adapted and control *E. coli* O157:H7 survived for 19 and 17 days at 50°C, respectively, and 6 and 4 days for both types of culture at 55 and 60°C, respectively. Overall, pathogen survival was non-

significant ($P > 0.05$) between control and acid-adapted cultures at all sampling intervals at all tested temperatures. In finished compost (Black Kow®), the survival of *E. coli* O157 was also non-significant ($P > 0.05$) at most of the sampling times between control and acid-adapted cultures at 55°C. However, the duration of survival for both cultures was short in comparison to that in fresh compost. In fresh compost with short come-up time (15 min) acid-adaptation provided *E. coli* O157 some cross-protection to heat at 55°C up to 30 min of exposure. In saline, acid-adapted *E. coli* O157 was inactivated at 55°C significantly slow as compared to control culture with short come-up time at 0.5 and 1 h of heat exposure.

To understand survival mechanisms for pathogens during composting, a two- step real-time PCR assay was used to evaluate expression of stress and virulent genes in *E. coli* O157:H7 heat-shocked in compost. *E. coli* O157:H7 (strain F07-020-1) was inoculated in autoclaved fresh dairy compost which was heat-shocked at 47.5°C for 10 min in water bath. Bacterial heat-shock was also done in tryptic soy broth (TSB) to serve as medium control. In compost heat-shock genes, *clpB*, *dnaK, groEL*, and alternative sigma factor (*rpoH*) were all up-regulated significantly ($P < 0.05$). There was no significant ($P > 0.05$) difference in the expression of trehalose synthesis genes. Virulent genes such as *stx1* and *fliC* were up-regulated while rest of the virulent genes was down-regulated with no significant difference ($P > 0.05$). In toxin-antitoxin system, toxin genes *mazF*, *hipA*, and *yafQ* were up-regulated with no significant difference ($P > 0.05$), whereas antitoxin gene *dinJ* was up-regulated with level of expression significantly different ($P < 0.05$). Most of the other antitoxin genes were down-regulated. In broth as

the heat-shock medium, all heat-shock genes were up-regulated with relative fold change significantly different ($P < 0.05$). There was no significant change ($P > 0.05$) in trehalose synthesis genes in broth medium either. Except for *eaeA*, rest of the virulent genes was down- regulated with no significant ($P > 0.05$) change. Majority of the toxin-antitoxin genes were down-regulated with relative fold change in toxin gene *hipA* and *chpB* only significantly different ($P < 0.05$).

These results suggest that the induction of heat-shock response in pathogens extended survival at temperatures typically considered lethal during composting process. Genetically, heat-shock genes play an important role in this process. Slow come-up time at the beginning of composting can be the reason for extended pathogen survival during composting due to better heat-adaptation. Additionally, both carbon to nitrogen (C/N) ratio and the initial moisture level in the compost mix affect the rate of pathogen inactivation. Thermal inactivation of pathogens during composting could be modeled by the mixed Weibulll model, with both moisture and come-up time identified as significant factors. Cross-protection against heat in *E. coli* O157:H7 due to acid adaptation was lost in fresh dairy compost during composting with 2 days of come-up time but was demonstrated in saline. In poultry compost, high nitrogen content is an additional factor contributing to *Salmonella* inactivation through ammonia volatilization during thermal exposure. Overall, physiological stage of pathogens, initial moisture level, composting come-up time, and controlled ammonia volatilization are some important parameters affecting the microbiological safety and quality of compost product.

DEDICATION

I would like to dedicate this work to my wife, Neetu Saini, daughter, Samridhi Saini, my parents, Amar Singh and Jagir Kaur for their moral support and encouragement. My wife and daughter's strength to carry on while I was away persuing higher education also motivated me to carry on this work with complete dedication.

ACKNOWLEDGMENTS

I would like to acknowledge the influence of my major advisor, Dr. Xiuping Jiang, on this work. Her direction and encouragement during the entire period of this work resulted in useful and encouraging findings for composting operations. My thanks are also due to the members of my advisory committee, Dr. Paul Dawson, Dr. Annel K. Greene, and Dr. Mike Henson for their suggestions. I would also like to sincerely thank all of my lab members for their support and advice for this work.

TABLE OF CONTENTS

Page

TITLE PAGE i

ABSTRACT ii

DEDICATION vii

ACKNOWLEDGMENTS viii

LIST OF TABLES xii

LIST OF FIGURES xiv

CHAPTER

I. LITERATURE REVIEW 1

Introduction 1
Foodborne Pathogens 5
E. coli O157:H7 5
Salmonella spp 7
L. monocytogenes 9
Animal Wastes and Compost 10
Dairy compost 10
Poultry Compost 13
Composting Process 14
Factors Affecting Composting Process 16
Re-growth of pathogen in compost 20
Microbiological Hygiene Criteria for Composting 22
Microbial Stress Response 24
Microbial Cross-protection Mechanisms to Stress 26
Composting Stress and its Influence on Microbial Inactivation 28
Microbial Survival Mechanisms during Heat-Stress 30
Microbial Cell Acid-shock/adaptation and Cross Protection to Heat 31
Toxin-antitoxin Role in Stress Response 33
Predictive Microbiology 35
Summary 37
References 39

II. THERMAL INACTIVATION OF HEAT-SHOCKED *ESCHERICHIA COLI* O157:H7, *SALMONELLA*, AND *LISTERIA MONOCYTOGENES* IN DAIRY COMPOST 53

Abstract 53
Introduction 54
Materials and Methods 56
Results 61
Discussion 64
Conclusions 70
Acknowledgements 71
References 72
Tables 78

III. DETERMINING THERMAL INACTIVATION OF *ESCHERICHIA COLI* O157:H7 IN FRESH COMPOST BY SIMULATING EARLY PHASES OF COMPOSTING PROCESS 82

Abstract 82
Introduction 83
Materials and Methods 85
Results 89
Discussion 97
Conclusions 104
Acknowledgements 105
References 106
Figure legends 109
Tables and figures 110

IV. HEAT INACTIVATION OF *SALMONELLA* SPP. IN FRESH POULTRY COMPOST BY SIMULATING EARLY PHASE OF COMPOSTING PROCESS 117

Abstract 117
Introduction 118
Materials and Methods 120
Results 124
Discussion 127
Conclusions 131
Acknowledgements 131
References 132
Figure legends 135
Tables and figures 136

V. THERMAL INACTIVATION OF ACID-ADAPTED *ESCHERICHIA COLI* O157:H7 IN FRESH DAIRY COMPOST 139

Abstract 139
Introduction 140
Materials and Methods 142
Results 146
Discussion 149
Conclusions 153
Acknowledgements 154
References 155
Tables 158

VI. EXPRESSION OF STRESS AND VIRULENCE GENES IN *ESCHERICHIA COLI* O157:H7 HEAT-SHOCKED IN FRESH DAIRY COMPOST 163

Abstract 163
Introduction 164
Materials and Methods 166
Results 174
Discussion 179
Conclusions 188
Acknowledgements 189
References 190
Figure legends 196
Tables and figures 197

VII. CONCLUSIONS 204

LIST OF TABLES

Table Page

2.1 Survival of control and heat-shocked *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* in compost at 50°C 78

2.2 Survival of control and heat-shocked *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* in compost at 55°C 79

2.3 Survival of control and heat-shocked *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* in compost at 60°C 80

2.4 Parameters of double Weibulll distribution of *E. coli* O157:H7, *Salmonella* & *L. monocytogenes* thermal inactivation curves at 50, 55 & 60°C 81

3.1 Thermal inactivation of *Escherichia coli* O157:H7 in fresh compost at 50°C under different conditions 110

3.2 Parameters of mixed Weibulll distribution of *E. coli* O157:H7 inactivation curves at 50°C 111

3.3 Thermal inactivation of *Escherichia coli* O157:H7 in fresh compost at 55°C under different conditions 112

3.4 Parameters of mixed Weibulll distribution of *E. coli* O157:H7 inactivation curves at 55°C 113

3.5 Thermal inactivation of *Escherichia coli* O157:H7 in fresh compost at 60°C under different conditions 114

3.6 Parameters of mixed Weibulll distribution of *E. coli* O157:H7 inactivation curves at 60°C 115

4.1 Changes of ammonia concentration in poultry compost with different moisture content during early phase of composting 136

5.1 Thermal inactivation of acid-adapted *E. coli* O157:H7 at 55°C in saline 158

5.2 Thermal inactivation of acid-adapted (AA) *Escherichia coli* O157:H7 in fresh dairy compost at 50°C 159

List of Tables (Continued)

Table Page

5.3 Thermal inactivation of acid-adapted (AA) *Escherichia coli* O157:H7 in fresh dairy compost at 55°C 159

5.4 Thermal inactivation of acid-adapted (AA) *Escherichia coli* O157:H7 in fresh dairy compost at 60°C 160

5.5 Thermal inactivation of acid-adapted (AA) *Escherichia coli* O157:H7 in fresh dairy compost at 55°C with short come-up time 160

5.6 Thermal inactivation of acid-adapted (AA) *Escherichia coli* O157:H7 in finished commercial dairy compost at 55°C.................................. 161

5.7 Moisture contents of the compost mix for different trials 162

6.1 Primers used in real-time PCR expression assays along with their efficiency.. 197

6.2 Heat-shock optimization of *E. coli* O157:H7 in saline 198

LIST OF FIGURES

Figure Page

3.1 Regression coefficient of four factors, i.e. temperature, moisture, come-up time and C:N, for four different model parameters 116

4.1a Thermal inactivation of *Salmonella* spp. in fresh poultry compost at 50 °C with different moisture content and 2 days of come-up time 137

4.1b Thermal inactivation of *Salmonella* spp. in fresh poultry compost at 55 °C with different moisture content and 2 days of come-up time 137

4.1c Thermal inactivation of *Salmonella* spp. in fresh poultry compost at 60°C with different moisture content and 2 days of come-up time 138

6.1 Thermal inactivation of *E. coli* O157:H7 heat-shocked in autoclaved fresh dairy compost 199

6.2 Expression of stress genes in *E. coli* O157:H7 heat-shocked in autoclaved fresh dairy compost and in broth 200

6.3 Expression of trehalose synthesis genes in *E. coli* O157:H7 heat-shocked in autoclaved fresh dairy compost and in broth 201

6.4 Expression of virulent genes in *E. coli* O157:H7 heat-shocked in autoclaved fresh dairy compost and in broth 202

6.5 Expression of toxin-antitoxin genes in *E. coli* O157:H7 heat-shocked in autoclaved fresh dairy compost and in broth 203

CHAPTER ONE

LITERATURE REVIEW

Introduction

According to US Senate Agriculture Committee report, the United States produces an estimated 1.3 billion tons (ca. 1179 billion kg) of animal waste or five tons of manure for each American annually (Grossfield, 1998). However, livestock manure, one of the important organic ingredients for the composting, may be a source of human pathogens, including *Escherichia coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes*. These pathogens are present in the gastro-intestinal tract of healthy farm animals (Doyle et al., 2001; Elder et al., 2000). Presence of these pathogens in animal waste that is not properly contained and treated can lead to foodborne and waterborne diseases. Such diseases have a negative impact on the health and economy of the country; for example, foodborne *Salmonella* infection alone causes approximately $365 million in direct medical expenditures annually (USDAERS, 2010), and the cost of a single fatal case of shiga toxin producing *E. coli* (STEC) O157 infection has been estimated at $7 million (Frenzen et al., 2005).

In the United States, foodborne illnesses are responsible for approximately 1,000 outbreaks, which accounts for an estimated 48 million illnesses, 128,000 hospitalizations, and 3,000 deaths annually (CDC, 2010; Scallan et al., 2011a, 2011b). In 2010, a total of 19,089 foodborne infection cases, 4,247 hospitalization and 68 deaths were reported from FoodNet surveillance sites. *Salmonella* accounted for the majority of the illnesses resulting in 17.6 cases per 100,000 people with 2,290 hospitalizations and 29 deaths,

whereas 125 cases, 112 hospitalizations and 16 deaths were the result of listeriosis (CDC, 2011b). Shiga toxin-producing *E. coli* O157 infection caused 0.9 illnesses per 100,000 people (CDC, 2011b). The Center for Disease Control (CDC) has listed STEC and *Salmonella* among the top 5 foodborne illnesses that require hospitalization in the United States (CDC, 2011c).

Foodborne diseases due to animal waste occur when improperly treated manure is used as a soil amendment or organic fertilizer to grow fresh fruits and vegetables. Manure runoff from agricultural field also may contaminate irrigation and water sources which results in waterborne illnesses directly or foodborne illness indirectly. There have been a number of outbreaks associated with manure contamination directly or indirectly. For example, the large *E. coli* O157:H7 spinach outbreak in 2006 was associated with baby spinach linked to cattle feces present in a field of one of four California farms implicated in the outbreak (CDC, 2006). Besides *E. coli* O157:H7, other *E. coli* serotypes also have been involved in major foodborne outbreaks (ECDC, 2011a). Recently a major outbreak due to *E. coli* O104:H4 was reported in several European countries as initially reported in Germany. The outbreak itself caused 908 hemolytic uremia syndrome (HUS) and 3,167 non-HUS cases with 34 and 16 deaths, respectively. The source of the outbreak was suspected to be sprouted seeds (ECDC 2011a, 2011b, WHO, 2011). In another outbreak, raw Jalapeno peppers as well as Serrano peppers and tomatoes were implicated as a possible source of nationwide *S*. Saintpaul outbreak, due to contaminated irrigation water (CDC, 2008).

Efficient treatment of livestock and poultry waste is not only critical to prevent such outbreaks, it is also imperative to tap huge amounts of organic waste as an important source of organic fertilizer for agricultural use. Composting is commonly used for treating organic wastes (livestock manure and food waste) as it makes it easier to dispose of animal waste on agricultural fields and for home garden, applications for soil conditionings. Composted organic waste also serves as an important organic fertilizer, which is rich in nutrients, circumventing the need for chemical fertilizers (USEPA, 2008). Furthermore composting is considered an important tool for ensuring microbiological safety of the waste as higher temperature reached during this process brings about inactivation/killing of the pathogens (Erickson 2010).

There are official standards for meeting the microbiological quality of bio-solid based compost (USEPA, 1999; EC, 2001). According to the United States Environmental Protection Agency (USEPA) standard, class A bio-solid compost the maximum limits for *Salmonella* spp. and fecal coliforms is less than 3 most probable number (MPN)/g and less than 1,000 MPN/g, respectively (USEPA, 1999). Inadequately composted livestock manure has been considered a potential source of contamination of fresh produce. Although there is no official standard for animal waste based compost, there is a temperature-time requirement for composting in order to bring about inactivation of pathogens that may be present in the raw compost ingredients (USEPA, 1999).

Temperature is a critical factor during composting as it causes inactivation of pathogens. Despite high temperatures reached during the composting process survival of pathogens in the compost heaps has been reported (Droffner & Brinton, 1995;

Hutchinson et al., 2005).There is possible heat-adaptation of pathogens during the prolonged mesophilic phase of the composting process which can influence their duration of survival. Various studies have shown that heat-shocked cultures exhibit higher thermal resistance than the non-stressed cells in various sample matrixes including compost (Linton et al., 1990; Mackey and Derrrick, 1986; Shepherd et al., 2010). Microbial cells exposed to stress at sub-lethal levels can resist the same stress better at lethal levels in comparison to non-stressed cells (Murano and Pierson, 1992). Microbial populations also have the ability to tolerate other unrelated stresses once they have been exposed to a stress (Rowe and Kirk, 2000; Singh et al., 2006); this phenomenon is termed cross-protection. Pathogens in animal manure might be exposed to different stresses before composting such as acid-adaptation in the gastro-intestinal tract, or being adapted to low nutrients/starvation. Cross-protection mechanism may also extend the survival of certain microbial populations. In addition, sub-optimal conditions, such as slow heat-up of compost, low moisture content and inadequate carbon to nitrogen ration (C:N) may extend the mesophilic phase and delay the onset of thermophilic phase during composting process. This slow temperature increase from mesophilic to thermophilic range can induce heat-shock response in some microbial populations, thereby resulting in increased heat resistance of pathogens that may survive composting.

Foodborne Pathogens

***E. coli* O157**

E. coli O157:H7 was first reported in 1982 as an emerging foodborne pathogen transmitted from animals to humans during an investigation of bloody diarrhea (hemorrhagic colitis) as a result of consumption of contaminated hamburger (Riley et al., 1983). *E. coli* O157:H7, a Gram-negative rod is a member of *Enterobacteriaceae* family. It is also called enterohemorrhagic *E. coli* (EHEC) which is one of the six classes of diarrheagenic *E. coli* strains (Nataro and Kaper, 1998). Like other *E. coli*, *E. coli* O157:H7 is a normal inhabitant of intestinal tracts of cattle without any apparent symptoms (Nataro and Kaper 1998). *E. coli* O157:H7 are shed in feces and therefore abundant in manure of these animals (Sobsey et al., 2006). Presence of this microorganism in the feces of animals has become a major public health concern because of its potential to cause human infection from fecal contamination of food and water. In the United States alone *E. coli* O157:H7 is responsible for an estimated 70,000 illnesses and 60 deaths annually (Mead et al., 1999). In latest estimates the level of infection has reduced from previous estimates with the annual infection rate now at 0.9 illnesses per 100,000 people (CDC, 2011b).

E. coli O157:H7 has been detected in cattle of different ages, as low as 10^2 CFU/g (positive after enrichment) and as high as 10^5 CFU/g of fecal sample (Zhao et al., 1995). In one experimental study when *E. coli* O157:H7 was inoculated orally in cattle it was shed in feces in three intervals, approximately for 1 week, 1 month or 2 or more months (Grauke et al., 2002). Although *E. coli* O157:H7 has been associated mainly with cattle

population, it has also been isolated from other animal hosts such as sheep, pigs and wild birds (Foster et al., 2006; Kaufmann et al., 2006; Ogden et al., 2005). The shedding of *E. coli* O157:H7 has been reported to be intermittent, however it is based on animal age, diet and seasonal variations. The calves are considered a source of these pathogens in feces as their rumen is not well developed to limit pathogen survival (Grauke et al., 2002). Laegreid et al. (1999) in their study reported 6.5 % of the calves carried *E. coli* O157:H7 in their feces tested across 15 different herds. The highest prevalence of *E. coli* O157:H7 was 21.7% reported in cattle 4 and 12 months of age (Heuvelink et al., 1998).

Impact of diet on the shedding of *E. coli* O157:H7 has been reported by some researchers in their study based on grain or forage feeding. Hovde et al. (1999) found that *E. coli* O157:H7 was shed in the feces of cattle for longer duration of time when fed forage than grain. In contrast, in another study, feedlot cattle with natural *E. coli* O157 infection had prevalence of 52% and 18% in grain and hay fed groups, respectively (Callaway et al., 2003). These types of findings have lead to vigorous debate among scientific community on impact of grain vs. hay feeding on the shedding of *E. coli* O157 in feces. The effect of feeding and fasting on the fecal shedding of *E. coli* also has been studied. Fasting increased total coliform counts in both rumen and fecal samples (Cray et al., 1998). However, fasting effect on the population dynamics of *E. coli* O157 in cattle have not been observed as with other microbes (Cray et al., 1998). The change in diet and fasting of animals can change physiological conditions of the animal gastrointestinal tract creating conditions suitable for pathogen survival (Cray et al., 1998; Heuvelink et al., 1998).

The incidence of *E. coli* O157:H7 infection has been suggested to be higher in summer from July through September due to increased grazing of animals and contamination of pasture (Heuvelink et al., 1998). In another study, it was reported that *E. coli* O157:H7 was isolated at higher rate in spring than in winter months (Chapman et al., 1997).

Understanding that this pathogen is harbored in a wide range of animal hosts and is shed in their feces is important because of its ability to cause life threatening illness in humans. Furthermore, the infective dose of *E. coli* O157:H7 is also very low, ca. 50 organisms, which make it even more severe in terms of causing disease (Fratamico and Smith, 2006). All these factors offer a great deal of challenges for the prevention of food contamination from this pathogen.

***Salmonella* spp.**

Salmonella spp. is another important pathogen that is enteric in origin and belongs to family of *Enterobacteriaceae*. These are Gram-negative rods that can grow under facultative anaerobic conditions. Over the years, there have been changes in the classification and nomenclature of *Salmonella* spp. Earlier, each *Salmonella* serotype was treated as separate species. However, according to a new taxonomic scheme all *Salmonella* spp. has been grouped into two species, namely *S. enterica* and *S. bongori. S. enterica* which represents 99% of the *Salmonella* isolated so far is further divided into six subspecies, i.e. *enterica, salamae, arizonae, diarizonae, houtenae* and *indica*, while *S. bongori* has no subspecies under it (Popoff et al., 2003). Each subspecies have different serotypes. The serotyping of the *Salmonella* spp. is based on the lipopolysaccharide (O),

flagellar (H) and sometimes capsular (Vi) antigens (Popoff et al., 2003). Additionally, based on the type of risk they pose to human health *Salmonella* bacteria can be broadly classified into two groups: typhoidal group (*S.*Typhi and *S*. Paratyphi) that are responsible for enteric fevers (typhoid) and the non-typhoidal group, which causes gastrointestinal illnesses (Sobsey et al., 2006). This study is mainly focused on *Salmonella* spp. (non-typhoidal) that infect human as well as animals. Poultry, cattle, sheep and pigs are the main reservoir of these zoonotic pathogens (Sobsey et al., 2006). Poultry are the major source of these pathogens yet they exhibit no apparent symptoms; however, other hosts may or may not suffer illnesses such as a gastrointestinal disturbance such as diarrhea and fever. The non-typhoidal group of *Salmonella* spp. contains over 2,500 different serotypes that are found in different hosts (Molbak et al., 2006). Although salmonellosis has been associated with poultry (O'Flynn, 2006), there are several outbreaks due to *Salmonella* that have been associated with fresh produce such as tomatoes, carrot, lettuce, and fruits (Sivapalasingam et al., 2004). In a study where data for different food borne outbreaks in the United States were analyzed from 1973-1997, about 48% of the produce associated outbreaks reported during that period were associated with *Salmonella* spp. Trace back investigation of these outbreaks revealed that fecal contamination of the produce at farm directly or indirectly was the key reason for illnesses (Sivapalasingam et al., 2004). *Salmonella* still remains as the leading cause of foodborne outbreak accounting for the majority of the illnesses with 8256 cases, 2,290 hospitaliztion and 29 deaths annually (CDC, 2011b). *Salmonella* serotype Typhimurium and serotype Enteritidis are the most common that are associated with

illness in the United States, beside serotypes Heidelberg and Newport (CDC, 2011a; Finke et al., 2002).

Fecal contamination of food (meat and fresh produce) and water is an important source of this pathogen. The organism can gain entry into a healthy host through the fecal-oral route. The infection may be caused by as low as 10 cells of *Salmonella*, but this number can vary depending upon the serotype, type of food consumed and the immune status of the host (Mobak et al., 2006). The disease symptoms caused by this organism are due to enterotoxin produced in the intestine which results in diarrhea and other clinical manifestations (Mobak et al., 2006). Presence of this organism in the fecal material of healthy animals and their ability to survive in the environment make them important organisms for food safety. Islam et al. (2004) in their study found that *S*. Typhimurium survived 203 days in soil when applied to irrigation water and also resulted in contamination of radish and carrot. In 2005, iceberg lettuce contaminated with *S*. Typhimurium was the cause of outbreak in Spain which was irrigated with the contaminated waste water (Takkinen et al., 2005).

Listeria monocytogenes

L. monocytogenes is a Gram-positive rod, which is widely distributed in the environment (Sobsey et al., 2006). This organism is capable of growing at wide temperature ranges. The disease listeriosis mainly affects elderly, immunocompromised, or pregnant women. The disease usually results in abortion, encephalitis, and septicemia (Altekruse et al., 1997). In the United States approximately 255 people die each year due to listeriosis (CDC, 2011c). Like *Salmonella* spp. and *E. coli* O157:H7, *L. monocytogenes* is also

carried by animals without any apparent illness. Humans are infected with this organism through consumption of contaminated food such as meat, cheese and other dairy products (Sobsey et al., 2006). Fresh produce can be contaminated with this organism from soil or from manure used as fertilizer. *Listeria* was reported to survive for 182 days in soil that has not been exposed to sun (Fenlon, 1999). It also has been reported to survive in liquid manure for 36 and 106 days, respectively, in summer and winter season (Fenlon, 1999). The wide distribution of this organism and its ability to survive at different conditions make it a potential pathogen for foodborne illnesses.

Animal Wastes and Compost

Dairy compost

According to the estimate of the United States Department of Agriculture, 335 million tons (ca. 304 billion kg) of dry matter waste (dairy waste after removing water) is produced annually on farms in the United States (USDA, 2006). Dairy operations produce about 100 times more manure than human sewage waste processed in municipal waste water plants (Gerba and Smith, 2004). Some of this waste is directly spread on land where animals graze. However, treating the rest of the waste is a problem. Composting of animal waste has been used as a practical way of treating dairy animal waste. Dairy animal waste is a known source of human pathogens such as *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* (Doyle et al., 2001; Elder et al., 2000). Therefore, proper treatment of animal waste becomes all the more important. Inactivation and survival of human pathogens during composting process has been reported from various

studies (Erickson et al., 2009; Shepherd et al., 2010; Jiang et al., 2003). In a study to determine the fate of high levels of *E. coli* O157:H7 (ca. 7 log CFU/g) in bovine manure that was composted at 21 and 50°C in a bioreactor, *E. coli* O157:H7 was detected at all the sampling locations until 7 days when external temperature of the bioreactor was maintained at 50°C. Whereas, when the temperature of the reactor was held at 21°C, the pathogen was detected at all sampling locations through 36 days of composting (Jiang et al. 2003). To study the effect of different C:N ration on inactivation of pathogen and temperature rise in dairy compost in a bioreactor, Erickson et al. (2009) reported ca. 7 log reduction within the first 2 days of composting process in compost mix formulated to 20:1 and 40:1, C:N ratio. However, there was only 5 log reduction in compost with a 30:1 C:N ratio. Temperature rise was gradual from day 1 to day 4 in compost with a 30:1 C:N ratio and it did not increase until 2nd day in compost with a 20:1 C:N ratio, whereas in compost with a 40:1 C:N ratio temperature reached maximum level on day 1 and then started to decline. In another study, fresh cow manure was composted with saw dust in a bioreactor with forced aeration, held at external temperature of 25 and 45°C (Lung et al., 2001). They reported *E. coli* O157:H7 and *Salmonella* level below the detection limits 3 and 2 days after the onset of composting process at 45°C, respectively. However, pathogen population remained constant when composting was done at 25°C.

Composting in a bioreactor under controlled condition does not represent a true picture of the actual composting process that occurs under a natural setting. Composting under field conditions is subjected to environmental variations such as rainfall, UV

exposure, and different seasons. All these factors can affect the final outcome of the composting process.

In a field study, Toth et al. (2011) studied *S.enterica* serovar Newport (multi drug resistant) survival in dairy manure compost. In static compost pile at 64°C, *S*. Newport was eliminated within 18 h, whereas, in lagoon and field soil it persisted for > 137 and 276 days, respectively. Therefore, composting process was more effective in destroying the multi drug resistant pathogen than lagoon treatment or spreading on soil. Shepherd et al. (2007), based on their study in the field, suggested turning of compost heap for inactivation of pathogens on the surface. They found that *E. coli* O157:H7 survived for 4 months on the surface of dairy manure compost heap. In contrast, the pathogen was detected inside the heap through 14 days by enrichment. Nicholson et al. (2005) on the other hand reported no difference in the survival of *E. coli* O157:H7, *Salmonella* and *Listeria* in dairy manure compost maintained as static or static aerated heaps. Metabolic state of pathogens during composting also affected their inactivation (Shepherd et al., 2010). In that study, researchers reported that during summer heat-shocked *E. coli* O157:H7 and *S*. Typhimurium survived 7 and 2 days longer at the surface and bottom location of the dairy compost heap, respectively, than the non heat-shocked culture. Whereas, in winter season both heat-shocked and non heat-shocked cultures were detectable for more than 60 days in all the samples. This study indicated that metabolic state of the pathogen during composting and season can affect their survival.

Field based studies are much more complicated than laboratory based study as they are subjected to several variations. The studies above also show that composting process

is complex as several factors govern final outcome of this process. Therefore, it is important to study as many factors as possible which can influence compost safety. There is need for conducting studies in order to determine various factors affecting this process and validate lab research findings using field studies as well.

Poultry Compost

The poultry industry in the United States produces between 13-25 million tons (ca. 12-23 billion kg) of waste each year (Nachman et al., 2005). Poultry waste is a rich source of nitrogen, phosphorus and other nutrients and is preferred fertilizer in agricultural practices (Moore et al., 1995). Poultry waste however is known to harbor human pathogens such as *Salmonella* spp., *Staphylococcus* (Martin et al., 1998; Terzich et al., 2000), *Campylobacter* (Montrose et al., 1985) and *Clostridium perfringens* (Ogonowski et al., 1984). If poultry waste is not properly treated, then it has the potential to introduce pathogens into soil, water and produce. Livestock and poultry waste treatment is important since studies have shown the persistence of pathogens for extended period of time in feces, soil and manure amended soil (Islam et al., 2005; You et al., 2006)

Composting of poultry waste is one such method to treat the ever increasing volume of poultry waste. The end product of proper composting process is stable, safe and conducive for soil amendment. Studies have shown effectiveness of composting process in eliminating pathogens from the poultry waste. Brodie et al (1994) reported elimination of *Salmonella*, *C. jejuni* and *L. monocytogenes* from poultry compost with

compost temperature in excess of 55°C. In another study, none of the 64 samples of composted poultry litter were positive for *E. coli* O157:H7 and *Salmonella* Spp. (Martin et al., 1998). However, this process may not be effective every time in inactivating pathogens in compost heap despite high temperatures. Hutchison et al. (2005) demonstrated *Salmonella*, *E. coli* O157:H7 and *Listeria* survived for 8 days in poultry compost despite temperature of the compost was above 55°C. Therefore, it is important to study various factors that can affect survival of pathogens in poultry compost. Most of the research to date had focused on dairy manure-based composting process, findings from these studies have been extended to poultry manure based composting. Owing to the differences in physical, chemical and nutritional properties of poultry and dairy manure it would be appropriate to focus on waste treatment and safety issues of these two types of waste separately.

Composting Process

Composting is a microorganism driven process done under aerobic conditions that brings about decomposition of organic matter/waste such as animal manure and agricultural waste into stable humus-like material that can be used as a soil conditioner for agricultural production and home gardening. During composting microbial metabolism also generates heat that can bring about inactivation of pathogens present in the compost material. The process of composting is broadly divided into three main phases based on the temperature and active microbial community (USEPA 1999). The mesophilic phase is the first phase of the composting process where temperature of the

compost heap begins to rise slowly from ambient temperature to 40°C. Mesophiles are abundant during this phase. The thermophilic phase is the critical phase of the composting process where temperature reaches as high as 55-70°C. This phase lasts for weeks to months and is dominated by the activity of the thermophilic microorganisms (Erickson et al., 2010). The Cooling/maturation phase occurs when most of the nutrients in the compost mix have been used and the microbial activity starts to slow down. In the mean time the temperature slowly and gradually reduces to ambient temperature resulting in a stable product. During this phase, there is reemergence of mesophilic bacteria (Hassen et al., 2002).

Typically, composting of waste material can be done in three ways:

1. Static piles, which are actively or passively aerated.
2. Windrow system, where compost ingredients are mixed and stacked in long narrow rows which are turned regularly.
3. In-vessel system, where compost materials are contained in a vessel for composting process with some means of aeration.

Studies have shown that diversity of mesophilic, thermophilic and thermotolerant organisms are part of this composting process, which include bacteria, actinomycetes, fungi and yeast. The microbial communities active during different phases of the composting process are not the same and they change as the phase of the compost process proceeds. The 16S rRNA (ribosomal ribonucleic acid) analysis of the microbial community by Density Gradient Gel Electrophoresis (DGGE) revealed that 87% of the microorganisms in the thermophilic phase of the composting process were of the genus

Bacillus (Ishii et al., 2000). Also, temperature and nutrients that were available during the composting process affected the microbial communities that were different from the onset of composting. Besides, *Bacillus*, species of genus *Thermonospora* and *Micropolyspora* have also been reported to be active during the thermophilic phase of composting (Herrmann and Shann 1996; Strom 1985).

Analysis of compost microbial communities using techniques other than 16S RNA such as gas chromatography-fatty acid methyl ester (GC-FAME) also revealed *Bacillus* as the most prevalent genus during the thermophilic phase (Ryckeboer et al., 2003). In another study, microbial community analysis by GC-FAME during composting of household waste further confirmed genera of *Bacillus* and *Thermus* to be the predominant microbial communities during the thermophilic phase (Steger et al., 2005).

Predominance of the genus *Bacillus* during the thermophilic phase and at the end of the composting process underlines the importance of this organism during composting. This is in agreement to the wide environmental prevalence of this genus and its ability to withstand high temperatures due to spore formation. Therefore, presence of thermophiles is important during composting as the thermophilic phase is characterized by elevation of temperature necessary for pathogen inactivation.

Factors Affecting Composting Process

The heat generated during composting is a primary factor responsible for inactivation of pathogens during composting of animal manure, providing the minimum threshold temperature during that process reaches 55°C and is maintained for 3 days in a

static pile or vessel and 15 days for windrow system of composting (USEPA, 1999). In order to ensure that composting follows the proper process, certain factors need to be controlled or monitored. The composting materials that are mixed for this purpose should have balanced nutrient level, which is measured as carbon to nitrogen (C:N) ratio. The C:N ratio of 20 - 40:1 is acceptable for active composting, but a range of 25:1 -30:1 is preferred (Sherman, 2005). The carbon source in the compost mix is used by indigenous microbial population for growth, while nitrogen is essential for synthesis of their proteins and for reproduction. When microbial communities grow and reproduce, heat is generated that sanitizes compost and inactivates the pathogens. However when the C:N ratio level is imbalanced, microbial growth is hindered or slowed which can prolong pathogen survival by not generating enough heat. In one study, Erickson et al. (2009) reported effect of C:N ratio on *Salmonella* spp. inactivation in dairy manure-based mixture composted in a bioreactor under controlled conditions. They found that *Salmonella* spp. of 7 log CFU/g in compost mix with C:N ratio of 20:1, 30:1 and 40:1, survived for 4, 5 and 7 days, respectively. Also, cumulative heat exposure required for pathogen inactivation in compost mix with C:N of 20:1 was 15 fold less than the compost mix with C:N ratio of 40:1. The longer survival of *Salmonella* spp. in compost mix with C:N ratio of 40:1 was believed to be due to nitrogen limitation. As a result temperature increased to maximum within 1 day of composting and then started to decline, whereas in compost with 20:1 and 30:1 C:N ratio temperature increase during composting was gradual.

Moisture is important for the growth of microorganisms. Therefore, moisture content of the compost ingredients is another important parameter that can affect composting process. Moisture content of the compost mix in the range of 40-65% is acceptable, whereas, 50-60% is preferred (Sherman, 2005). Compost mix with high initial moisture level will turn composting condition anaerobic due to insufficient available oxygen and therefore, slow decomposition. On the other hand, compost mix with too low moisture will not have sufficient available water for the microorganisms to grow and generate heat through microbial metabolism. Effect of moisture on thermal inactivation of microorganism in compost has been reported. Ceustermans (2007) observed that temperature and moisture are the critical factor for composting. In their study, complete inactivation of *S*. Senftenberg was achieved within 7 days at a temperature of 50-55°C in garden waste compost. Whereas, in bio-waste compost *S.* Senftenberg survived for 10 h at 60°C with moisture content varying between 60-65%. It was also observed that reduction in moisture content of the compost ingredient extended survival of *Salmonella,* in addition to microbial antagonism.

The pH of the compost mix is another factor that can affect the composting outcome. A pH range of 6.5-8.0 is considered optimal for the composting process. Fluctuation above pH range can retard composting process from entering into the thermophilic phase (Sundberg et al., 2004). However, there is wide range of microbial communities that can allow continuation of the composting process in spite of pH fluctuations (Cochran and Carney, 2006).

The type of raw ingredients and their size used for composting also influence composting process. For example, carbon from straw is more available than the carbon from woody material, therefore, compost heap made of straw as one of the raw ingredient would heat up more quickly than wood chips as the raw ingredient (Erickson et al., 2010). Smaller particle size of raw ingredient increases surface area for microbial activity, however, smaller particle size can reduce aeration during composting. Therefore, particle size of 1/8 to 2 inches is considered optimal for efficient composting (Sherman 2005). Manure used during composting process is the primary source of microbial community (Green et al., 2004). High microbial diversity is the key to efficient and effective composting process, however, microbial composition varies in different types of manure. For example, diverse microbial community associated with poultry manure is more capable of utilizing diverse substrate than the microbes associated with cattle manure (Wang et al., 2007). The age of manure used for composting can also affect final outcome of composting process as aged manure would have undergone change in nutritional status thereby affecting rate of heat generation versus the use of manure in its fresh initial state as compost ingredient (Erickson et al., 2010).

Due to the outdoor nature of composting process, weather conditions such as ambient temperature, UV irradiation, drying and rainfall further add to the variability that can affect composting outcome. Shepherd et al. (2010) in their study reported effect of seasonality on the survival of pathogens in dairy compost. They found that *E. coli* O157:H7 and *S*. Typhimurium survived 21 to 30 days of composting as detected by enrichment on the surface during summer season. The same organisms were able to

survive for almost the entire period of composting experiment (60 days) as detected by enrichment during winter season. Variability in pathogen survival within heaps during field composting trial has also been reported. Shepherd et al. (2007) found temperature stratification within the heap and reported extended survival of *E. coli* O157:H7 on surface of the compost heap for about 4 months, whereas they were positive within the heap by enrichment through 14 days of the process.

Based on above information it can be concluded that key to successful composting depends on several factors. Therefore, in order to achieve effective and safe composting it is important to achieve right level of nutrients, moisture, pH, particle size, and microbial community to ensure that compost reaches at least minimum temperature threshold to make it pathogen free.

Re-growth of pathogen in compost

Re-growth of pathogens after the completion of the composting process is a major concern. This concern is valid as the thermophilic phase of the compost is believed to inactivate pathogens present in the compost ingredients. However, there are several factors that can influence pathogen re-growth after population of the microorganisms has dropped below detectable limits. Temperature stratification within compost heap can affect pathogen re-growth in compost. Shepherd et al. (2007) reported cold spots inside the compost heaps in a field study with pathogens surviving longer on the surface with potential to re-grow to higher levels. In another study, *E. coli* O157:H7 re-grew during composting of bovine manure by 1 and 2 log CFU/g with composting temperature of >

50°C for 2 days and 40 -50°C for 1 days, respectively (Hess et al., 2004). Deportes et al. (1998) in their study detected *Salmonella* in the cooling phase of windrow composting process, even though temperature during the thermophilic phase exceeded 55°C for 16 days and *Salmonella* were undetectable during 12 days of composting. This could be due to selection of heat resistant strains during composting.

Presence of higher moisture has been found to be another factor responsible for re-growth of pathogens in compost. Kim et al. (2009) reported re-growth of *E. coli* O157 in dairy manure compost with moisture content as low as 20% (water activity of 0.986). The number of *E. coli* O157:H7 level increased from ca. 1 to 4.85 log CFU/g in autoclaved compost. In a similar study re-growth of *Salmonella* and enteropathogenic *E. coli* was reported in sterilized compost to a significant level with moisture content of 10, 40 and 80% (Pietronave et al., 2004). These results also point out to the importance of background microflora in controlling re-growth of pathogens in the compost environment. This was evident in the findings of Kim et al. (2009) where they found background micro flora was able to suppress re-growth of *E. coli* O157 by ca. 6.5 log CFU/g in dairy manure compost, however, background miroflora suppression was weak with higher levels of pathogenic *E. coli* O157:H7 (ca. 3 log CFU/g) in the compost. From the above information it can be concluded that the composting process that has followed EPA composting guidelines may not be enough to ensure compost safety. Besides that, handling of the composted material with proper care is equally important to prevent pathogen re-growth and subsequent contamination of environment.

Microbiological Hygiene Criteria for Composting

Microbiological hygiene criteria for compost vary throughout the world and are set or established by various organizations, public and private. In the United States, composting involving animal manure is not specifically under the regulation of a federal agency. Requirements for the composting operation vary from state to state. However, for the microbiological hygiene of the compost, states rely on the federal guidelines for biosolid based compost set by EPA in 40 CFR part 503. The EPA has recommended regulations for land application of different classes of compost based on their microbiological quality (Erickson 2010). According to EPA hygiene criteria for Class A compost, coliform and *Salmonella* should be less than 1,000 CFU/g and 3 MPN/4g of dry compost, respectively, and is recommended for application on agricultural land including fresh produce field. Whereas, Class B biosolid can have coliform and *Salmonella* numbers more than Class A biosolid, but with restrictions to use at places with less chances of human exposure, such as forest land. The composting process criteria recommended in 40 CFR part 503 for obtaining Class A compost consist of minimum temperature of 55°C or above for 3 days for static aerated piles or in vessel composting, and 15 days for windrow system with at least five turnings (USEPA 1999). EPA also recommend covering static composting piles with 0.3 m layer of insulating materials such as finished compost to ensure each and every particle of the compost material is subjected to the time-temperature criteria. In uncovered compost piles, particles at the surface are more close to ambient temperature than temperatures inside the compost pile which could lead to incomplete inactivation of the pathogens at the

surface (USEPA, 2003). Therefore, mixing or turning of the compost heap frequently is recommended to ensure all compost particles are exposed to the required time-temperature (NOSB, 2006).

Despite these guidelines, achieveing microbiological hygiene criteria for every compost facility as per the recommendation of the EPA does not occur. A survey of 16 composting facilities in Massachusetts revealed that five of the facilities had *E. coli* levels higher than the standard for EPA's class A compost and one even exceeded class B standards where a majority of the facilities used aerated static piles for their composting process (Soares et al., 1995). Hussong et al. (1985) in their study on sewage sludge composts in the United States found that in two samples fecal coliforms numbers were 1.3×10^7 MPN/g and 1.1×10^5 MPN/g, with three samples positive for *Salmonella* at a level of 1.7×10^4 MPN/g. In another study on testing microbiological quality of the market-ready recycled organic matter (ROM) composts, Brinton et al. (2009) revealed that 23, 44 and 20 % of the samples exceeded the EPA guidelines for fecal coliforms, in Washington, Oregon, and California states, respectively. *E. coli* O157:H7 also was detected in some facilities in the same study. Based on the results from different studies reported above it can be said that relying solely on time-temperature guidelines is not sufficient to ensure microbial safety of the composting process. There is need for a broader look into various factors that can influence compost hygiene.

Microbial Stress Response

Stress is broadly defined as factors such as physical, chemical and biological that affects microbial behavior and growth (Wesche et al., 2009). The behavior of the microbial cell exposed to the stress depends on the magnitude of the stress. Exposure to sublethal stress causes sublethal-celluar injury and temporary cessation of growth, but does not kill a microorganism, whereas, lethal stress causes permanent injury and death of the cell (Rodriguez et al., 2005).

Microorganisms once exposed to sublethal stress are subsequently able to survive lethal levels of the same stress better, this phenomenon is called as stress adaptation (Rodriguez et al., 2005). Microorganisms that are important for food safety are exposed to different types of stress in their journey from farm to fork. The most commonly used stresses in food industry include heating, drying, freezing, acidification, salts, preservatives, antimicrobial compounds, high hydrostatic pressure, radiation etc (Wesche et al., 2009).

Microorganisms can be induced to starvation/low nutrient stress. Adaptation to starvation/low nutrient occurs when microorganisms are present in an environment with adequate oxygen but with low or no available nutrient to support their growth and multiplication (Dickson and Frank, 1993). In natural environments microorganisms are exposed to growth limiting conditions and rapidly changing nutrient availability (Hengge-Aronis, 1993). Presence of microorganisms in different physiological states such as lag phase, exponential, stationary or death phase also makes them either susceptible or resistant to a particular stress. Induction of starvation stress in *E. coli*

O157:H7 was reported when they were kept overnight in water (Rowe and Kirk, 2000). Similarly, low nutrient level has also been observed to induce stress in nonpathogenic *E. coli* and S. Typhimurium when they were exposed to glucose starvation for 4 h in minimal media and minimal media with 0.02% glucose for 10 h, respectively (Jenkins et al., 1988; Tolker-Nielsen and Molin, 1996)

Acid-adaption is also a common stress that foodborne pathogens are exposed to in food processing environment. Acid-adaption of microorganism usually occurs when they are exposed to pH variations in the environment, such as acidic food, during processing or when they pass through gastrointestinal tract of animals and humans (Sharma et al., 2003). Acid-adaption response of the microorganisms in food industry is the result of biological effect of low pH caused by organic acids such as acetic, propionic and lactic acid (Abee and Wouters 1999). Induction of acid-adapted response has been observed in *E. coli* O157:H7, *S*. Typhimurium and *L. monocytogenes*, when they were grown in tryptic soy broth (TSB) supplemented with 1% glucose, grown in medium E with 0.4% glucose acidified with HCL and in TSB with yeast extract acidified with HCL or acetic acid, respectively (Farber and Pagotto, 1992; Leyer and Johnson, 1993 and Singh et al., 2006).

E. coli O157:H7 is considered to be more acid resistant than other pathogenic *E. coli* and this additional property makes it more virulent with low infective dose (Grauke et al., 2003; Hancock et al., 1994). Acid-adaptation as a result of type of diet has been considered a potentially important factor that influences gut microbial population and that of *E. coli* O157:H7 (Diez-Gonzalez et al., 1998). Ability to divide, acid resistance, and

diet variation also influence shedding of *E. coli* O157:H7 in environment. Impact of diet especially grain feeding on fecal shedding of *E. coli* O157:H7 in ruminants is undergoing intense debate. Some suggest that grain feeding creates an acidic environment in the gut of cattle due to production of acetic, propionic, butyric and lactic acid. This leads to the selection of acid-tolerant *E. coli* including *E. coli* O157 strains. The selected strains survive harsh gastric pH after having adapted to low pH in rumen. Other studies have revealed no difference in *E. coli* O157:H7 prevalence between grain fed and hay-fed cattle (Hancock et al., 1994).

In addition to heat, starvation/low nutrients and acid stresses, microorganism can also be induced to stress such as osmotic stress (high salt, sugar or dry environment), oxidative stress (exposed to hydrogen peroxide or ozone) and cold stress (Wesche et al., 2009). Induction of stress response mechanisms in microbial cells make them more resistant to the environment they are present. This behavior makes their inactivation difficult and raises public health concern.

Microbial Cross-protection Mechanisms to Stress

Microbial cell adaptation to a stress may also enhance cell tolerance to multiple lethal stresses. This multiple stress-adaptation response is called as cross-protection. Microorganisms, especially foodborne pathogens, are frequently exposed to stresses that cross-protect them against various other lethal stresses (Rodriguez-Romo and Yousef, 2005).

There are studies showing cross- protection of microbial cells to high inactivation temperature once adapted to acid (Ryu and Beuchat, 1998). Acid-adapted *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* survived heat-inactivation in media and food system through cross protection mechanisms (Roering et al., 1999; Ryu and Beuchat, 1998; Singh et al., 2006). The heat tolerance of *E. coli* O157:H7, acid-adapted in Tryptic soya broth (TSB) with 1% glucose, was high in comparison to non-adapted one when inactivated at 52, 54 or 56°C in TSB (Ryu and Beuchat, 1999). In a similar study *E. coli* O157:H7 in apple and orange juice was found to have higher *D*-value at 52°C when acid-adapted in TSB acidified with organic acids such as acetic and lactic acid and fruit juices such as apple cider and orange juice (Ryu and Beuchat, 1998). Leyer and Johnson (1993) reported that *S*. Typhimurium acid-adaptation in Medium E acidified with 10 N HCL had increased thermal resistance in comparison to non-adapted culture when inactivated in phosphate buffer saline (PBS) at 50, 55 and 57.5°C. Enhanced thermal tolerance of *L. monocytogenes* acid-shocked with HCL has also been reported (Farber and Pagotto, 1992). From these studies we can conclude that cross protection due to acid-adaptation is universal in microbial populations and can be induced with different conditions. Therefore, enhanced resistance of microorganisms due to cross protection is another concern that needs detailed attention in order to avoid food safety and public health issue.

Stress due to starvation or low nutrients has also been reported to provide cross-protection for microorganisms to heat. Rowe and Kirk (2000) observed resistance of *E. coli* O157:H7 to high temperature of 56°C for 90 min when they were starved for 24 h in

water. *S*. Typhimurium was able to resist high temperature of 52°C when grown in minimal medium with 0.02% glucose for 10 h (Tolker-Nielsen and Molin, 1996). Clearly, microorganisms present in nature are exposed to an environment of rapid changing nutrient levels resulting in starvation stress that can enhance their tolerance to other stresses.

Composting Stress and its Influence on Microbial Inactivation

Microbial inactivation during composting generally relies on high temperatures reached during this process. High temperature achieved during composting process builds slowly, from ambient temperature to mesophilic range and then to thermophilic phase. This temperature transition from mesophilic to themophilic phase of composting, consequently, has potential to cause some population of pathogenic bacteria to become acclimatized before lethal temperature is reached or even survive at higher temperature for extended period of time (Amner et al., 1991; Davis et al., 1992). A slow increase in the temperature of compost mixture during mesophilic phase can also induce heat-shock response, thereby resulting in increased heat resistance of pathogens during subsequent composting. Extended survival of *E. coli* O157:H7 in heaped mixture of dairy cattle manure and beef cattle manure for 32 and 93 days, respectively, has been reported despite heap temperature in excess of 50°C (Hutchison et al., 2005), suggesting the role of heat acclimatization for pathogens.

Generally bacterial cells are able to survive longer under lethal temperature treatment after they have been exposed to sub-lethal temperature for a brief period of

time, as commonly called heat-shock response (Murano and Pierson, 1992). Various studies have demonstrated that heat-shocked cells exhibit higher thermal resistance than the control cells in lab culture media (Linton et al., 1990; Mackey and Derrick, 1986; Murano and Pierson, 1992). In some studies thermo-tolerance in *E. coli* O157:H7 was increased by 2 and 1.5 fold in *D*-value at 55 and 60°C, respectively, due to heat-shock response in culture broth and ground beef (Juneja et al., 1998; Murano and Pierson, 1992). Similarly, *Salmonella* has also been reported to develop thermal resistance at 52 or 57.8°C in lab media, and at 60°C in turkey meat after heat-shocked at various temperatures (42, 48, 52 and 54°C) (Bunning et al., 1990; Wesche et al., 2005). After heat-shock at 46°C for 30 min to 1 h or 48°C for 120 min, *L. monocytogenes* was able to survive for 20.1 to 22.3 min at 57°C in TSB with 0.6% yeast extract and for 8 min at 64°C in pork and beef mix sausage (Faber and Brown, 1990 and Skandamis et al., 2008).

There are time-temperature combinations that produce maximum thermo tolerance following heat shock. Temperatures between 45 and 50°C are optimal for development of the heat shock response in mesophilic bacteria although there is no specific temperature for particular species of bacteria that can induce heat-shock response (Lindquist, 1986). In earlier studies, different sub-lethal temperatures have been used for inducing heat-shock response in different species of bacteria, which varied from as low as 32°C to as high as 54°C (Bunning et al., 1990; Lin and Chou, 2004; Murano and Pierson, 1992; Wesche et al., 2005).

Compost although is a nutrient rich environment but microorganisms present in compost may be protected by compost matrix or they may exist in biofilms. Cells in

biofilm are exposed to starvation/low-nutrient and other types of stress, which could induce stress adaptation and cross-protective responses (Rodriguez-Romo and Yousef, 2005). Additionally, microorganisms present in compost having animal origin might be acid adapted as they are exposed to different acids when they pass through gastrointestinal tract. This could also increase their tolerance to compost high temperature extending their survival.

Microbial Survival Mechanisms during Heat-Stress

Microorganisms have inherent ability to sense stress and respond to it by triggering protective responses (Rodriguez-Romo and Yousef, 2005). One of the responses of microorganism to stress includes synthesis of protective proteins that participate in damage repair, cell maintenance, or eradication of stress agents. The regulation of the general stress response has been well characterized in several microorganisms. The general stress response in most gram-negative bacteria, including *E. coli* and *Salmonella,* is under the control of the alternative sigma factors such as RpoS (Abbe and Wouters, 1999). In *E. coli*, RpoS (σ^s) controls the expression of more than 50 genes involved in the general stress response (Loewen et al., 1998). In *L. monocytogenes* and other gram-positive bacteria, the alternative sigma subunit σ^B regulates the general stress response and controls the expression of several genes (Abbe and Wouters, 1999). Under non-stress condition housekeeping sigma factor is responsible for gene regulation, as its level is high in comparison to alternative sigma factor (Chung et al., 2006). In *E. coli* housekeeping sigma factor 70 (σ^{70}) is responsible for housekeeping function

whereas alternative sigma factors 32 (σ^{32}), 38 (σ^{38}) are responsible for heat and general stress, respectively (Abee and Wouters, 1999). Exposure of bacterial cell to stress results in rapid accumulation of alternative sigma factors to high levels, and subsequent expression of more than 50 genes involved in stress adaptation (Hengge-Aronis, 2000). Stress proteins behave as molecular chaperones that function to bind and stabilize cellular proteins that are generated during protein synthesis or by denaturation of existing proteins, modulate proteins-folding pathway to prevent misfolding or aggregation of proteins, and promote protein refolding and proper assembly (Chung et al., 2006). Like the general stress response, several specific stress responses also induce some additional stress proteins that allow bacteria to survive under stressful conditions (Abbe and Wouters, 1999).

The heat-shock response and associated heat-shock proteins have been studied extensively. A number of heat-shock proteins have been identified in *E. coli* O157:H7 including DnaK, DnaJ, GrpE, GroEL and GroES, and most of them are heat inducible (Carruthers and Minton, 2009). Heat-shock proteins have also been reported in *Salmonella* spp. and *L. monocytogenes* that are induced on heat stress (Sergelidis and Abrahim, 2009; Wesche et al., 2009).

Microbial Cell Acid-shock/adaptation and Cross Protection to Heat

Exposure to low pH often induces an acid-shock/acid-adaptation response or acid tolerance mechanism. This result in the expression of acid-shock proteins that protects the cells against lethal acid conditions or these proteins may cross-protect the

microorganism against high temperature. Acid-shock proteins have been identified in *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* (Audia et al., 2001; Bergholz et al., 2009; Ferreira et al., 2003).

Acid Adaptation in microorganisms especially *E. coli* O157:H7 and *Salmonella* has been divided into two types, one which is induced in log phase, and the other one in stationary phase (Foster, 2000; Small et al., 1994). The log phase acid-adapted system is triggered when microbial cells are grown in moderately acidic conditions and involves the synthesis of acid-shock proteins. The induction of these acid-shock proteins are under the control of σ^S factor (Abee and Wounters 1999; Sharma et al., 2003). These acid-shock proteins protect the cells from lethal acid conditions as well as cross protecting cells from other unrelated stress such as temperature, oxidative damage and high osmolarity (Foster 2000). The stationary phase acid-adapted system is further divided into two σ^S-independent and σ^S-dependent mechanisms. The first mechanism requires induction by acid and involves participation of response regulator, outer membrane proteins to control synthesis of acid-shock proteins. The σ^S-dependent mechanism is triggered by entry of the cells in the stationary phase and does not require previous exposure to acid for induction (Beales 2004; Foster, 2000; Yousef and Courtney, 2003). Stationary phase σ^S-dependent acid-adaptation system in *E. coli* has been divided into three (Audia et al., 2001; Bearson et al., 1997; Lin et al., 1995, 1996). The three acid resistance (AR) mechanisms include an oxidative system (AR1) and two other systems that involve glutamate decarboxylase (AR2) and arginine decarboxylase (AR3) and are fermentative in nature (Lin et al., 1995, 1996). AR 1 is induced in stationary phase under the control of

Rpo*S* and is repressed by glucose. Whereas, AR 2 and AR 3 systems are partially dependent on σ^S. AR 2 & 3 are associated with accumulation of proteins in the growth medium. AR 2 and 3 systems maintain bacterial pH homeostasis by consuming proton during decarboxylation in exchange of decarboxylated end product for new substrate through membrane proteins. Glutamine decarboxylation system has three genes that are an essential component of AR 2. Genes *gadA* and *gadB*, encode glutamate decarboxylase isomers in *E. coli* and the third gene *gadC* encodes for the antipoter protein (Smith et al., 1992). In AR 3 gene *adiA* is involved in acid tolerance response (Lin et al., 1995).

Toxin-antitoxin Role in Stress Response

Prokaryotic toxin-antitoxin (TA) systems are also hypothesized to play role in general stress responses. TA systems are commonly found both on plasmids, where they increase effective stability of plasmid, and on bacterial chromosomes, where their function has been the subject of considerable debate (Magnuson, 2007).

TA systems are ubiquitous in microbial genome and have been proposed to play possible role in many cellular functions such as gene regulation, growth control, stabilization of foreign genetic element, and persisters formation (Magnuson, 2007). These systems consist of a toxic gene that encodes stable toxic proteins that target an essential cellular function (transcription and translation) and an antitoxin gene encoding unstable antitoxin that binds and inhibits toxin. So far toxins characterized include gyrase inhibitors, phosphotransferase, site-specific ribonucleases, ribosome-dependent ribonucleases, and a possible riboexonuclease (Megnuson, 2007). Differential stability of

the toxin-antitoxin gene regulates toxin activity (Lewis, 2010). So far 15 TA modules have been identified in *E. coli* (Pandey and Gerdes, 2005). Some well-known TA system identified in *E. coli* K-12 (MG1655) chromosome includes *mazEF*, *relBE*, *yefM-yoeB*, *chpB*, and *dinJ-yafQ* (Tsilibaris et al., 2007). In one study, Hansen et al. (2008) identified several mutants showing 10-fold decrease in persister formation, these mutants were deficient in either of the global regulator genes such as *dnaKJ*, *dkaA*, *hupAB*, and *IhAB*. This study also suggested that there is high degree of redundancy in persister genes. It has also been proposed that there are several parallel and independent mechanisms that operate in persister cell formation (Lewis, 2010).

Induction of specific and general stress responses usually leads to a temporary and reversible reduction or cessation of growth. Chromosomally encoded TA systems are good candidates for participating in a general stress response. It has been found that activation of TA system by stress inhibit protein synthesis without affecting viability (Christensen et al., 2003; Pedersen et al., 2002). TA systems are therefore also believed to be important in the generation of "persisters," a subfraction of the population that is characterized by low growth rate and high resistance to stress such as antibiotics (Hansen et al., 2008). In one study done on *E. coli* it was also proposed that TA *mazEF* function as a programmed cell death when induced in response to high temperature (50°C) in minimal media (Kolodkin-Gal and Engelberg-Kulka, 2006). However, some studies reported that TA *mazEF* does not kill but induce stasis by inhibiting translation (Pedersen et al., 2002). It is therefore believed that chromosomal TA systems are growth modulators that induce a reversible dormancy upon stressful condition that enhance

fitness. A majority of the studies have been done in studying persisters formation on exposure to antibiotic and very few on other stress such as elevated temperature. Some experiments have been performed in liquid media, therefore, It would be interesting to see how TA system would respond when stress (heat) is applied in different environment such as compost.

During composting process temperature in compost heaps reaches as high as 55°C or above, placing great stress on microbial populations present in the compost mixture. However, despite high temperature during composting survival of pathogens has still been reported from those operations (Hutchison et al., 2005). Besides, possible role of heat-shock response providing protection to pathogens during composting process, role of TA system cannot be ruled out either.

Predictive Microbiology

Predictive microbiology is currently widely used in microbiological field, especially food microbiology to predict bacterial inactivation for safe processing of food products (Coroller et al., 2006; Juneja et al., 1997; Peleg, 2000). Predictive microbiology discipline combines microbiology, mathematics and statistics with the goal of predicting growth and inactivation of microorganism under different environmental conditions. Historically it has been assumed that reduction of microbial population by heat inactivation is log-linear in nature, i.e. at a given temperature the logarithm of bacterial number declines linearly with heating time (Stumbo, 1973; Tomlins and ordal, 1976). However, deviation from the linear decline in the log values with time has been

frequently observed (Juneja et al., 1997; 2001). This is due to the fact that bacterial survival curve exhibits an initial lag period or shoulder followed by exponential decline. Also a subpopulation of more persistent bacteria that decline at a slower rate than the majority of the population resulting in tailing. Persistent microbial population also influence and account for deviation in log-linear reduction. Application of log-linear regression approach for calculating thermal inactivation time results in inadequate estimation of thermal processing time that can compromise the safety of the food products.

A bacterial population under stress condition can produce different shapes of curves based on the type of stress (Coroller et al., 2006; Whiting, 1993). Different mathematical models have been proposed to simulate the inactivation of pathogens with different types of surviving curve. Among these models are the Vitalistic models (Cole et al., 1993), Modified Gompertz model (Membre et al., 1997) and Whiting model (Whiting, 1993). However, these individual models are unable to deal with all shapes of curves, and most of them are based on log-linear inactivation. The Weibull model is being extensively used in thermal and non-thermal treatment studies of the microorganism (Coroller et al., 2006). This model has been useful in describing most of the surviving curve such as biphasic with a nonlinear decrease, sigmoidal, concave, linear, convex, biphasic and linear with a tail curve. Application of such modeling in composting has not received must of attention.

Predictive microbiology is considered as the new paradigm of food safety management. It provides essential support to widely accepted food safety management

system such as Hazard Analysis Critical Control Point (HACCP). It relies on data that is extensively generated on study of microbial behaviour under different conditions. Such data is then put into mathematical equation for that particular process. Effect of different factors on microorganism is also incorporated in model to further fine tune it. A predictive model developed for a particular process can then be used to estimate the microbial safety without using traditional microbiological enumeration techniques. The existence of different composting techniques and types of composting material necessitates development of a model for composting in real-world application, thereby ensuring composting safe and effective.

Summary

Composting process no doubt is an important method that can take care of ever increasing waste and convert it into a useful byproduct for agricultural use. It is also believed to be effective in treating livestock and poultry manure of human pathogens that are of great public health problem. Composting is now being widely used by different farmers to treat their farm waste and is also being used for commercial purpose. Regulations and guidelines for the composting are not comprehensive. Also, composting process is complex influenced by several intrinsic and extrinsic factors that can determine its outcome. As a result there are chances for this process to be ineffective at certain occasions despite following time-temperature guidelines, creating a potential public health scare. It is therefore important to study various factors that can affect composting process outcome and influence survival of pathogenic microorganisms. In order to

elucidate complexity of the pathogen inactivation during composting process, the present study was designed with the following objectives:

1. To study thermal inactivation of heat-shocked *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* in dairy compost under isothermal condition
2. To evaluate thermal inactivation of *E. coli* O157:H7 and *Salmonella* spp. in different types of compost at elevated composting temperatures by simulating early stage of on-farm composting
3. To determine thermal inactivation of acid-adapted *E. coli* O157:H7 in fresh dairy compost by simulating early phase of composting process.
4. To develop predictive mathematical model for thermal inactivation of stress-adapted *E. coli* O157:H7, *Salmonella* spp. & *L. monocytogenes* in compost.
5. To study stress and virulence gene expression of *E. coli* O157:H7 in fresh dairy compost using real-time PCR.

References

1. Abee, T., J. A. Wouters. 1999. Microbial stress response in minimal processing. *International Journal of Food Microbiology.* 50:65-91.

2. Altekruse, S. F., M. L. Cohen, and D. L. Swerdlow. 1997. Emerging foodborne diseases. *Emerging Infectious Diseases.* 3:285-293.

3. Amner, W., A. J. Mccarthy, and C. Edwards. 1991. Survival of a plasmid-bearing strain of *Bacillus subtilis* introduced into Compost. *Journal of General Microbiology.* 137:1931-1937.

4. Audia, J. P., C. C. Webb, and J. W. Foster. 2001. Breaking through the acid barrier: An orchestrated response to proton stress by enteric bacteria. *International Journal of Medical Microbiology.* 291:97-106.

5. Beales, N. 2004. Adaptation of microorganisms to cold temperatures, weak acid preservatives, low pH, and osmotic stress: A review. *Comprehensive Reviews in Food Science and Food Safety.* 3:1-20.

6. Bearson, S., B. Bearson, and J. W. Foster. 1997. Acid stress responses in enterobacteria. *FEMS Microbiology Letters.* 147:173-180.

7. Bergholz, T. M., S. K. Vanaja, and T. S. Whittam. 2009. Gene expression induced in *Escherichia coli* O157:H7 upon exposure to model apple juice. *Applied and Environmental. Microbiology.* 75:3542-3553.

8. Brinton, W.F., P. Storms, and T.C. Blewett. 2009. Occurrence and levels of fecal indicators and pathogenic bacteria in market-ready recycled organic matter composts. *Journal of Food Protection*. 72: 332-339.

9. Brodie, H.L., Donald, J.O., Conner, D.E., Tucker, J.K. and Harkin, H.D. 1994. Field evaluation of mini-composting of poultry carcasses. *Poultry Science*. 73(Suppl 1), 41.

10. Bunning, V. K., R. G. Crawford, J. T. Tierney, and J. T. Peeler. 1990. Thermotolerance of *Listeria monocytogenes* and *Salmonella* Typhimurium after sublethal heat shock. *Applied and Environmental Microbiology.* 56:3216-3219.

11. Callaway, T. R., R. O. Elder, J. E. Keen, R. C. Anderson, and D. J. Nisbet. 2003. Forage feeding to reduce preharvest *Escherichia coli* populations in cattle, a review. *Journal of Dairy Science.* 86:852-860.

12. Carruthers, M. D., C. Minion. 2009. Transcriptome analysis of *Escherichia coli* O157:H7 EDL933 during heat shock. *FEMS Microbiology Letters.* 295:96-102.

13. Centers for Disease Control and Prevention (CDC). 2006. Ongoing multistate outbreak of *Escherichia coli* serotype O157:H7 infections associated with consumption of fresh spinach--United States, September 2006. *Morbidity and Mortality Weekly Report.* 55:1045-1046.

14. Centers for Disease Control and Prevention (CDC). 2008. Outbreak of *Salmonella* serotype Saintpaul infections associated with multiple raw produce items--United States, 2008. *Morbidity and Mortality Weekly Report.* 57:929-934.

15. Centers for Disease Control and Prevention (CDC). 2010. Surveillance for foodborne disease outbreaks --- United States, 2007. *Morbidity and Mortality Weekly Report.* 59:973-979.

16. Centers for Disease Control and Prevention (CDC), 2011a. *Salmonella*. http://www.cdc.gov/salmonella/general/ (Accessed July 2011)

17. Centers for Disease Control and Prevention (CDC). 2011b. Vital signs: incidence and trends of infection with pathogens transmitted commonly through food --- foodborne diseases active surveillance network, 10 U.S. Sites, 1996--2010. *Morbidity and Mortality Weekly Report.* 60:749-755.

18. Centers for Disease Control and Prevention (CDC), 2011c. http://www.cdc.gov/foodborneburden/PDFs/FACTSHEET_A_FINDINGS_updated4-13.pdf (Accessed July 2011)

19. Ceustermans, A., D. De Clercq, A. Aertsen, C. Michiels, A. Geeraerd, J. Van Impe, J. Coosemans, and J. Ryckeboer. 2007. Inactivation of *Salmonella* Senftenberg strain W 775 during composting of biowastes and garden wastes. *Journal of Applied Microbiology.* 103:53-64.

20. Chapman, P. A., C. A. Siddons, J. Manning, and C. Cheetham. 1997. An outbreak of infection due to verocytotoxin-producing *Escherichia coli* O157 in four families: the influence of laboratory methods on the outcome of the investigation. *Epidemiology and Infection.* 119:113-119.

21. Christensen, S. K., K. Pedersen, F. G. Hansen, and K. Gerdes. 2003. Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. *Journal of Molecular Biology.* 332:809-819.

22. Chung, H. J., W. Bang, and M. A. Drake. 2006. Stress Response of *Escherichia coli*. *Comprehensive Reviews in Food Science and Food Safety* 5:52-64.

23. Cochran, B. J. and W. A. Carney. 2005. Basic principles of composting. Louisiana State University Agricultural Center. Available at: http://www.lsuagcenter.com/NR/rdonlyres/1A247D4F-4E94-4021-B09E-2DF1043E179E/2908/pub2622compost.pdf. (Accessed on September, 2011)

24. Cole, M. B., K. W. Davies, G. Munro, C. D. Holyoak, and D. C. Kilsby. 1993. A Vitalistic model to describe the thermal inactivation of *Listeria monocytogenes*. *Journal of Industrial Microbiology and Biotechnology.* 12:232-239.

25. Coroller, L., I. Leguerinel, E. Mettler, N. Savy, and P. Mafart. 2006. General model, based on two mixed Weibulll distributions of bacterial resistance, for describing various shapes of inactivation curves. *Applied and Environmental Microbiology.* 72:6493-6502.

26. Cray, W. C., T. A. Casey, B. T. Bosworth, and M. A. Rasmussen. 1998. Effect of dietary stress on fecal shedding of *Escherichia coli* O157: H7 in calves. *Applied and Environmental Microbiology.* 64:1975-1979.

27. Davis, C. L., S. A. Hinch, C. J. Donkin, and P. J. Germishuizen. 1992. Changes in microbial-population numbers during the composting of pine bark. *Bioresource Technology.* 39:85-92.

28. Deportes, I., J. L. Benoit-Guyod, D. Zmirou, and M. C. Bouvier. 1998. Microbial disinfection capacity of municipal solid waste (MSW) composting. *Journal of Applied Microbiology.* 85:238-246.

29. Dickson, J., J. Frank. 1993. Bacterial Starvation Stress and Contamination of Beef. *Food Microbiology.* 10:215-222.

30. Diez-Gonzalez, F., T. R. Callaway, M. G. Kizoulis, and J. B. Russell. 1998. Grain feeding and the dissemination of acid-resistant *Escherichia coli* from cattle. *Science* 281:1666-1668.

31. Doyle, M. P., T. Zhao, J. Meng, and S. Zhao. 2001. *Escherichia coli* O157:H7. *In* M. P. Doyle, L. R. Beuchat, and T. J. Montville (ed.), Food microbiology: fundamentals and frontiers. ASM Press, Washington, D.C.

32. Droffner, M. L., W. F. Brinton. 1995. Survival of *E. coli* and *Salmonella* populations in aerobic thermophilic composts as measured with DNA gene probes. *Zentralbl. Hyg. Umweltmed.* 197:387-397. [Abstract Pubmed]

33. European Centers for Disease Control and Prevention (ECDC). 2011a. Outbreak of shiga toxin-producing *E. coli* (STEC) in Germany. http://www.ecdc.europa.eu/en/publications/Publications/1105_TER_Risk_assessment_EColi.pdf (Accessed on July 2011)

34. European Centers for Disease Control and Prevention (ECDC). 2011b. Understanding the 2011 EHEC/STEC outbreak in Germany. http://ecdc.europa.eu/en/aboutus/organisation/Director%20Speeches/201109_MarcSprenger_STEC_ICAAC.pdf (Accessed October 2011)

35. Elder, R. O., J. E. Keen, G. R. Siragusa, G. A. Barkocy-Gallagher, M. Koohmaraie, and W. W. Laegreid. 2000. Correlation of enterohemorrhagic *Escherichia coli* O157:H7 prevalence in feces, hides and carcasses of beef cattle during processing. *Proceedings of National Academy of Science.* 97:2999–3003.

36. Erickson, M. C., F. Critzer, and M. P. Doyle. 2010. Produce safety project. http://www.producesafetyproject.org/admin/assets/files/PEW-PSP-Composting-Manure-Narrative-v4-2.pdf (Accessed on July, 2010)

37. Erickson, M. C., J. Liao, L. Ma, X. Jiang, and M. P. Doyle. 2009. Inactivation of *Salmonella* spp. in cow manure composts formulated to different initial C:N ratios. *Bioresource Technology.* 100:5898-5903.

38. European Community. 2001. Working document—biological treatment of biowaste, 2nd draft. http://www.compost.it/www/pubblicazioni_on_line/biod.pdf (Accessed on July 2011)

39. Farber, J. M., B. E. Brown. 1990. Effect of prior heat shock on heat resistance of *Listeria monocytogenes* in meat. *Applied and Environmental Microbiology.* 56:1584-1587.

40. Farber, J. M., F. Pagotto. 1992. The Effect of acid shock on the heat-resistance of *Listeria monocytogenes*. *Letters in Applied Microbiology.* 15:197-201.

41. Fenlon, D. R. 1999. *Listeria monocytogenes* in the natural environment. *In:* Ryser, E. T. and E. H. Marth (ed.), *Listeria*, listeriosis, and food safety. Marcel Decker, Inc, New York.

42. Ferreira, A., D. Sue, C. P. O'Byrne, and K. J. Boor. 2003. Role of *Listeria monocytogenes* sigma (B) in survival of lethal acidic conditions and in the acquired acid tolerance response. *Applied and Environmental Microbiology.* 69:2692-2698.

43. Finke, M., P. Shillam, T. McGiven, X. Wang, D. Vugla, S. Segler, D. Gerber, S. Stenzel,S. Zansky, M. Kennedy, R. Marcus and the EIP FoodNet Working Group. 2002. Hospitalizations among cases with the most common serotypes of *Salmonella*:FoodNet 1996 – 2000. International Conference on Emerging Infectious Diseases. Atlanta, GA.

44. Foster, G., J. Evans, H. I. Knight, A. W. Smith, G. J. Gunn, L. J. Allison, B. A. Synge, and T. W. Pennycott. 2006. Analysis of feces samples collected from a wild-bird garden feeding station in Scotland for the presence of verocytotoxin-producing *Escherichia coli* O157. *Applied and Environmental Microbiology.* 72:2265-2267.

45. Foster, J. W. 2000. Microbial responses to acid stress. In: Storz, G. and Hengge-Aronis, R. (ed.), Bacterial stress responses. American Society for Microbiology, Washington, D.C.

46. Fratamico, P. M. and J. L. Smith. 2006. *Escherichia coli* infections. In: H. P. Riemann and D. O. Cliver (ed.), Foodborne infections and intoxications, 3rd ed. Elsevier Inc., San Diego, California.

47. Frenzen, P. D., A. Drake, F. J. Angulo, and Emerging Infections Program. 2005. Economic cost of illness due to *Escherichia coli* O157 infections in the United States. *Journal of Food Protection.* 68:2623-2630.

48. Gerba, Charles P., and James E. Smith, Jr. 2004. Sources of pathogenic microorganisms and their fate during land application of wastes. *Journal of Environmental Quality*, 34: 42-48.

49. Grauke, L. J., I. T. Kudva, J. W. Yoon, C. W. Hunt, C. J. Williams and C. J. Hovde.2002. Gastrointestinal tract location of *Escherichia coli* O157:H7 in ruminants. *Applied and Environmental Microbiology*. 68:2269 – 2277.

50. Grauke, L. J., S. A. Wynia, H. Q. Sheng, J. W. Yoon, C. J. Williams, C. W. Hunt, and C. J. Hovde. 2003. Acid resistance of *Escherichia coli* O157: H7 from the gastrointestinal tract of cattle fed hay or grain. *Veterinary Microbiology.* 95:211-225.

51. Green, S.J., F.C. Michel, Jr., Y. Hadar, and D. Minz. 2004. Similarity of bacterial communities in sawdust- and straw-amended cow manure composts. *FEMS Microbiology Letters*. 233:115-123.

52. Grossfeld, S., 1998. Animal waste emerging as US problem regulations fall short in protecting water quality, report finds. The Boston Globe. http://www.highbeam.com/doc/1P2-8502299.html (Accessed July 2011).

53. Hancock, D. D., T. E. Besser, M. L. Kinsel, P. I. Tarr, D. H. Rice, and M. G. Paros. 1994. The prevalence of *Escherichia coli* O157.H7 in dairy and beef-cattle in Washington-State. *Epidemiology and Infection.* 113:199-207.

54. Hansen, S., K. Lewis, and M. Vulic. 2008. Role of global regulators and nucleotide metabolism in antibiotic tolerance in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy.* 52:2718-2726.

55. Hassen, A., K. Belguith, and N. Jedidi. 2002. Microbial characterization during composting of municipal solid waste. Proceedings of International Symposium on Environmental Pollution Control and Waste Management. 357-368.

56. Hengge-Aronis, R. 1993. Survival of hunger and stress - the role of Rpos in early stationary phase gene-regulation in *Escherichia coli*. *Cell* 72:165-168.

57. Hengge-Aronis R. 2000. The general stress respondent in *Escherichia coli*, p. 161-178. *In:* Storz G and Hengge-Aronis R (ed.), Bacterial stress response. American Society for Microbiology, Washington, D.C.

58. Herrmann, R.F. and J.F. Shann. 1997. Microbial community changes during the composting of municipal solid waste. *Microbial Ecology*. 33: 78-85.

59. Hess, T.F., I. Grdzelishvili, H. Sheng, and C.J. Hovde. 2004. Heat inactivation of *E. coli* during manure composting. *Compost Science and Utilization*. 4: 314-322.

60. Heuvelink, A. E., F. L. A. M. van den Biggelaar, J. T. M. Zwartkruis-Nahuis, R. G. Herbes, R. Huyben, N. Nagelkerke, W. J. G. Melchers, L. A. H. Monnens, and E. de Boer. 1998. Occurrence of verocytotoxin-producing *Escherichia coli* O157 on Dutch daily farms. *Journal of Clinical Microbiology.* 36:3480-3487.

61. Hovde, C. J., P. R. Austin, K. A. Cloud, C. J. Williams, and C. W. Hunt. 1999. Effect of cattle diet on *Escherichia coli* O157: H7 acid resistance. *Applied and Environmental Microbiology.* 65:3233-3235.

62. Hussong, D., W.D. Burge, and N.K. Enkiri. 1985. Occurrence, growth and suppression of *Salmonellae* in composted sewage sludge. *Applied and Environmental Microbiology*. 50: 887-893.

63. Hutchison, M. L., L. D. Walters, S. M. Avery, and A. Moore. 2005. Decline of zoonotic agents in livestock waste and bedding heaps. *Journal of Applied Microbiology.* 99:354-362.

64. Ishii, K., M. Fukui and S. Takii. 2000. Microbial succession during a composting process as evaluated by denaturing gradient gel electrophoresis analysis. *Journal of Applied Microbiology*. 89:768-777.

65. Islam, M., J. Morgan, M. P. Doyle, S. C. Phatak, P. Millner, and X. P. Jiang. 2004. Fate of *Salmonella enterica* serovar Typhimurium on carrots and radishes grown in fields treated with contaminated manure composts or irrigation water. *Applied and Environmental Microbiology.* 70:2497-2502.

66. Islam, M., M. P. Doyle, S. C. Phatak, P. Millner, and X. P. Jiang. 2005. Survival of *Escherichia coli* O157: H7 in soil and on carrots and onions grown in fields treated with contaminated manure composts or irrigation water. *Food Microbiology.* 22:63-70.

67. Jenkins, D., J. Schultz, and A. Matin. 1988. Starvation-induced cross protection against heat or H_2O_2 challenge in *Escherichia coli*. *Journal of Bacteriology.* 170:3910-3914.

68. Jiang, X., J. Morgan, and M. P. Doyle. 2003. Fate of *Escherichia coli* O157:H7 during composting of bovine manure in a laboratory-scale bioreactor. *Journal of Food Protection.* 66:25-30.

69. Juneja, V. K., B. S. Eblen, and H. M. Marks. 2001. Modeling non-linear survival curves to calculate thermal inactivation of *Salmonella* in poultry of different fat levels. *International Journal of Food Microbiology.* 70:37-51.

70. Juneja, V. K., O. P. Snyder, and B. S. Marmer. 1997. Thermal destruction of *Escherichia coli* O157:H7 in beef and chicken: Determination of D- and z-values. *International Journal of Food Microbiology.* 35:231-237.

71. Juneja, V. K., P. G. Klein, and B. S. Marmer. 1998. Heat shock and thermotolerance of *Escherichia coli* O157:H7 in a model beef gravy system and ground beef. *Journal of Applied Microbiology.* 84:677-684.

72. Kaufmann, M., C. Zweifel, M. Blanco, J. E. Blanco, J. Blanco, L. Beutin, and R. Stephan. 2006. *Escherichia coli* O157 and non-O157 Shiga toxin-producing *Escherichia coli* in fecal samples of finished pigs at slaughter in Switzerland. *Journal of Food Protection.* 69:260-266.

73. Kim, J., F. Luo, and X. Jiang. 2009. Factors impacting the regrowth of *Escherichia coli* O157:H7 in dairy manure compost. *Journal of Food Protection.* 72:1576-1584.

74. Kolodkin-Gal, I., H. Engelberg-Kulka. 2006. Induction of *Escherichia coli* chromosomal *mazEF* by stressful conditions causes an irreversible loss of viability. *Journal of Bacteriology.* 188:3420-3423.

75. Laegreid, W. W., R. O. Elder, and J. E. Keen. 1999. Prevalence of *Escherichia coli* O157: H7 in range beef calves at weaning. *Epidemiology and Infection.* 123:291-298.

76. Lewis, K. 2010. Persister cells. *Annual Review of Microbiology*. 64:357-372.

77. Leyer, G. J., E. A. Johnson. 1993. Acid adaptation induces cross-protection against environmental stresses in *Salmonella* Typhimurium. *Applied and Environmental Microbiology.* 59:1842-1847.

78. Lin, J. S., I. S. Lee, J. Frey, J. L. Slonczewski, and J. W. Foster. 1995. Comparative-analysis of extreme acid survival in *Salmonella* Typhimurium, *Shigella flexneri*, and *Escherichia coli*. *Journal of Bacteriology.* 177:4097-4104.

79. Lin, J. S., M. P. Smith, K. C. Chapin, H. S. Baik, G. N. Bennett, and J. W. Foster. 1996. Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. *Applied and Environmental Microbiology.* 62:3094-3100.

80. Lindquist, S. 1986. The heat-shock response. *Annual of Review of Biochemistry*. 55:1151-1191.

81. Lin, Y. D., C. C. Chou. 2004. Effect of heat shock on thermal tolerance and susceptibility of *Listeria monocytogenes* to other environmental stresses. *Food Microbiology.* 21:605-610.

82. Linton, R. H., M. D. Pierson, and J. R. Bishop. 1990. Increase in heat-resistance of *Listeria monocytogenes* Scott-A by sublethal heat-shock. *Journal of Food Protection..* 53:924-927.

83. Loewen, P. C., B. Hu, J. Strutinsky, and R. Sparling. 1998. Regulation in the rpoS regulon of *Escherichia coli*. *Canadian Journal of Microbiology.* 44:707-717.

84. Lung, A. J., C. M. Lin, J. M. Kim, M. R. Marshall, R. Nordstedt, N. P. Thompson, and C. I. Wei. 2001. Destruction of *Escherichia coli* O157:H7 and *Salmonella* Enteritidis in cow manure composting. *Journal of Food Protection.* 64:1309-1314.

85. Mackey, B. M., C. M. Derrick. 1986. Elevation of the heat-resistance of *Salmonella* Typhimurium by sublethal heat-shock. *Journal of Applied Bacteriology.* 61:389-393.

86. Magnuson, R. D. 2007. Hypothetical functions of toxin-antitoxin systems. *Journal of Bacteriology.* 189:6089-6092.

87. Martin, S. A., M. A. McCann, and W. D. Waltman II. 1998. Microbiological survey of Georgia poultry litter. *Journal of Applied Poultry Research*. 7:90–98.

88. Mead, P. S., L. Slutsker, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States - Reply. *Emerging Infectious Diseases.* 5:841-842.

89. Membre, J. M., J. Thurette, and M. Catteau. 1997. Modelling the growth, survival and death of *Listeria monocytogenes*. *Journal of Applied Microbiology.* 82:345-350.

90. Mølbak, K., J. E. Olsen and H. C. Wegener. 2006. *Salmonella* Infections, p.57 – 136. In H. P. Riemann and D. O. Cliver (ed.), Foodborne infections and intoxications, 3rd ed. Elsevier Inc., San Diego, California.

91. Montrose, M. S., S. M. Shane, and K. S. Harrington. 1985. Role of litter in the transmission of *Campylobacter jejuni*. *Avian Diseases.* 29:392-399.

92. Moore P.A. Jr., T.C. Daniel, A.N. Sharpley, and C.W. Wood. 1995. Poultry manure management: Environmentally sound options. *Journal of Soil and Water Conservation*. 50:321-327.

93. Murano, E. A., M. D. Pierson. 1992. Effect of heat-shock and growth atmosphere on the heat-resistance of *Escherichia coli* O157-H7. *Journal of Food Protection.* 55:171-175.

94. Nachman, K. E., J. P. Grahamn, L. B. Price and E. K. Silbergeld. 2005. Arsenic – a roadblock to potential animal waste management solutions. Environmental Health Perspectives. May 2005. Pages 1 – 12.

95. Nataro, J. P., J. B. Kaper. 1998. Diarrheagenic *Escherichia coli*. *Clinical and Microbiological. Reviews.* 11:403-403.

96. Nicholson, F.A., S. J. Groves and B. J. Chambers. 2005. Pathogen survival during livestock manure storage and following land application. *Bioresource Technology*. 96:135 – 143.

97. NOSB. 2006. National Organic Standards Board Crops Committee recommendation for guidance use of compost, vermicompost, processed manure, and compost teas, October 19, 2006. Available at:http://www.ams.usda.gov/AMSv1.0/getfile?dDocName=STELPRDC5057102. (Accessed June 2011).

98. O'Flynn, G., A. Coffey, G. F. Fitzgerald, and R. P. Ross. 2006. The newly isolated lytic bacteriophages st104a and st104b are highly virulent against *Salmonella enterica*. *Journal of Applied Microbiology.* 101:251-259.

99. Ogden, I. D., M. MacRae, and N. J. C. Strachan. 2005. Concentration and prevalence of *Escherichia coli* O157 in sheep feces at pasture in Scotland. *Journal of Applied Microbiology.* 98:646-651.

100. Ogonowski, K., Barnard, M. and Glesecke, W. 1984. Bacteriological findings regarding the hygienic safety of poultry litter intended as an ingredient of feeds for ruminants. *Onderstepoort Journal of Veterinary Research*. 51, 249–252.

101. Pandey, D. and K. Gerdes. 2005. Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. *Nucleic Acids Research*. 33:966-976.

102. Pedersen, K., S. K. Christensen, and K. Gerdes. 2002. Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. *Molecular Microbiology.* 45:501-510.

103. Peleg, M. 2000. Microbial survival curves - the reality of flat "shoulders" and absolute thermal death times. *Food Research International.* 33:531-538.

104. Pietronave, S., L. Fracchia, M. Rinaldi, and M. G. Martinotti. 2004. Influence of biotic and abiotic factors on human pathogens in a finished compost. *Water Research.* 38:1963-1970.

105. Popoff, M. Y., J. Bockemuhl, and L. L. Gheesling. 2003. Supplement 2001 (no. 45) to the Kauffmann-White scheme. *Research in Microbiology.* 154:173-174.

106. Riley, L. W., R. S. Remis, S. D. Helgerson, H. B. Mcgee, J. G. Wells, B. R. Davis, R. J. Hebert, E. S. Olcott, L. M. Johnson, N. T. Hargrett, P. A. Blake, and M. L. Cohen. 1983. Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *New England Journal of Medicine.* 308:681-685.

107. Rodriguez-Romo, L and A. Yousef. 2005. Cross –protective effect of bacterial stress, p. 128-151. In M**.** Griffiths (ed), Understanding pathogen behaviour : virulence, stress response and resistance. CRC Press, Washington D.C.

108. Roering, A. M., J. B. Luchansky, A. M. Ihnot, S. E. Ansay, C. W. Kaspar, and S. C. Ingham. 1999. Comparative survival of *Salmonella* Typhimurium DT 104, *Listeria monocytogenes*, and *Escherichia coli* O157:H7 in preservative-free apple cider and simulated gastric fluid. *International Journal of Food Microbiology.* 46:263-269.

109. Rowe, M. T., R. B. Kirk. 2000. Effect of nutrient starvation on the resistance of *Escherichia coli* O157: H7 to subsequent heat stress. *Journal of Food Protection.* 63:1745-1748.

110. Ryckeboer, J., J. Mergaert, J. Coosemans, K. Deprins and J. Swings. 2003. Microbiological aspects of biowaste during composting in monitored compost bin. *Journal of Applied Microbiology*. 94:127 – 137.

111. Ryu, J. H., L. R. Beuchat. 1999. Changes in heat tolerance of *Escherichia coli* O157: H7 after exposure to acidic environments. *Food Microbiology.* 16:317-324.

112. Ryu, J. H., L. R. Beuchat. 1998. Influence of acid tolerance responses on survival, growth, and thermal cross-protection of *Escherichia coli* O157: H7 in acidified media and fruit juices. *International Journal of Food Microbiology.* 45:185-193.

113. Scallan, E., P. M. Griffin, F. J. Angulo, R. V. Tauxe, and R. M. Hoekstra. 2011a. Foodborne Illness Acquired in the United States-unspecified agents. *Emerging Infectious Diseases* 17:16-22.

114. Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011b. Foodborne illness acquired in the United States-major pathogens. *Emerging Infectious Diseases* 17:7-15.

115. Sergelidis, D., A. Abrahim. 2009. Adaptive response of *Listeria monocytogenes* to heat and its impact on food safety. *Food Control* 20:1-10.

116. Sharma, M., P. J. Taormina, and L. R. Beuchat. 2003. Habituation of foodborne pathogens exposed to extreme pH conditions: Genetic basis and implications in foods and food processing environments. *Food Science and Technology Research* 9:115-127.

117. Shepherd, M. W., Jr, P. Liang, X. Jiang, M. P. Doyle, and M. C. Erickson. 2007. Fate of *Escherichia coli* O157:H7 during on-farm dairy manure-based composting. *Journal of Food Protection.* 70:2708-2716.

118. Shepherd, M.W., R. Singh, J. Kim, and X. Jiang. 2010. Effect of heat-shock treatment on the survival of *Escherichia coli* O157:H7 and *Salmonella enterica* Typhimurium in dairy manure co-composted with vegetable wastes under field conditions. *Bioresource Technology*. 101: 5407-5413.

119. Sherman, R. 2005. Large-scale organic materials composting. North Carolina Cooperative Extension Service. Pages 1 – 16. Available at: http://www.bae.ncsu.edu/topic/vermicomposting/pubs/ag593.pdf. (Accessed August 2011).

120. Singh, M., S. M. Simpson, H. R. Mullins, and J. S. Dickson. 2006. Thermal tolerance of acid-adapted and non-adapted *Escherichia coli* O157:H7 and *Salmonella* in ground beef during storage. *Foodborne Pathogens and Diseases.* 3:439-446.

121. Sivapalasingam, S., C. R. Friedman, L. Cohen, and R. V. Tauxe. 2004. Fresh produce: A growing cause of outbreaks of foodborne illness in the United States, 1973 through 1997. *Journal of Food Protection.* 67:2342-2353.

122. Skandamis, P. N., Y. Yoon, J. D. Stopforth, P. A. Kendall, and J. N. Sofos. 2008. Heat and acid tolerance of *Listeria monocytogenes* after exposure to single and multiple sublethal stresses. *Food Microbiology.* 25:294-303.

123. Small, P., D. Blankenhorn, D. Welty, E. Zinser, and J. L. Slonczewski. 1994. Acid and base resistance in *Escherichia coli* and *Shigella flexneri* - Role of Rpos and Growth pH. *Journal of Bacteriology.* 176:1729-1737.

124. Smith, D. K., T. Kassam, B. Singh, and J. F. Elliott. 1992. *Escherichia coli* has 2 homologous glutamate-decarboxylase genes that map to distinct loci. *Journal of Bacteriology.* 174:5820-5826.

125. Sobsey, M. D., L. A. Khatib, V. R. Hill, E. Alocilja, and S. Pillain. 2006. Pathogens in animal wastes and the impacts of waste management practices on their survival, transport and fate, p. 609-666. *In:* J. M. Rice, D. F. Caldwell and F. J. Humenik (ed.), Animal Agriculture and the Environment: National Center for Manure and Animal Waste Management White Papers. st. Joseph, Michigan.

126. Sorares, H.M., B. Cardenas, D. Weir, and M.S. Switzenbaum. 1995. Evaluating pathogen regrowth in biosolids compost. *Biocycle*. 36: 70-74.

127. Steger, K., Y. Eklind, J. Olsson and I. Sundh. 2005. Microbial community growth and utilization of carbon constituents during thermophilic composting at different oxygen levels. *Microbial Ecology*. 50:163 – 171.

128. Strom, P.F. 1985. Identification of thermophilic bacteria in solid waste composting. *Applied and Environmental Microbiology*. 50: 906-913.

129. Stumbo, C. R. 1973. Thermobacteriology in food processing. , Academic Press, New York.

130. Sundberg, C., S. Smärs and H. Jonsson. 2004. Low pH as an inhibiting factor in the transition from mesophilic to thermophilic phase in composting. *Bioresource Technology*. 95: 145 – 150.

131. Takkinen, J., Nakari, U-M., Johansson, T., Niskanen, T., Siitonen, A. and Kuusi, M. (2005) A nationwide outbreak of multiresistant *Salmonella* Typhimurium var. *Copenhagen* DT104B infection in Finland due to contaminated lettuce from Spain, May 2005. Eurosurveillance Weekly 10, http://www.eurosurveillance.org/ew/2005/050630.asp (Accessed July 2011).

132. Terzich, M., M. J. Pope, T. E. Cherry, and J. Hollinger. 2000. Survey of pathogens in poultry litter in the United States. *Journal of Applied Poultry Research* 9:287-291.

133. Tolker-Nielsen, T., S. Molin. 1996. Role of ribosome degradation in the death of heat-stressed *Salmonella* Typhimurium. *FEMS Microbiology Letters.* 142:155-160.

134. Tomlins and Ordal, 1976. R.I. Tomlins and Z.J. Ordal. *In*: F.A. Skinner and W.B. Hugo, (Ed), Thermal injury and inactivation in vegetative bacteria, inhibition and inactivation of vegetative microbes, Academic Press, New York.

135. Toth, J. D., H. W. Aceto, S. C. Rankin, and Z. Dou. 2011. Survival characteristics of *Salmonella enterica* serovar Newport in the dairy farm environment. *Journal of Dairy Science.* 94:5238-5246.

136. Tsilibaris, V., G. Maenhaut-Michel, N. Mine, and L. Van Melderen. 2007. What is the benefit to *Escherichia coli* of having multiple toxin-antitoxin systems in its genome? *Journal of Bacteriology.* 189:6101-6108.

137. United States Department of Agricultural Research Service. 2006. *"FY-2005 Annual Report Manure and Byproduct Utilization*. (Accessed August 2011).

138. United States Environmental Protection Agency. 1999. Standards for the use or disposal of sewage sludge (40 CFR Parts 403 and 503). Revised August 4, 1999. Available at: http://www.epa.state.oh.us/portals/35/sludge/503_08_04_99.pdf (Accessed June 2011).

139. United States Environmental Protection Agency. 2003. Control of pathogens and vector attraction in sewage sludge. http://www.epa.gov/nrmrl/pubs/625r92013/625R92013.pdf (Accessed September 2011).

140. United States Environmental Protection Agency. 2008. Wastes - resource conservation - reduce, reuse, recycle – composting http://www.epa.gov/waste/conserve/rrr/composting/basic.htm. (Accessed July 2011).

141. United States Department of Agriculture, Economic Research Service. 2010. Foodborne illness cost calculator: *Salmonella*. Washington, DC: US Department of Agriculture, Economic Research Service. Available at http://www.ers.usda.gov/data/foodborneillness/salm_intro.asp. (Accessed May 2011)

142. Wang, C-M., C-L. Shyu, S-P. Ho, and S-H Chiou. 2007. Species diversity and substrate utilization patterns of thermophilic bacterial communities in hot aerobic poultry and cattle manure composts. *Microbial. Ecology*. 54:1-9.

143. Wesche, A. M., J. B. Gurtler, B. P. Marks, and E. T. Ryser. 2009. Stress, sublethal injury, resuscitation, and virulence of bacterial foodborne pathogens. *Journal of Food Protection.* 72:1121-1138.

144. Whiting, R. C. 1993. Modeling bacterial survival in unfavorable environments. *Journal Industrial Microbiology and Biotechnology.* 12:240-246.

145. World Health Organization. 2011. Outbreaks of *E. coli* O104:H4 infection: update 30. http://www.euro.who.int/en/what-we-do/health-topics/emergencies/international-health-regulations/news/news/2011/07/outbreaks-of-e.-coli-o104h4-infection-update-30 (Accessed September 2011)

146. You, Y., S. C. Rankin, H. W. Aceto, C. E. Benson, J. D. Toth and Z. Dou. 2006. Survival of *Salmonella enterica* serovar Newport in manure and manure-amended soils. *Applied and Environmental Microbiology*. 72:5777 – 5783.

147. Yousef, A. E. and Courtney, P. D. 2003. Basics of stress adaptation and implications in new-generation foods, p. 1-30. *In:* Yousef, A. E. and Juneja, V. K. (ed.), Microbial stress adaptation and food safety. CRC Press, Boco Raton, FL.

148. Zhao, T., M. P. Doyle, J. Shere, and L. Garber. 1995. Prevalence of enterohemorrhagic *Escherichia coli* O157-H7 in a survey of dairy herds. *Applied and Environmental Microbiology.* 61:1290-1293.

CHAPTER TWO

THERMAL INACTIVATION OF HEAT-SHOCKED *ESCHERICHIA COLI* O157:H7, *SALMONELLA*, AND *LISTERIA MONOCYTOGENES* IN DAIRY COMPOST

Abstract

Thermal resistance of heat-shocked *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* was compared to non-heat-shocked (control) strains in finished dairy compost. A three strain mixture for each pathogen was heat-shocked at 47.5°C for 1 h and inoculated into the compost at a final concentration of 10^7 CFU/g. The inoculated compost was placed inside an environmental chamber set at 50, 55, or 60°C with humidity at ca. 70 %. The heat-shocked *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* survived better ($P < 0.05$) at 50°C with reductions of 2.7, 3.2 & 3.9 log CFU/g within 4 h in compared with reductions of 3.6, 4.5, and 5.1 log CFU/g, respectively, in control cultures. The heat-shocked cultures of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* had 1.2, 1.9 & 2.3 log reductions, respectively, within 1 h at 55°C, whereas the corresponding control cultures had 4, 5.6 & 4.8 log reductions, respectively. At 60°C, a rapid population reduction was observed during the come-up time of 14 min in control cultures of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* with 4.9, 4.8 & 2.3 log reductions, respectively, compared with 2.6, 2.4 & 1.7 log reduction, respectively, in heat-shocked cultures. *L. monocytogenes* survival curves for all three temperatures had extensive tailing. The double Weibull distribution model was a good fit for the survival curves of pathogens, with difference in the shape parameter of

heat-shocked and control cultures. Our results suggest that the heat-shocked pathogens may have extended survival at lethal temperatures during composting process.

Introduction

Composting is an important and practical method for converting livestock and agricultural wastes into organic fertilizer. A self-heating process is generated metabolically by microbial action. During composting, the initial mesophilic phase (ambient temperature to 40°C) is followed by a thermophilic phase (from 55 to 65°C), and these temperatures are high enough to potentially inactivate some pathogenic microorganisms by affecting their microbial rate and population structure (19). Therefore, thermophilic phase of the composting is the critical step for pathogen inactivation.

Organic wastes added to compost may contain human pathogens such as *Salmonella*, *Escherichia coli* O157:H7, *Listeria monocytogenes*, which have been detected in municipal waste, sewage sludge (3, 13, 17) and animal manure (22, 23). Inactivation of these pathogens has been achieved during composting of several types of waste, such as animal manure, sewage sludge, and food scraps (1, 7, 36, 46). However, the persistence and survival of these human pathogens during composting has also been reported (12, 21, 24).

Application of compost with persistent pathogens can introduce these biological hazards to soil, from which may then gain access to crops (26, 27, 40, 42). Some of the foodborne disease outbreaks have been associated with contamination of fresh produce with *E. coli* O157:H7 and *Salmonella* form manure. An outbreak of *E. coli* O157:H7

among members of four families was associated with potatoes fertilized with manure (8). In another instance, a woman acquired *E. coli* O157:H7 after eating inadequately washed vegetables from garden fertilized with manure (9). Recently, raw Jalapeno and Serrano peppers were the source of a nationwide outbreak of *Salmonella* Saintpaul infection associated with contaminated irrigation water (6).

Some composting practices are less effective because of infrequently turning of material or because the carbon to nitrogen ratio, moisture content, and/or pH of the compost material are inadequate for optimal microbial activity (46, 49, 54), in slow heat-up of the compost pile. When the heating process is too slow, some bacterial populations become acclimatized to heat before the lethal temperature is reached or may be able to survive at usually lethal temperatures (4, 11). A slow increase in the temperature of compost during the mesophilic phase may induce a heat-shock response, resulting in the increased heat resistance of pathogens during subsequent composting. Extended survival of *E. coli* O157:H7 in heaped mixture of dairy cattle manure and beef cattle manure for 32 and 93 days, respectively, was reported despite heap temperature in excess of 50°C (24), suggesting heat acclimatization by this pathogen.

Generally, bacterial cells are able to survive longer at a lethal temperature after they have been exposed to sub-lethal temperatures for a brief period, i.e., these bacteria develop a heat-shock response (41). Various researchers have demonstrated that heat-shocked cells exhibit higher thermal resistance than control cells in lab cultures (34, 37, 41). However, previous composting studies conducted under laboratory conditions or in the field have used only non-stressed cultures (28, 29, 36, 46). To simulate real-world

composting, the stressed culture should be evaluated as they are present in the compost, i.e. as a part of the complex composting system.

The objectives of this study were to investigate thermal inactivation of heat-shocked *E. coli* O157:H7, *Salmonella*. and *L. monocytogenes* in dairy compost under controlled conditions and to develop a mathematical model to analyze the thermal inactivation data.

Materials and Methods

Culture preparation. A three-strain mixture of rifampin-resistant (Rif^r) *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* was used as inoculum: *E. coli* O157:H7 strains F06M-0923-21 (spinach outbreak strain from the California Department of Health), F07M-020-1(Taco John's outbreak strain from the California Department of Health), and avirulent B6914 (Dr. Fratamico, U.S. Department of Agriculture, Agricultural Research Services, Eastern Regional Research Centre, Wyndmoor, PA); *Salmonella* serovars Newport H9119, Poona H9301 (Dr. M. Doyle, University of Georgia, Griffin), and avirulent Typhimurium 8243 (Dr. R. Curtis, Washington University, St. Louis, MO); and *L. monocytogenes* strains LCDC, Scott A and 101M from our stock cultures.

All three strains of *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* were grown separately overnight at 37°C in 10 ml of tryptic soya broth (Difco™, Becton, Dickinson & company, Sparks, MD) containing 100 µg/ml of rifampin (TSB-R). These overnight cultures were further propagated in 10 ml of TSB-R by inoculating a loopful of

culture and incubating overnight at 37°C. The bacterial subcultures were washed and resuspended in TSB-R to an optical density (at 600 nm) of ca. 0.6~0.7 (ca. 10^9 CFU/ml) for heat-shock treatment. An equal volume of the three strains for each pathogen was mixed to provide the inocula for the following experiments.

Heat-shock treatment. For each pathogen, the three-strain mixed culture in TSB-R (15 ml) was heat-shocked at 47.5°C for 60 min in a water bath (Precision® shaking water bath, Precision Scientific Inc., Chicago, IL) excluding the come-up time (3 min). Three temperatures of 45, 47.5, & 50°C, with 30 and 60 min of heating time were used initially to select the optimal heat-shock temperature for the study and 47.5°C with 60 min of heat-shock time was selected based on the results (data not shown). The non-heat-shocked culture was used as control and kept in a refrigerator for the same duration of time. Immediately after the heat-shock treatment, each culture tube containing 15 ml of bacterial culture was put in an ice bath for 10 min to stop further effect of heat. Heat-shocked and control cultures were washed twice with 0.85% saline and resuspended to their respective optical density with saline.

Inoculation of dairy manure compost. Heat-shocked and control cultures were inoculated separately into 1.4 kg of commercial dairy compost (Black Kow®, Black Gold Compost Co., Oxford, Fla.) purchased from a local store. The cultures were inoculated with a spray nozzle (sterilized with 70% ethanol and rinsed with sterile saline) at a 1:100 (vol/wt) ratio to a final level of ca. 10^7 CFU/g. The composition of the compost as printed on the compost bag was ≥ 0.5% total nitrogen, 0.5% available phosphate, ≥ 0.5% soluble potash, and ≤ 1% chlorine. The compost moisture was measured with a moisture

analyzer (model IR-35, Denver Instrument, Gottingen, Germany) and adjusted to ca. 40% with sterile tap water.

Thermal inactivation study. A thin layer (ca. 0.5 cm) of the inoculated compost (heat-shocked and controlled) was spread evenly (60 g) inside Tyvek pouches (5.25 by 10 in. [13.3 by 25.4 cm], SPS Medical, Rush, NY).The pouches were then kept in a single layer on shelf inside the environmental chamber (model 9005L**,** VWR International Inc., Batavia, IL) with humidity set at ca. 70% to mimic the conditions inside a compost heap. The temperatures used for the study were 50, 55, and 60°C. The temperature was monitored with type-T thermocouples (DCC Corporation, Pennsauken, NJ) with one cord inserted inside the compost and other in the chamber. The temperature of the chamber was initially set at a higher set point (target temperature +7°C) to reduce the come-up time and prevent control culture from getting acclimatized. The temperature inside the compost was maintained within ± 0.5°C of the set temperature. Although temperature of the chamber was set high, the temperature inside the compost was not allowed to exceed the desired inactivation temperature ± 0.5°C. When the interior of the compost reached the desired temperature, the temperature setting of the chamber was readjusted and maintained at the designated experimental temperature. If the temperature inside the compost exceeded the temperature range, the door of the chamber was opened briefly to bring the temperature down to the desired range. These adjustments were made until the temperature on the panel of environmental chamber was stabilized. At predetermined intervals (excluding the come-up time), two duplicate sample bags were withdrawn for each treatment, and 25 g of compost sample

was added to 225 ml of universal pre-enrichment broth (UPB; Acumedia manufacturers Inc., Lansing, MI) in a stomacher bag and homogenized. Serial dilutions of sample homogenates were plated in duplicate onto tryptic soy agar (Difco, Becton Dickinson) containing 100 µg/ml rifampin (TSA-R) to analyze the surviving populations. Two samples were included per replicate, and the experiment was done in duplicate. Detection limit for plating was 25 CFU/g. Samples for which *E. coli* O157:H7 was not detected after direct-plating were pre-enriched in UPB, selectively enriched in TSB-R at 37°C overnight, and then streaked onto TSA-R. Detection limit for enrichment was 0.02 CFU/g. Presumptive-positive colonies on the plates were confirmed as *E. coli* O157:H7 using immunolatex agglutination test (Oxoid, Basingtoke, UK). The *Salmonella* and *Listeria* detection was performed in a similar manner except that for selective enrichment Rappaport-Vassiliadis medium broth (50) with 100 µg/ml rifampin and Frazer broth (Difco, Becton Dickinson, MD), respectively, were used as enrichment media. Enriched samples were plated on xylose lysine tergitol agar (Difco, Becton Dickinson, MD) with rifampin and modified oxford agar (Difco, Becton Dickinson, MD) with rifampin for selective detection of *Salmonella* and *L. monocytogenes,* respectively.

Identification of the predominant heat resistant strains of *E. coli* O157:H7 by PCR. Heat-resistant *E. coli* O157:H7 colonies from compost samples inoculated with either control or heat-shocked culture (five colonies for each) that survived heat treatment and were detected only by enrichment at all three temperatures were isolated. *E. coli* O157:H7 test strain B6914 is missing both stx_1 and stx_2 genes, strain F06m-0923-21 has

the stx_2 gene only, and strain F07m-020-1 has both stx_1 and stx_2. A PCR method was used to identify the predominant strain of *E. coli* O157:H7 in the sample (25).

Curve fitting for isothermal inactivation. To describe the inactivation of microorganisms in compost, we applied a model of double Weibull distributions (10) to our thermal inactivation data. In the double Weibull model, the population is assumed to be composed of two groups that differ in their levels of resistance to stress, and the resistance of each subpopulation is assumed to follow a Weibull distribution (10). The double Weibull model can describe different shapes of inactivation kinetics curves. The size of the surviving population can be described by the following equation:

$$N(t) = \frac{N_0}{1+10^{\alpha}}\left(10^{-(\frac{t}{\delta_1})^p+\alpha} + 10^{-(\frac{t}{\delta_2})^p+\alpha}\right)$$

Where N_0 is the initial number of bacteria, α is model parameter, p is the shape parameter, and δ_1 and δ_2 are the decimal reduction time in subpopulation 1 (sensitive subpopulation) and subpopulation 2 (resistant subpopulation), respectively. Because inactivation of our inoculated pathogens was rapid at 55 and 60°C, enrichment data was used in order to increase the accuracy of curve fitting.

Statistical analysis. To compare the difference in bacterial populations between control and heat-shocked cultures, plate count data were log transformed and subjected to an analysis of variance with a test criterion (*F* statistic) and type I error controlled at P = 0.05. The Tukey's multiple comparison procedure of the Statistical Analysis System (2001, SAS, Cary, N.C.) was used.

Results

The thermal inactivation of *E. coli* O157:H7, *Salmonella,* and *L. monocytogenes* in compost occurred inside an environmental chamber with the come-up time of 12 min at 50°C. The initial population reduction was 0.2 log CFU/g due to come-up time only for *E. coli* O157:H7 (Table 2.1). Both *E. coli* O157:H7 and *Salmonella* survived for at least 8 h at 50°C as detected by enrichment. *L. monocytogenes* was detectable up to 24 h by enrichment. The control cultures of these three pathogens were more quickly inactivated at 50°C ($P < 0.05$) than were the corresponding heat-shocked cultures at the 2 and 4 h of sampling times. The reduction in heat-shocked and control population at 4 h were ca. 2.7 and 3.6 log CFU/g for *E. coli* O157:H7, 3.2 and 4.5 log CFU/g for *Salmonella*, and 3.9 and 5.1 log CFU/g for *L. monocyotogenes,* respectively.

The come-up time for thermal inactivation at 55°C was 12 min for all three pathogens. For *E. coli* O157:H7 and *Salmonella*, during come-up time there were initial population reductions of 0.1 and 0.1 log CFU/g for control cultures, and 0.05 and 0.2 log CFU/g for heat-shocked cultures, respectively (Table 2.2). Heat-shocked *E. coli* O157:H7 and *Salmonella* survived at least 5 and 2 h, respectively, whereas the control cultures were detectable until only 3 and 1 h, respectively. The difference in the survival of control and heat-shocked *Salmonella* and *E. coli* O157:H7 cultures was significant ($P<0.05$) at 0.5, 1, and 2 h. *L. monocytogenes* was detectable by enrichment for at least 5 h for both control and heat-shocked populations. Pathogen survival was significantly different ($P < 0.05$) for control and heat-shocked cultures within 1 h of thermal exposure,

with reduction being 4.9 and 2.3 log CFU/g, respectively. Survival was not significantly different ($P > 0.05$) at sampling time 2, 3, 4 & 5 h.

At 60°C, during the come-up time of 14 min control and heat-shocked population reductions was 4.9 and 2.6 log CFU/g for *E. coli* O157:H7, 4.8 and 2.4 log CFU/g for *Salmonella*, and 2.3 and 1.7 log CFU/g for *L. monocytogenes,* respectively. Heat-shocked *E. coli* O157:H7 and *Salmonella* survived for at least 20 and 10 min of the heat exposure, respectively, as detected by enrichment, whereas control populations of *E. coli* O157:H7 and *Salmonella* were detectable at 10 and 0 min, respectively (Table 2.3). The difference in the survival of control and heat-shocked *E .coli* O157:H7 was significant ($P < 0.05$) only at time 0, whereas for *Salmonella* the difference was significant also at 10 min. *L. monocytogenes* control and heat-shocked populations survived for at least 60 min as detected by enrichment, and the difference in the survival of the control and heat-shocked cultures was significant ($P < 0.05$) only at sampling time 0.

Thirty colonies of *E. coli* O157:H7 that were picked from enrichment cultures after heat inactivation at 50, 55, and 60°C were subjected to PCR analysis. The Spinach outbreak strain (F06-0923-21) was the predominant surviving strains. At 50 and 60°C, five of five *E. coli* O157:H7 isolates of either control or heat-shocked cultures were identified as F06-0923-21, whereas at 55°C, three of five isolates were that strain (data not shown).

Mathematical modeling was used to model thermal inactivation of *E. coli* O157:H7, *Salmonella,* and *L. monocytogenes* in compost. We applied the double Weibull

distributions model (10) to the thermal inactivation data. The parameters of the mixed Weibull model are given in Table 2.4.

At 50, 55, & 60°C, the heat-shocked populations of all three pathogens (except for *Salmonella* at 60°C where double Weibull model could not be fitted) had shape parameter $p > 1$, i.e., greater than or equal to those for control populations (Table 2.4). The α values of control culture for all three species at all three temperatures were very close to respective values of heat-shocked cultures. The δ_1 values (sensitive subpopulation) of all heat-shocked pathogens at all three temperatures were also greater than those of the control cultures. The δ_2 values (resistant subpopulation) had the same trend as the δ_1 values for most pathogen and temperature combinations except for *Salmonella* at 55°C and *L. monocytogenes* at 60°C, for which both control and heat-shocked cultures had almost the same δ_2 values. The shape of the survival curves at 50°C was biphasic for both control and heat-shocked populations of all three pathogens (data not shown). However, at 55°C the shape of the curve was sigmoidal for heat-shocked and biphasic for control cultures of *E. coli* O157:H7 and *Salmonella,* and sigmoidal for both control & heat-shocked population of *L. monocytogens* (data not shown). Biphasic and concave curve (with $p < 1$) for heat-shocked and control *E. coli* O157:H7, respectively, and sigmoidal curves for both heat-shocked and control *L. monocytogenes* were evident at 60°C (data not shown).

Discussion

The microbial safety of compost is determined by whether potential pathogens likely to be present are killed during composting. The thermophilic phase is critical for pathogen inactivation because of high temperatures generated during composting (19). However, a microorganism that becomes acclimatized or heat-shocked at sub-lethal temperature during extended mesophilic phase may survive lethal temperatures during the subsequent thermophilic phase of the composting process (41). Thus, the heat-shock response of pathogens is of substantially practical importance for compost production.

Temperatures between 45 and 50°C are optimal for development of the heat-shock response in mesophilic bacteria (33). In other studies, various sub-lethal temperatures from 32 to 54°C have been used for inducing a heat-shock response in different species of bacteria (5, 32, 41, 53). In this study, 47.5°C for 1 h was optimal for inducing the heat-shock response in these three pathogens.

To better understand the survival kinetics of heat-shocked microbial population in compost, we investigated the effect of heat-shock treatment of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* on subsequent survival in dairy manure compost at several composting temperatures. Because of the heterogeneous of the fresh compost, we used finished compost these experiments because it would allow more uniform heat distribution and give more consistent results. Our results indicate that all three species that adapted to sub-lethal temperature (heat-shocked) were able to resist higher composting temperatures (50, 55 and 60°C) for longer periods ($P < 0.05$) than were the non-heat adapted control populations. Under recommended guidelines for composting,

the process should ensure temperature of 55°C for three consecutive days to provide pathogen inactivation (15). However, in the present study, the heat-shocked populations of all three species were inactivated in less than 1 day at 55°C. This shorter period may be the result of the more uniform and controlled laboratory conditions compared with composting situation in the field. During field composting, the come-up time is usually days rather than minutes. In field compost piles, temperature is not uniformly distributed, leading to cold spots that further extend pathogen survival (47).

Numerous studies on thermal inactivation of heat-shocked *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* have been conducted in culture media and food matrices (30, 37, 41). Thermotolerance in *E. coli* O157:H7 in culture broth and ground beef was increased by 2 and 1.5 fold based on *D*-values at 55 and 60°C, respectively, due to heat-shock response (30, 41). Similarly, *Salmonella* developed heat resistance at 57.8 or 52°C in laboratory media and at 60°C in turkey meat after being heat-shocked at various temperatures (42, 48, 52 and 54°C) (5, 53). After heat-shock at 46°C for 30 to 60 min and 48°C for 2 h, *L. monocytogenes* survived at 57°C in TSB with 0.6% yeast extract for 20.1 to 22.3 min and at 64°C in pork and beef mix sausage for 8 min (16, 48).

Published information on the thermal inactivation of heat-shocked microorganisms in compost is scarce. Recently, our field study revealed that heat-shocked *E. coli* O157:H7 and *S*. Typhimurium survived 7 and 16 days longer, respectively ($P < 0.05$), at the surface of the compost than did non heat-shocked population during a summer trial (47). However, at the bottom of the compost heap heat-shocked population of both *E. coli* O157:H7 and *S*. Typhimurium were inactivated in 5

days, whereas non heat-shocked population were inactivated in 3 and 1 day, respectively. In that study, the temperature of the compost heap reached 55°C in 3 to 4 days.

In addition to the impact of heat-shock response in bacteria, the length of pathogen survival during thermophilic composting differs among studies because of differences in experimental conditions. Jiang *et al*. (29) reported thermal inactivation of non-stressed *E. coli* O157:H7 that survived for 24, 6, and 0.5 h at 50, 55, and 60°C, respectively, in dairy compost under isothermal conditions. In the present study, *E. coli* O157:H7 inactivation occurred more rapidly. The difference in the survival of *E. coli* O157:H7 between these two studies can be explained by the fact that in the present study thermal inactivation was carried out in an environmental chamber with 70% humidity instead of in a water bath. The cultures used in these studies also were different, and the inoculated compost in this study was immediately put in the environmental chamber for the thermal inactivation study without letting them sit overnight due to the concern of the loss of heat-shock response during room temperature acclimatization (5).

In this present study, *Salmonella* was inactivated in less than 12 h at all three temperature. However, extended survival of *Salmonella* in compost has also been reported. Droffner and Brinton (12) found that *Salmonella* Typhimurium survived for 9 and 5 days in food biowaste compost and waste water sludge compost, respectively, at 60°C. Ceustermans et al. (7) found that *Salmonella* Senftenberg, considered the most heat resistant of the *Salmonella* serovars, survived for 10 h at 60°C in biowaste with a moisture content of 60 to 65%. In garden waste compost, complete inactivation of *S*. Senftenberg was achieved within 7 days at 50 to 55°C and a 5% reduction in moisture

content of the compost ingredient extended survival of *Salmonella*. Other factors such as microbial antagonism and toxic waste also influenced the survival of *Salmonella*.

Extensive persistence of *Listeria* spp. during composting has been reported. Grewal et al. (21) compared persistence of *L. monocytogenes* in different types of swine manure treatments, such as aerated liquid, unaerated liquid, compost mixed with sawdust (ca. 60% moisture), and pack storage at 20 to 25°C, with thermophilic composting at 55°C. These researchers found that *L. monocytogenes* was culturable after 28 days in all types of manure treatments and after 56 days in thermophilic composting, indicating the role of compost heterogeneity.

First-order kinetics of thermal inactivation assumes that microorganisms in the population have the same probability of dying (51, 52). However, in nature microbial populations are heterogeneous. Therefore, the survival curve with shoulders and tailing deviates from linearity. The first-order kinetics model is not compatible with survival curves in the present study. In our study, cultures were heat-shocked at 47.5°C for 60 min and then exposed to higher temperatures. As a result, the microbial population could consist of cells with different sensitivities to heat, and the application of a heat-shock treatment may further increase that variation in the population (38).

Despite the number of proposed models, none is flexible enough to reflect all changes of thermal inactivation curve shapes with the intensity of the stress or with the physiological state of the cells (20). Albert and Mafart (2) used the modified Weibull model to analyze bacterial inactivation data from various studies with different food matrices and different temperature conditions. Although Albert and Mafart obtained a

good fit for survival curves, they did not take into consideration the heterogeneity of microbial population with different susceptibilities. In the present study, the double Weibull model considered was used for two populations of pathogens with different rate of inactivation in compost. The two-population concept is also reflected in the shape of the survival curves. Shape parameter *p*, which had a higher value for the thermal inactivation curves of heat-shocked than for control populations, indicated initial resistance in the surviving population leading to slow inactivation. Apparently, the shape parameter was dependent on physiological state of the population (heat-shock versus non heat-shock). The shape parameter was either > 1 or < 1, indicating that thermal inactivation curves for all three species at all three temperatures were nonlinear. The δ parameter in the model was close to the classical concept of the *D*- value. The δ_1 and δ_2 values represented first decimal reduction times of the more sensitive and more resistant population, respectively. Our results revealed that δ_1 and δ_2 were also dependent on the physiological state of the microbial population; δ values were higher for the heat-shocked than for the control population, especially for the sensitive subpopulation. Heat-shock treatment appears to increases the thermal resistance of sensitive populations but not of inherently resistant populations.

In this study, the difference in survival of heat-shocked and control populations of *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* was significant only during initial stages of heat treatment; later, on both populations had similar survival rates with tailing at all three temperatures. Extensive tailing was observed in the survival curves of *L. monocytogenes* at all three lethal temperatures. Tailing has also been observed during

heat treatment of various *L. monocytogenes* strains at 55°C in TSB with yeast extract (31). Some researchers have reported that non-stressed population of *L. monocytogenes* has a slow thermal inactivation rate compared with *E. coli* O157:H7 and *Salmonella*. For example, the *D*-values for non-stressed *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* at 55 to 70°C were 32.11 to 0.08, 69.48 to 0.29 and 150.46 to 4.30 min in ready-to-eat breaded pork patties, respectively, and 27.62 to 0.04, 67.68 to 0.22 and 81.37 to 0.37 min in ready-to-eat chicken fried patties, respectively (43, 44). The *D*- values in that study were calculated based on the log-linear inactivation curve. However, in our study we could not apply log linear inactivation kinetics because inactivation curves for *L. monocytogenes* and the other two pathogens were not linear. In the present study, a 4-log reduction for heat-shocked *L. monocytogenes* at 50, 55, and 60°C was achieved in 4.3 h, 1.6 h, and 16 min, respectively, whereas a 7-log reduction for this pathogen was achieved in 24, 5, and 1 h, respectively. If process calculation for composting were made based on the linear portion of the thermal inactivation curve under the present situation, under processing would result because the tailing in the survival curve was not considered. *L. monocytogenes* is ubiquitous in nature, can tolerate a wide range of pH (4.0 to 9.5) and temperature (<1 to 45°C) conditions, can tolerate high salt concentrations, and has the ability to grow in biofilms (35, 39). Thus, the resistant nature of this organism may explain the extensive tailing observed in the survival curves in the present study.

In natural settings, bacteria predominantly exist in multistrain combinations with variation in thermal sensitivity, resulting in the heterogeneity in the distribution of heat

resistance among strains (31). Our results revealed that *E. coli* O157:H7 strain F06m-0923-21, which has been isolated from the spinach outbreak, was the dominant strain surviving at 50, 55 & 60°C for longer than did the other strains. Strains that are very resistant to stressors in food or the environment may also have increased pathogenicity (18). The predominance of *E. coli* O157:H7 strain F06m-0923-21 in present composting trial is particularly interesting, because of its history of association with foodborne illness, indicating that this characteristic may have contributed to its survival. Duffy et al. (14) also found that different strains of *E. coli* O157 had different thermal resistance at specific temperature. Lin and Chou (32) found that heat-shock response of *L. monocytogenes* differed among strains, the type of heat shock, and the type of stress. These variations have important implications for the selection and use of strains in composting trials aimed at the establishment of adequate composting protocols.

Conclusions

Our results revealed that microorganisms when adapted at sub-lethal temperature can survive longer in a compost matrix when subsequently exposed to lethal temperatures. *L. monocytogenes* was the most heat resistant of the three pathogens tested. Overall, a 7-logs reduction in heat-shocked *E. coli* O157:H7 and *Salmonella* could be achieved in compost within 8 h, 5 h, and 20 min at 50, 55, and 60°C, respectively, whereas populations of heat-shocked *L. monocytogenes* were eliminated only after 24, 5 and 1 h at these three temperatures, respectively. Based on these thermal inactivation data, survival of heat-shocked pathogens inside compost heaps should be minimal as long

as the thermophilic composting phase can be maintained at 55°C throughout the heap for at least three consecutive days. However, in real-world composting situations the temperature is not uniformly distributed throughout the heap, and the existence of cold spots could extend pathogen survival times.

Acknowledgments

This project was funded by a grant from Fresh Express Produce Safety Initiative.

References

1. Aitken, M. D., M. D. Sobsey, N. A. Van Abel, K. E. Blauth, D. R. Singleton, P. L. Crunk, C. Nichols, G. W. Walters, and M. Schneider. 2007. Inactivation of *Escherichia coli* O157: H7 during thermophilic anaerobic digestion of manure from dairy cattle. *Water Res.* 41:1659-1666.

2. Albert, I., and P. Mafart. 2005. A modified Weibull model for bacterial inactivation. *Int. J. Food Microbiol*. 100:197-211.

3. Al-Ghazali, M. R., and S. K. Al-Azawi. 1986. Detection and enumeration of *Listeria monocytogenes* in a sewage treatment plant in Iraq. *J. Appl. Bacteriol.* 60:251-254.

4. Amner, W., A. J. Mccarthy, and C. Edwards. 1991. Survival of a plasmid-bearing strain of *Bacillus subtilis* introduced into compost. *J. Gen. Microbiol.* 137:1931-1937.

5. Bunning, V. K., R. G. Crawford, J. T. Tierney, and J. T. Peeler. 1990. Thermotolerance of *Listeria monocytogenes* and *Salmonella* Typhimurium after sublethal heat shock. *Appl. Environ. Microbiol.* 56:3216-3219.

6. Centers for Disease Control and Prevention (CDC). 2008. Outbreak of *Salmonella* serotype Saintpaul infections associated with multiple raw produce items--United States, 2008. *Morb. Mortal. Wkly. Rep.* 57:929-934.

7. Ceustermans, A., D. De Clercq, A. Aertsen, C. Michiels, A. Geeraerd, J. Van Impe, J. Coosemans, and J. Ryckeboer. 2007. Inactivation of *Salmonella* Senftenberg strain W 775 during composting of biowastes and garden wastes. *J. Appl. Microbiol.* 103:53-64.

8. Chapman, P. A., C. A. Siddons, J. Manning, and C. Cheetham. 1997. An outbreak of infection due to verocytotoxin-producing *Escherichia coli* O157:H7 in four families: the influence of laboratory methods on the outcome of the investigation. *Epidemiol. Infect.* 119:113-119.

9. Cieslak, P. R., T. J. Barrett, P. M. Griffin, K. F. Gensheimer, G. Beckett, J. Buffington, and M. G. Smith. 1993. *Escherichia coli* O157:H7 infection from a manured garden. *Lancet* 342:367.

10. Coroller, L., I. Leguerinel, E. Mettler, N. Savy, and P. Mafart. 2006. General model, based on two mixed Weibull distributions of bacterial resistance, for describing various shapes of inactivation curves. *Appl. Environ. Microbiol.* 72:6493-6502.

11. Davis, C. L., S. A. Hinch, C. J. Donkin, and P. J. Germishuizen. 1992. Changes in microbial population numbers during the composting of pine bark. *Bioresour. Technol.* 39:85-92.

12. Droffner, M. L., and W. F. Brinton. 1995. Survival of *Escherichia coli* and *Salmonella* populations in aerobic thermophilic composts as measured with DNA gene probes. *Zentralbl. Hyg. Umweltmed.* 197:387-397.

13. Dudley, D. J., M. N. Guentzel, M. J. Ibarra, B. E. Moore, and B. P. Sagik. 1980. Enumeration of potentially pathogenic bacteria from sewage sludges. *Appl. Environ. Microbiol.* 39:118-126.

14. Duffy, G., D. C. R. Riordan, J. J. Sheridan, B. S. Eblen, R. C. Whiting, I. S. Blair, and D. A. McDowell. 1999. Differences in thermotolerance of various *Escherichia coli* O157: H7 strains in a salami matrix. *Food Microbiol.* 16:83-91.

15. Environmental Protection Agency. Guide to the Part 503 Rule. Chapter 5: Pathogen and vector attraction requirements: A plain English guide to the EPA Part 503biosolids rule. September, 1994. United States Environmental Protection Agency, Washington, DC.

16. Farber, J. M., B. E. Brown. 1990. Effect of prior heat shock on heat resistance of *Listeria monocytogenes* in meat. *Appl. Environ. Microbiol.* 56:1584-1587.

17. Garrec, N., F. Picard-Bonnaud, and A. M. Pourcher. 2003. Occurrence of *Listeria spp.* and *L. monocytogenes* in sewage sludge used for land application: effect of dewatering, liming and storage tank on survival of *Listeria spp*. *FEMS Immunol. Med. Microbiol.* 35:275-283.

18. Gorden, J. and P. L. C. Small. 1993. Acid resistance in enteric bacteria. *Infect. Immun.* 61:364-367.

19. Gray, K. R., and A. J. Biddlest. 1973. Composting - Process Parameters. *Chem. Eng.-London* 71-76.

20. Greenacre, E. J., T. F. Brocklehurst, C. R. Waspe, D. R. Wilson, and P. D. G. Wilson. 2003. *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* acid tolerance response induced by organic acids at 20°C: optimization and modeling. *Appl. Environ. Microbiol.* 69:3945–3951.

21. Grewal, S., S. Sreevatsan, and F. C. Michel Jr. 2007. Persistence of *Listeria* and *Salmonella* during swine manure treatment. *Compost Sci. Util.* 15:53-62.

22. Haapapuro, E. R., N. D. Barnard, and M. Simon. 1997. Animal waste used as livestock feed: Dangers to human health - Review. *Prev. Med.* 26:599-602.

23. Hassen, A., K. Belguith, N. Jedidi, A. Cherif, M. Cherif, and A. Boudabous. 2001. Microbial characterization during composting of municipal solid waste. *Bioresour. Technol.* 80:217-225.

24. Hutchison, M. L., L. D. Walters, S. M. Avery, and A. Moore. 2005. Decline of zoonotic agents in livestock waste and bedding heaps. *J. Appl. Microbiol.* 99:354-362.

25. Ibekwe, A. M., P. M. Watt, C. M. Grieve, V. K. Sharma, and S. R. Lyons. 2002. Multiplex fluorogenic real-time PCR for detection and quantification of *Escherichia coli* O157:H7 in dairy wastewater wetlands. *Appl. Environ. Microbiol.* 68:4853-4862.

26. Islam, M., M. P. Doyle, S. C. Phatak, P. Millner, and X. P. Jiang. 2005. Survival of *Escherichia coli* O157: H7 in soil and on carrots and onions grown in fields treated with contaminated manure composts or irrigation water. *Food Microbiol.* 22:63-70.

27. Islam, M., J. Morgan, M. P. Doyle, S. C. Phatak, P. Millner, and X. P. Jiang. 2004. Fate of *Salmonella* enterica serovar Typhimurium on carrots and radishes grown in fields treated with contaminated manure composts or irrigation water. *Appl. Environ. Microbiol.* 70:2497-2502.

28. Jiang, X., J. Morgan, and M. P. Doyle. 2003a. Fate of *Escherichia coli* O157:H7 during composting of bovine manure in a laboratory-scale bioreactor. *J. Food Prot.* 66:25-30.

29. Jiang, X., J. Morgan, and M. P. Doyle. 2003b. Thermal inactivation of *Escherichia coli* O157:H7 in cow manure compost. *J. Food Prot.* 66:1771-1777.

30. Juneja, V. K., P. G. Klein, and B. S. Marmer. 1998. Heat-shock and thermotolerance of *Escherichia coli* O157:H7 in a model beef gravy system and ground beef. *J. Appl. Microbiol.* 84:677-684.

31. Lianou, A., J. D. Stopforth, Y. Yoon, M. Wiedmann, and J. N. Sofos. 2006. Growth and stress resistance variation in culture broth among *Listeria monocytogenes* strains of various serotypes and origins. *J. Food Prot.* 69:2640-2647.

32. Lin, Y. D., C. C. Chou. 2004. Effect of heat shock on thermal tolerance and susceptibility of *Listeria monocytogenes* to other environmental stresses. *Food Microbiol.* 21:605-610.

33. Lindquist, S. 1986. The heat-shock response. *Ann. Rev. Biochem*. 55:1151-1191.

34. Linton, R. H., M. D. Pierson, and J. R. Bishop. 1990. Increase in heat-resistance of *Listeria-monocytogenes* Scott-A by sublethal heat-shock. *J. Food Prot.* 53:924-927.

35. Liu, D., M. L. Lawrence, A. J. Ainsworth, and F. W. Austin. 2005. Comparative assessment of acid, alkali and salt tolerance in *Listeria monocytogenes* virulent and avirulent strains. *FEMS Microbiol. Lett.* 243:373-378.

36. Lung, A. J., C. M. Lin, J. M. Kim, M. R. Marshall, R. Nordstedt, N. P. Thompson, and C. I. Wei. 2001. Destruction of *Escherichia coli* O157:H7 and *Salmonella* Enteritidis in cow manure composting. *J. Food Prot.* 64:1309-1314.

37. Mackey, B. M., C. M. Derrick. 1986. Elevation of the heat-resistance of *Salmonella*-Typhimurium by sublethal heat-shock. *J. Appl. Bacteriol.* 61:389-393.

38. Mangalassary, S., I. Han, J. Rieck, J. Acton, X. Jiang, B. Sheldon, and P. Dawson. 2007. Effect of combining nisin and/or lysozyme with in-package pasteurization on thermal inactivation of *Listeria monocytogenes* in ready-to-eat turkey bologna. *J. Food Prot.* 70:2503-2511.

39. Moretro, T and S. Langsrud. 2004. *Listeria monocytogenes*: biofilm formation and persistence in food-processing environments. *Biofilms*. 1:107-121.

40. Mukherjee, A., D. Speh, E. Dyck, and F. Diez-Gonzalez. 2004. Preharvest evaluation of coliforms, *Escherichia coli*, *Salmonella*, and *Escherichia coli* O157: H7 in organic and conventional produce grown by Minnesota farmers. *J. Food Prot.* 67:894-900.

41. Murano, E. A., and M. D. Pierson. 1992. Effect of heat-shock and growth atmosphere on the heat-resistance of *Escherichia coli* O157:H7. *J. Food Prot.* 55:171-175.

42. Ogden, I. D., D. R. Fenlon, A. J. A. Vinten, and D. Lewis. 2001. The fate of *Escherichia coli* O157 in soil and its potential to contaminate drinking water. *Int. J. Food Microbiol.* 66:111-117.

43. Osaili, T., C. L. Griffis, E. M. Martin, B. L. Beard, A. Keener, and J. A. Marcy. 2006. Thermal inactivation studies of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in ready-to-eat chicken-fried beef patties. *J. Food Prot.* 69:1080-1086.

44. Osaili, T. M., C. L. Griffis, E. M. Martin, B. L. Beard, A. E. Keener, and J. A. Marcy. 2007. Thermal Inactivation of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in Breaded Pork Patties. *J. Food Sci.* 72:M56-M61.

45. Pourcher, A. M., P. Morand, F. Picard-Bonnaud, S. Billaudel, S. Monpoeho, M. Federighi, V. Ferre, and G. Moguedet. 2005. Decrease of enteric micro-organisms from rural sewage sludge during their composting in straw mixture. *J. Appl. Microbiol.* 99:528-539.

46. Shepherd, M. W. Jr, P. Liang, X. Jiang, M. P. Doyle, and M. C. Erickson. 2007. Fate of *Escherichia coli* O157:H7 during on-farm dairy manure-based composting. *J. Food Prot.* 70:2708-2716.

47. Shepherd, M. W. Jr, R. Singh, J. Kim, and X. Jiang. 2010. Effect of heat-shock treatment on the survival of *Escherichia coli* O157:H7 and *Salmonella enterica* Typhimurium in dairy manure co-composted with vegetable wastes under field conditions. Bioresour. Technol. 101:5407-5413

48. Skandamis, P. N., Y. Yoon, J. D. Stopforth, P. A. Kendall, and J. N. Sofos. 2008. Heat and acid tolerance of *Listeria monocytogenes* after exposure to single and multiple sublethal stresses. *Food Microbiol.* 25:294-303.

49. Sundberg, C., S. Smars, and H. Jonsson. 2004. Low pH as an inhibiting factor in the transition from mesophilic to thermophilic phase in composting. *Bioresour. Technol.* 95:145-150.

50. U. S.Food and Drug Administration. 2001. Bacteriological Analytical Manual. Available at: http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm063568.htm (Accessed Dec, 15, 2008).

51. van Boekel, M. A. J. S. 2002. On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. *Int. J. Food Microbiol.* 74:139-159.

52. Virto, R., D. Sanz, I. Alvarez, S. Condon, and J. Raso. 2006. Application of the Weibull model to describe inactivation of *Listeria monocytogenes* and *Escherichia coli* by citric and lactic acid at different temperatures. *J. Sci. Food Agric.* 86:865-870.

53. Wesche, A. M., B. P. Marks, and E. T. Ryser. 2005. Thermal resistance of heat-, cold-, and starvation-injured *Salmonella* in irradiated comminuted Turkey. *J. Food Prot.* 68:942-948.

54. Zavala, M. A. L., and N. Funamizu. 2005. Effect of moisture content on the composting process in a biotoilet system. *Compost Sci. Util.* 13:208-216.

Table 2.1 Survival of control and heat-shocked *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* in compost at 50°C

		log CFU/g at 50°C with heating time (h)[1]							
Species	Treatment	0	2	4	6	8	18	24	48
E. coli O157:H7	Control	6.81 ± 0.07A[2]	5.63 ± 0.14B	3.45 ± 0.41B	(+)A[3]	(+)A	ND[4]	–	–
	Heat-Shocked	6.81 ± 0.11A	5.91 ± 0.08A	4.33 ± 0.23A	(+)A	(+)A	ND	–	–
Salmonella	Control	7.16 ± 0.33A	5.08 ± 0.18B	2.69 ± 0.32B	(+)A	1.05 ± 0.65A	ND	–	–
	Heat-Shocked	7.13 ± 0.35A	5.47 ± 0.12A	3.92 ± 0.05A	(+)A	(+)A	ND	–	–
L. monocytogenes	Control	7.44 ± 0.03B	5.06 ± 0.49B	2.35 ± 0.49B	1.76 ± 0.54A	1.59 ± 0.36A	(+)A	(+)A	ND
	Heat-Shocked	7.33 ± 0.04A	6.15 ± 0.40A	3.44 ± 1.06A	1.89 ± 0.42A	1.66 ± 0.38A	1.05 ± 0.65A	1.05 ± 0.65A	ND

[1]The come-up time at 50°C was 12 min.

[2]Mean log CFU ± standard deviation with different upper case letters differs significantly ($P < 0.05$) within column for each pathogen.

[3](+), positive by enrichment.

[4]ND, not detected by enrichment.

Table 2.2 Survival of control and heat-shocked *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* in compost at 55°C

Species	Treatment	log CFU/g at 55°C with heating time (h)[1]							
		0	0.5	1	2	3	4	5	6
E. coli O157:H7	Control	6.86 ± 0.03B[2]	5.33 ± 0.62B	3.02 ± 0.65B	(+)B[3]	(+)A	NDB[4]	NDB	ND
	Heat-Shocked	6.95 ± 0.03A	6.17 ± 0.39A	5.83 ± 0.49A	4.78 ± 0.16A	(+)A	(+)A	(+)A	ND
Salmonella	Control	6.89 ± 0.06A	(+)B	(+)B	NDB	ND	–	–	–
	Heat-Shocked	6.83 ± 0.06A	6.36 ± 0.08A	5.15 ± 0.56A	(+)A	ND	–	–	–
L. monocytogenes	Control	7.19 ± 0.07A	5.12 ± 0.56B	2.30 ± 0.97B	1.70 ± 0.56A	(+)A	(+)A	1.05 ± 0.65A	ND
	Heat-Shocked	7.16 ± 0.04A	6.20 ± 0.37A	4.90 ± 0.58A	1.72 ± 0.60A	(+)A	(+)A	(+)A	ND

[1]The come-up time at 55°C was 12 min.

[2]Mean log CFU± standard deviation with different upper case letters differs significantly ($P< 0.05$) within column for each pathogen.

[3](+), positive by enrichment.

[4]ND, not detected by enrichment.

Table 2.3 Survival of control and heat-shocked *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* in compost at 60°C

Species	Treatment	log CFU/g at 60°C with heating time (min)[1]							
		0	10	20	30	40	50	60	70
E. coli O157:H7	Control	2.12 ± 0.51B[2]	(+)A[3]	NDA[4]	ND	–	–	–	–
	Heat-Shocked	4.44 ± 0.23A	(+)A	(+)A	ND	–	–	–	–
Salmonella	Control	2.23 ± 0.63B	NDB	ND	–	–	–	–	–
	Heat-Shocked	4.62 ± 0.33A	1.12 ± 0.72A	ND	–	–	–	–	–
L. monocytogenes	Control	4.68 ± 0.22B	(+)A	(+)A	(+)A	(+)A	(+)A	(+)A	ND
	Heat-Shocked	5.33 ± 0.12A	1.72 ± 0.46A	(+)A	(+)A	(+)A	(+)A	(+)A	ND

[1]The come-up time at 60°C was 14 min.

[2]Mean log CFU± standard deviation with different upper case letters differs significantly ($P< 0.05$) within column for each pathogen.

[3](+), positive by enrichment.

[4]ND, not detected by enrichment.

Table 2.4 Parameters of double Weibull distribution of *E. coli* O157:H7, *Salmonella* & *L. monocytogenes* thermal inactivation curves at 50, 55 & 60°C

Temperature (° C)	Treatment	*E. coli* O157:H7					*Salmonella*					*L. monocytogenes*				
		α	δ_1 (t)	δ_2 (t)	P	R^2	α	δ_1 (t)	δ_2 (t)	P	R^2	α	δ_1 (t)	δ_2 (t)	P	R^2
50	Control	5.16 ±0.20	1.87 ±0.24 h	12.55 ±1.61 h	1.56 ±0.23	0.99	5.39 ±0.13	1.17 ±0.11 h	10.87 ±1.10 h	1.19 ±0.08	0.99	6 ±0.35	0.80 ±0.29 h	25.26 ±7.19	1.01 ±0.21	0.99
	Heat-shocked	5.29 ±0.34	2.56 ±0.37 h	14.45 ±2.13 h	1.99 ± 0.43	0.99	5.26 ±0.45	1.80 ±0.41 h	12.14 ±3.08 h	1.43 ±0.32	0.99	5.71 ±0.19	1.82 ±0.26 h	33.81 ±3.95 h	1.62 ±0.26	0.99
55	Control	5.38 ±0.54	0.42 ±0.15 h	3.52 ±1.28 h	1.37 ±0.43	0.99	6.01 ±0.89	0.29 ±0.21 h	2.67 ±1.24 h	2.05 ±1.57	0.98	5.12 ±0.27	0.48 ±0.07 h	4.42 ±0.56 h	1.85 ±0.33	0.99
	Heat-shocked	4.85 ±0.58	1.59 ±0.27 h	4.88 ±0.96 h	2.46 ±0.80	0.98	4.57 ±0.69	0.90 ±0.05 h	2.09 ±0.19 h	2.03 ±0.33	0.99	5.09 ±0.39	0.87 ±0.16 h	5.10 ±0.99 h	2.42 ±1.20	0.99
60	Control	5.86 ±0.86	0.51 ± 0.61 min	36.39 ±51.24 min	0.49 ± 0.17	0.99	-[1]	-	-	-		5.80 ±0.34	11.91 ±1.23 min	80.63 ±3.96 min	6 ±3.71	0.99
	Heat-shocked	6.37 ±0.42	7.2 ±1.46 min	87.37 ±38.09 min	1.45 ±0.24	0.99	-	-	-	-		5.64 ±0.34	12.58 ±0.90 min	79.65 ±3.97 min	6 ±3.46	0.99

[1](-), data could not be fitted with double Weibull model.

'±' indicated standard deviation, R^2; adjusted R^2 value.

CHAPTER THREE

DETERMINING THERMAL INACTIVATION OF *ESCHERICHIA COLI* O157:H7 IN FRESH COMPOST BY SIMULATING EARLY PHASES OF COMPOSTING PROCESS

Abstract

A three-strain mixture of *Escherichia coli* O157:H7 was inoculated into fresh dairy compost (ca. 10^7 CFU/g) with 40 or 50% moisture and was placed in an environmental chamber (ca. 70% humidity) that was programmed to ramp from room temperature to selected composting temperatures in 2 and 5 days to simulate the early composting phase. The surviving *E. coli* O157:H7 population was analyzed by direct plating and enrichment. Optimal and suboptimal compost mixes, with carbon/nitrogen (C/N) ratio of 25:1 and 16:1, respectively, were compared in this study. In the optimal compost mix *E. coli* O157:H7 survived for 72, 48, and 24 h in compost with 40% moisture and for 72, 24, and 24 h with 50% moisture at 50, 55, and 60°C, respectively, following 2 days of come-up time (rate of heating up). However, in the suboptimal compost mix, the pathogen survived for 288, 72, and 48 h in compost with 40% moisture and 240, 72, and 24 h in compost with 50% moisture at the same temperatures, respectively. Pathogen survival was longer with 5 days of come-up time compared with 2 days of come-up. Overall, *E. coli* O157:H7 was inactivated faster in the compost with 50% moisture than in the compost with 40% at 55 and 60°C. Both moisture and come-up time were significant factors affecting Weibull model parameters. Our results suggest that slow come-up time at the beginning of composting can extend pathogen survival during composting.

Additionally, both C/N ratio and the initial moisture level in the compost mix affect the rate of pathogen inactivation as well.

Introduction

Livestock wastes, e.g., manures that have undergone appropriate treatment to inactivate human pathogens, can be a safe soil amendment for use in agriculture. However, inadequate treatment of such manure may lead to the survival of pathogens that could contaminate produce in the field and, ultimately result in food-borne illness. *Escherichia coli* O157:H7 is one of the most important and common pathogens which is responsible for many of the food-borne illnesses in the United States (18). Animals carry this pathogen without apparent symptoms and may also sporadically shed the bacteria (12), which can be disseminated to the environment. In the past, several outbreaks of foodborne illnesses have been linked to the contamination of produce, such as garden vegetables and baby spinach, via direct or indirect contact with animal wastes containing human pathogens (2, 4, 5).

Composting is commonly used for treating organic wastes (livestock manure, food wastes, etc.), which makes them easier to dispose of on agricultural fields and home gardens. Composted organic waste serves as an important organic fertilizer, which is rich in nutrients, circumventing the need for chemical fertilizers. Heat generated from the metabolic activity of the microbes present in compost mixture plays a major role in the inactivation of zoonotic pathogens. Therefore, composting is considered important in

bringing about inactivation/killing of pathogens that may be present in livestock wastes. However, the primary process criteria used for ensuring the microbiological safety of composts have been narrowly defined as time-temperature conditions. In the United States, EPA regulations for composting of biosolids include either a minimum temperature of 55°C for 3 days in aerated static piles or in-vessel systems, or 15 days with 5 turnings in windrow systems (24).

Although temperature is a critical factor during composting, extended survival of pathogens in compost has been reported. Droffner and Brinton (8) reported that in a bench-scale trials, *E. coli* B survived for at least 9 days at 60 to70°C in a biowaste (food waste) compost or a waste water sludge compost and *Salmonella* enterica serovar Typhimurium Q survived for at least 9 and 5 days over 60°C in the food biowaste compost and the waste water sludge compost, respectively. Hutchison et al. (14) has also reported the extended survival of pathogens in field studies of static compost piles. In that study, *Salmonella*, *E. coli* O157:H7, and *Listeria* survived for more than 8 days in poultry manure-based compost piles when exposed to temperature above 55°C. These studies suggest that the time-temperature criteria set by the EPA may not always be sufficient to ensure complete inactivation of pathogens within the entire compost pile.

Pathogen inactivation during composting is very complex. Besides elevated temperature, composting is affected by other factors such as moisture content, carbon/nitrogen ratio (C/N), particle size, aeration, heap size, pH, and types and populations of indigenous microflora. The optimal moisture and C/N ratio for active composting is 50 to 60% and 25:1 to 30:1, respectively, however, 40 to 65% and 20:1 to

40:1, respectively, are acceptable (22). Variations of these factors may affect the rapid onset of self-heating at the beginning of composting, causing slow heat-up and extend the transition time from the mesophilic to the thermophilic phase of composting. Consequently, some populations of pathogenic bacteria may become acclimatized before lethal temperatures are reached, or even survive, for an extended period of time (1, 7). Therefore, relying solely on time-temperature criteria for pathogen inactivation without taking into consideration other composting factors may not completely ensure compost safety.

The objectives of this study were to investigate the effect of some composting parameters, i.e., initial moisture level, C/N ratio and rate of heating-up (come-up time) on the thermal inactivation of *E. coli* O157:H7 in fresh dairy compost under a controlled environment, and develop predictive models to analyze the thermal inactivation data.

Materials and methods

Compost preparation. Fresh compost mixtures were prepared by mixing dairy manure (collected from LaMaster dairy farm, Clemson University), sawdust bedding, and hay at different ratios to yield compost mixture of C/N ratio 25:1 (optimal ratio) or 16:1 (suboptimal ratio). The C/N ratio of the compost was analyzed by the Agricultural Service Laboratory (Clemson University, Clemson, SC). The compost mixture was stored under refrigeration conditions until used. Two days prior to the experiment, the refrigerated compost mixture was split into two lots and dried under an airflow supreme fume hood (Kewaunee Scientific Equipment corp., Michigan) to reduce the moisture to

ca. 50 % (for optimal composting conditions) and 40 % (for suboptimal composting conditions). The compost moisture was measured by an IR-35 moisture analyzer (Denver Instrument, Germany).

Bacterial culture preparation. *E. coli* O157:H7 strains F06M-0923-21 (spinach outbreak strain from the California Department of Health), F07M-020-1(Taco John's outbreak strain from the California Department. of Health) and avirulent strain B6914 (Kindly provided by Dr. Pina Fratamico, Eastern Regional Research Center, USDA-Agricultural Research Station) were induced to rifampin resistance as described previously (21).

E. coli O157:H7 cultures were grown separately overnight at 37°C in 10 ml of tryptic soya broth (Difco™, Becton, Dickinson & company, Sparks, MD) containing 100 μg/ml of rifampin (TSB-R). These overnight-grown cultures were further propagated in 30 ml each of TSB-R (young culture [YC]) and at a 1:10 strength of TSB-R (low nutrient adapted culture [LC]) by inoculating a loopful of culture and incubating overnight at 37°C. After another subculturing, the bacterial cultures were sedimented by centrifugation at 5000 x g for 10 min, washed twice, and then resuspended in sterile 0.85% saline to an optical density at 600 nm (OD_{600}) of ca. 0.6 to 0.7 (ca. 10^9 CFU/ml). Equal volume of the three *E. coli* O157:H7 cultures were mixed as inoculums for the following experiments.

Inoculation of fresh dairy manure compost. Mixed cultures of *E. coli* O157:H7 were inoculated into 2.2 kg (each) of fresh compost with 40 or 50% moisture at a final concentration of ca. 10^7 CFU/g separately, with the use of a spray nozzle sanitized with

70% ethanol and rinsed with sterile saline. The compost was mixed continuously for 10 min on sterile polypropylene trays by hand wearing sterile gloves. The inoculated levels of the pathogens in compost were enumerated by serial dilutions of the compost in 0.85% saline and plating on tryptic soya agar (TSA) (BD, MD) containing 100 μg/ml of rifampin (TSA-R).

Thermal inactivation study. About 100 g of inoculated compost with 40 or 50% moisture was put inside a Tyvek pouch (size 5.25 in. by 10 in.; SPS Medical, Rush, NY) and spread evenly into a thin layer (ca. 1 cm in depth). Tyvek pouches were then kept in a single layer on the shelf of an environmental chamber (model No. EC2047N; Thermo Scientific, Barnstead International, Dubuque, IA) with the humidity set at ca. 70% to mimic conditions inside of the composting heap. The temperatures used for this study were 50, 55, and 60°C, which were monitored constantly using type-T thermocouples (DCC Corporation, New Jersey) with one cord inserted inside the compost pouch and others were kept in the chamber. The temperature rise of the environmental chamber during the study was programmed to ramp step-wise from room temperature (ca. 26°C) to the target temperature in 2 days (representing a normal temperature rise during composting process) or 5 days (slow heat-up). After the temperature of the compost inside the bag reached the target temperature, sample bags were removed at predetermined time intervals and cooled immediately in an ice water bath.

A 25 g portion of compost sample was taken and mixed with 225 ml of universal preenrichment broth (UPB) (Acumedia manufacturers Inc., Lansing, MI) in a stomacher bag and homogenized. Serial dilutions of sample homogenates were plated in duplicate

on TSA-R to analyze the surviving population of *E. coli* O157. The detection limit for plating was 25 CFU/g. The samples, which were negative for *E. coli* O157:H7 after direct-plating, were preenriched in UPB, followed by selective enrichment in TSB-R at 37°C overnight, and then streaked on TSA-R and Sorbitol MacConkey agar with rifampin (SMAC-R). The detection limit for enrichment was 0.02 CFU/g. The presumptive colonies on the plates were confirmed to be *E. coli* O157:H7 using an immuno-latex agglutination test (Oxoid, Hampshire, United Kingdom). Two or three trials were conducted for each experiment.

Statistical analysis. To compare the difference in bacterial populations of different treatments, plate count data were converted to log_{10} values, and subjected to analysis of variance with a test criterion (F statistic) and type I error controlled at a *P* value of 0.05. The Tukey's multiple comparison procedure of the Statistical Analysis System (2001, SAS, Cary, N.C.) was used.

Data fitting for thermal inactivation of *E. coli* O157:H7 in fresh compost. The plate count data for the isothermal inactivation study were converted to log_{10} values and were subjected to a mixed Weibull distribution as described previously (23). The inactivation rate of *E. coli* O157 at different temperatures was reported as the 4 *D* value (time required to reduce 4 logs of pathogen at different temperature).

Analyzing factors on parameters of the mixed Weibull model. In order to examine the effects of different factors, including temperature, moisture, come-up time and C/N ratio on each parameter in the mixed Weibull model, two regression studies were performed using the fitting results of different experimental results. First, the

stepwise regression tool in the MATLAB software program (The MathWorks Inc. Natick, MA) was used to identify statistically significant factors. For each factor, a t-statistics test was performed on regression coefficients and the *P*-value threshold 0.05 was used to determine the significant factors. Then, the rstool in MATLAB was used to fit the interaction response surfaces in order to understand the relationship between factors.

Results

Thermal inactivation of *E. coli* O157:H7 in fresh compost was performed inside an environmental chamber by simulating the early phase of optimal composting (2 days of come-up time), compared to the early phase of suboptimal composting (5 days of come-up time). The effects of the moisture content, i.e., 50% (optimal) and 40% (suboptimal), along with different compost C/N ratios of 25:1 (optimal) and 16:1 (suboptimal) were also compared.

Comparison of young culture and low nutrient-adapted culture for thermal resistance

Initially, the thermal inactivation of the young culture (YC) and the low nutrient-adapted culture (LC) (grown in 1:10 strength of TSB-R) were compared at different composting temperatures. With 2 days of come-up time, both YC and LC of *E. coli* O157:H7 in compost with 50 and 40% moisture survived for 72 and 24 h at 50 and 60°C, respectively, and 24 and 48 h with 50 and 40% moisture, respectively, at 55°C (Tables 3.1, 3.2 and 3.3). The compost mix with 50% moisture content and with 2 days of come-

up time had a slightly quicker decline (lower 4 *D* value) in the LC population than that of the YC (higher 4 *D* value) at 60°C (see Table 3.6). The same trend was observed for YC and LC at 60°C in compost with 40% moisture content as well. In the compost mix with 50 and 40% moisture, the differences in survival between YC and LC were not significant ($P > 0.05$) for most of the sampling times at 50 and 55°C with 2 days of come-up time, except at times 0 and 2 h of inactivation at 50°C in compost mix with 40% moisture and 2 days of come-up time (Tables 3.1 and 3.2). At 60°C, there was a significant ($P < 0.05$) difference in the survival of the YC and that of the LC at most of the sampling times in compost with 40% moisture and only at 0 and 0.5 h in compost with 50% moisture (Table 3.3). Since the YC survived equally or slightly better than the LC, the YC was used for the rest of the composting trials.

<u>*E. coli* O157 inactivation in fresh compost with a C/N ratio of 25:1</u>

For 2 days of come-up time, *E. coli* O157:H7 was inoculated to the compost with a C/N ratio of 25:1 and moisture of 50% at levels of ca. 7.16±0.16, 7.23±0.13, and 7.04±0.28 log CFU/g for the thermal inactivation trials conducted at 50, 55, and 60°C, respectively, and ca.7.11±0.13, 7.13±0.11, and 7.04±0.08 log CFU/g, respectively, for compost with 40% moisture. For the same compost with 5 days of come-up time, the levels of *E. coli* O157 in the compost with 50% moisture were ca. 7.34±0.77, 7.11±0.07, and 7.47±0.08 log CFU/g for the thermal inactivation at 50, 55, and 60°C, respectively, and ca. 7.36±0.44, 7.09±0.77, and 7.14±0.04 log CFU/g, respectively, in the compost with 40% moisture. The level of mesophilic background microflora that was present in

the optimal compost mixture (C/N ratio of 25:1) was ca. 7.08±0.15 log CFU/g as enumerated by plating on TSA.

During the come-up time, there was a rapid decline in the surviving population of *E. coli* O157 just before the target composting temperature was reached (0 h) in all trials. With 2 days of come-up time, the *E. coli* O157 population in the compost with 50% moisture was reduced by ca. 4.64, 5.83 (enrichment positive), and 5.34 log reductions at 0 h of exposure to 50, 55, and 60°C, respectively, in comparison to population reductions of ca. 4.21, 4.90, and 3.86 log CFU/g, respectively, when the moisture of the compost was 40% at temperatures mentioned above (Table 3.1, 3.2, 3.3). When the come-up time was extended to 5 days, the pathogen decline was still quicker in compost with 50% moisture with ca. 5.85, 5.47, and 6.07 (enrichment positive) log reductions at 0 h of exposure to 50, 55, and 60°C, respectively, than ca. 5.12, 4.80, and 5.52 log reductions, respectively, in compost with 40% moisture.

After the designated temperatures were reached, the compost mix with a 25:1 C/N ratio, 50% moisture, and 2 days of come-up time had ca.5.60, 5.83 (enrichment positive), and 5.56 log reductions after 8, 4 and 1.5 h exposure at 50, 55, and 60°C, respectively, and compared with ca. 5.94 (enrichment positive), 5.70 and 6.07 (enrichment positive) log reductions at the same sampling intervals and temperatures, respectively, with 5 days of come-up time (Tables 3.1, 3.2 and 3.3). The same compost with 40% moisture and 2 days of come-up time, had ca. 5.55, 5.52 and 4.26 log reductions after 8, 4 and 1.5 h exposure at 50, 55, and 60°C, respectively, and with 5 days of come-up time, ca. 5.72,

5.37, and 5.74 (enrichment positive) log reductions at the same sampling interval and temperatures, respectively.

In all trials, the compost with a C/N ratio of 25:1, greater reductions in *E. coli* O157 populations were observed in composts with 50% moisture than were observed in composts with suboptimal moisture (40%) during the same come-up times to the desired temperatures in the environmental chamber. With 2 days of come-up time, the pathogen was detectable by enrichment ranging from 72 h, 24 to 48 h, and 24 h at 50, 55, and 60°C, respectively, and 96 to 144 h, 48 to 120 h, and 48 to 72 h, respectively, at the same temperature with 5 days of come-up time (Tables 3.1, 3.2 and 3.3). Overall, the pathogen survived longer in compost with low moisture (40%) and an extended come-up time (5 days) than in compost mix with 50% moisture and 2 days of come-up time. These results suggest heat-adaptation of *E. coli* O157:H7 that may occur during an extended come-up time during the early phase of composting.

The difference in *E. coli* O157 survival between 2 and 5 days of come-up time in fresh compost with 50% moisture was significant ($P < 0.05$) at sampling times 0, 2, 4, 6, and 8 h at 50°C, 0 h at 55°C, and 0 and 0.5 h at 60°C (Tables 3.1, 3.2, and 3.3). The difference in survival between 2 and 5 days of come-up time for the compost mix with 40% moisture was also significant ($P < 0.05$) at sampling times of 0 h at 50°C, and most of the sampling times at 60°C. Between composts with 40 and 50% moisture, *E. coli* O157 survival was significantly different ($P < 0.05$) from 0 to 4 h of the sampling times at both 55 and 60°C with 2 days of come-up time and at most of the sampling time at 50, 55, and 60°C with 5 days of come-up time.

<u>*E. coli* O157 inactivation in fresh compost with C/N ratio of 16:1</u>

For 2 days of come-up time, the compost with 50% moisture was inoculated with *E. coli* O157:H7 at levels of ca. 7.23±0.09, 7.18±0.08, and 7.14±0.06 log CFU/g for thermal inactivation at 50, 55, and 60°C, respectively, and the compost with initial moisture of 40% had ca. 7.20±0.04, 7.12±0.05, and 7.12±0.08 log CFU/g of *E. coli* O157:H7, respectively. For the come-up time of 5 days, the same compost with 50% moisture was inoculated with *E. coli* O157 at levels of ca. 7.21±0.07, 7.10±0.08, and 7.11±0.05 log CFU/g for the inactivation study at 50, 55, and 60°C, respectively, and the compost with 40% moisture had ca. 7.15±0.07, 7.09±0.04, and 7.13±0.03 log CFU/g of *E. coli* O157:H7, respectively. The level of mesophilic background microflora that was present in this suboptimal compost mixture (C/N ratio of 16:1) was ca. 8.27±0.08 log CFU/g as enumerated by plating on TSA.

The declines in the surviving populations of *E. coli* O157 were also rapid when the temperature of the compost reached the target level (0 h). The fresh compost mix with a 16:1 C/N ratio, and a moisture content of 50% with 2 days of come-up time, had ca. 3.32, 3.66, and 4.53 log reductions in *E. coli* O157 at 0 h exposure to 50, 55, and 60°C, respectively, and ca. 3.03, 4.30, and 5.40 log reductions, respectively, with 5 days of come-up time (Tables 3.1, 3.2 and 3.3). Declines in *E. coli* O157 were even slower during the same target composting temperature when the moisture content of the compost was reduced to 40% with 2 and 5 days of come-up time.

After designated temperatures were reached, with 2 days of come-up time, the compost mix with a 16:1 C/N ratio and 50% moisture had ca. 5.38, 5.41, and 5.65 log

reductions after 72, 12 and 4 h at exposure to 50, 55, and 60°C, respectively, and ca. 5.10, 5.61, and 5.71 (enrichment positive) log reductions at the same time and temperatures, respectively, with 5 days of come-up time (Tables 3.1, 3.2 and 3.3). For the same compost with 40% moisture, with 2 days of come-up time, *E. coli* O157:H7 was reduced for ca. 4.43, 3.69, and 4.13 log CFU/g after 72, 12, and 4 h of exposure to 50, 55, and 60°C, respectively, and with 5 days of come-up time, the reductions were ca. 4.38, 5.45, and 5.73 (enrichment positive) log CFU/g at the same sampling intervals and temperatures, respectively. When the come-up time was extended to 5 days, survival of *E. coli* O157 was extended in compost with both levels of moisture in comparison to that in compost with 2 days of come-up time. This was more pronounced at 55°C, where *E. coli* O157 in compost with 50 and 40% moisture survived 4 and 5 days, respectively. However, *E. coli* O157 survival was 3 days in compost with both types of moisture and 2 days of come-up time. At 60°C, survival of *E. coli* O157 in compost with 40% moisture was 2 and 3 days with 2 and 5 days of come-up time, respectively. Overall, pathogen survival was longer in the compost with the 16:1 C/N ratio than in the compost with a C/N ratio of 25:1 under most of the experimental conditions at the respective moisture levels and come-up times. The compost (C/N ratio of 16:1) with low moisture and 5 days of come-up time, survival of pathogens was more extended than in compost with 50% moisture or 2 days of come-up time.

Between 2 and 5 days of come-up time trials, pathogen survival in fresh compost (C/N ratio of 16:1) with 50% moisture was not significantly different ($P > 0.05$) at most of the sampling times at 50°C, whereas it was significantly different ($P < 0.05$) at most of

the sampling times at 55°C and until the 4-h sampling time at 60°C (Tables 3.1, 3.2, and 3.3). There was a significant difference ($P < 0.05$) in *E. coli* O157 survival in the compost with 40% moisture at 50°C between 2 and 5 days of come-up time at the 0-, 2-, 4-, 8- and 24-h sampling times and most of the sampling times at 55 and 60°C. For the composting treatment with 5 days of come-up time, the difference in pathogen survival in compost with 40 and 50% compost moisture was significant ($P < 0.05$) at most of the sampling times at 50°C and initial sampling intervals at 55 and 60°C.

Weibull modeling of thermal inactivation data

Inactivation kinetics of *E. coli* O157:H7 at different temperatures and different composting conditions were fitted with a mixed Weibull model. Parameters of the Weibull model for different temperatures and composting conditions are given in Tables 3.4, 3.5, and 3.6. In the Weibull model, α was model parameter (indicating the change in the ratio of the subpopulation resistant to stress), p was the shape parameter, and δ_1 and δ_2 were the decimal reduction times in subpopulation 1 (sensitive subpopulation) and subpopulation 2 (resistant subpopulation), respectively, that would rise due to stress.

All experimental conditions of composts with 25:1 C/N ratios were fit to the model with an $r^2 > 0.98$. However, only 8 out of 12 experiments under the 16:1 C/N ratio compost trials were fit to the model with an $r^2 > 0.97$. The remaining 4 trials which could not be modeled were probably due to more inactivation in the surviving populations of the pathogen during the come-up time than the other trials. At 50, 55, and 60°C, shape parameters were greater than 1 ($p > 1$) for all the tested conditions except at 50°C in

compost with C/N ratio, moisture, and come-up time 16:1, 40% and 2 days, respectively (Tables 3.4, 3.5 and 3.6). In composts with an optimal C/N ratio (25:1), δ_1 and δ_2 values of the compost with 5 days of come-up time was greater than the corresponding δ1 and δ_2 values of composting with 2 days of come-up time with the same moisture at all three temperatures. Similarly, the δ_1 and δ_2 values of the optimal compost with 40% moisture was greater than the respective δ_1 and δ_2 values at 50% moisture with the same come-up time. In composts with a suboptimal C/N ratio, similar trends were observed for δ_1 and δ_2 values between 40 and 50% moisture within 2 days of come-up time at 55 and 60°C.

The regression coefficients of four factors, i.e. C/N ratio, temperature, moisture and come-up time, were further examined for each parameter in the mixed Weibull distribution to determine whether the variable has statistically significant predictive capability in the presence of other factors using stepwise regressions in MATLAB. The results revealed that the C/N ratio is a significant factor for α; the moisture and come-up time are significant for δ_1; the temperature, moisture, and come-up time are significant for δ_2; and moisture and come-up time are significant for *p* (shape parameter) value (Fig. 3.1). To examine the interactions between those four factors, these parameters were fit to the model with interaction response surfaces. The coefficient was obtained as follows:

$$\ln(\alpha) = 0.77+0.015*T+1.91*M-0.01*CN+0.04*H-0.03*T*M+0.00001*T*CN-0.002*T*H+0.0003*M*CN-0.13*M*H+0.01*CN*H \quad (1)$$

$$\ln(\delta_1) = 7.92-0.08*T-3.24*M-0.35*CN+2.31*H+0.01*T*M+0.01*T*CN-0.02*T*H+0.17*M*CN-1.31*M*H-0.01*CN*H \quad (2)$$

$$\ln(\delta_2) = 0.09+0.04*T+14.45*M+0.06*CN+0.75*H-0.20*T*M-0.00004*T*CN-0.001*T*H-0.13*M*CN-0.80*M*H-0.002*CN*H \quad (3)$$

$$\ln(p) = 18.19+0.24*T+31.51*M+0.10*CN+2.96*H-0.44*T*M+0.001*T*CN-0.02*T*H-0.11*M*CN-2.48*M*H-0.028*CN*H \quad (4)$$

In the above equations, *T, M,* CN, and *H* represent temperature, moisture, C/N ratio and come-up time, respectively. The r^2 values of regressions for equations 1to 4 are 0.86, 0.96, 0.99, and 0.88, respectively. The high r^2 values indicate that parameters can be well-described by the interaction response surfaces of four factors. The interaction terms of response surfaces showed that moisture (*M*) and come-up time (*H*) have significantly ($P < 0.05$) high negative interactions for all four parameters.

Discussion

During composting, the rate and level of heat generation by microbial activities and subsequent pathogen inactivation depends on various factors. In this study we investigated the effect of moisture, come-up time, and C/N ratio on the survival of *E. coli* O157:H7 in fresh dairy compost at several composting temperatures in a controlled environment. Our results indicated that an optimal moisture level (50%) in fresh compost mix expedited *E. coli* O157:H7 inactivation in comparison to the suboptimal moisture (40%) under various conditions and temperatures examined in the study. In this study *E. coli* O157:H7 survived in fresh compost with 40% moisture for at least 12 and 5 days at 50 and 55°C, respectively, compared with 12 and 4 days in compost with 50% moisture at 50 and 55°C, respectively, under suboptimal composting conditions (C/N, 16:1, 5 days

of come-up time). Under current guidelines, a moisture range of 50-60% is preferred; however, a range of 40-65% is also acceptable for composting (22). Based on our results, initial composting moisture needs to be defined in much narrower range, since low initial moisture can extend the survival of pathogens.

Several studies have examined the impact of moisture level affecting pathogen inactivation during composting. Ceustermans et al. (3) demonstrated that *Salmonella enterica* serovar Senftenberg strain W 775 was inactivated within 10 h of composting at 60°C with moisture varying between 60-65%; however, when the moisture content of the compost was reduced by 5% the survival rate was increased by 0.50 log/h. The moisture content of the compost mixture affects temperature distribution within the compost heap (9, 20). When the moisture content of the compost mix is too high, conditions may turn anaerobic and the temperature of the compost heap will not rise, or the temperature rise is very slow, thereby increasing the duration of the mesophilic composting phase. On the other hand, if the initial moisture level of the compost mix is too low, the microbial metabolic rate is reduced for the microorganisms involved in composting, leading to slow temperature increases. In the present study, compost mixes with initial moisture levels of 40 and 50% were used. During temperature ramping in the environmental chamber, the compost mixes with optimal moisture (50%) would tend to lose more moisture in comparison to the compost with suboptimal (40%) level. As a result, *E. coli* O157 in compost with optimal moisture may be inactivated more quickly due to development of more moist heat. In addition, pathogen populations in the compost with suboptimal moisture compost may have became adapted to heat stress compared to the pathogen

levels in compost with optimal moisture content. Gotaas (11) suggests that composts with optimal initial moisture content have a higher temperature zone that extends within most of the compost pile, with less stratification observed than when the initial moisture is suboptimal. Furthermore, differences in moisture levels could have arisen with the different treatments. Our moisture data revealed that the compost samples were drier at the end of 5-day come-up time than at the end of 2-day come-up time (data not shown), which may be the contributing factor stressing the pathogen and increasing its survival rate under thermal conditions of extended mesophilic composting.

In the present study we found that at 55°C, *E. coli* O157:H7 survival was as short as 1 day and as long as 5 days depending upon the moisture level, come-up time, and C/N ratio. Despite this study being done under controlled conditions in a lab, the length of *E. coli* O157:H7 survival exceeded EPA recommended guidelines of 3 days of composting at 55°C. Our results suggest inadequacy of time-temperature guidelines for composting when optimal composting conditions are not met. It is expected that field composting under similar conditions can further extend pathogens survival beyond the time limit observed in this study due to exposure to environmental variations.

In this study we found that when the composting process had a long mesophilic phase (5 days of come-up time) before it reaches the thermophilic phase, *E. coli* O157:H7 was inactivated slowly during come-up time and survived for a longer time at specific composting temperatures in comparison to the situation where temperature rise was quick (2 days of come-up time). These results imply that an extended mesophilic phase of the composting process should be avoided for producing microbial safe compost. Previous

studies have suggested that an extended mesophilic phase may allow the pathogens in compost to adapt to rising temperature, thereby surviving lethal temperatures by heat-shock response induction (16, 23). Singh et al. (23), reported that *E. coli* O157:H7 heat-shocked at 47.5°C survived for 5 h and 20 min at 55 and 60°C, respectively, compared with 3 h and 10 min, respectively, at the same temperature when not heat-shocked. Lafond et al. (16) observed a cycle of appearance/disappearance of Gram-negative bacteria until day 32 of composting of duck excreta with C/N of 67.5:1. Since the composting process had a slow temperature rise, the authors suggest that Gram-negative bacteria developed heat-resistance. In a field study, Shepherd et al. (21) found that heat-shocked *E. coli* O157:H7 survived 5 days at the bottom of the dairy compost, whereas non heat-shocked culture survived for only 1 day. Apparently, to ensure complete inactivation of pathogens within the entire compost heap, it is necessary to take into consideration or monitor other stages of the composting process, such as the time (come-up time) required for the temperature of the compost heap to reach the thermophilic phase.

Carbon to nitrogen ratio is among one of the important factors affecting compost quality (10, 14). Generally, microorganisms use carbon for both energy and growth and available nitrogen for protein synthesis and reproduction. As a result, the generation of metabolic heat inactivates pathogens during the thermophilic phase of the composting process. Initial C/N ratios of 25:1 to 30:1 are considered ideal for compost degradation, although C/N ratios of 20:1 to 40:1 are considered acceptable (19). In the present study, we found that compost with a suboptimal C/N ratio supported longer survival for *E. coli*

O157:H7 than compost with the optimal C/N ratio within respective come-up times and moisture levels. Although, in our experimental setting, heat for microbial inactivation was produced by an environmental chamber rather than the self-heating from microbial metabolism, the difference in compost nutrient composition and microbial flora may be the reason for longer survival of *E. coli* O157:H7 in compost with a 16:1 C/N ratio. The importance of the C/N ratio during composting was highlighted in a field study by Huang (13). That study showed that outdoor composting of pig manure under a windrow system with 30:1 C/N ratio (optimal) entered the thermophilic phase on day 3 of composting, indicating quick establishment of microbial activities in the composting pile, whereas about 7 days were required to reach thermophilic phase in compost with a C/N ratio of 15:1 (sub-optimal). Such conditions are critical during composting, since composting under suboptimal conditions may facilitate microbial adaptation to a slow rise of temperature by mounting heat-shock response, thereby extending their survival. Also, in composting situations where the C/N ratio is high, e.g., > 40:1, the temperature rise is slow too and may not reach thermophilic phase, thereby affecting pathogen survival (16). Lafond et al. (16) found that compost with a C/N ratio of 67.5:1 had partial elimination of fecal streptococci, total coliform, and Gram-negative bacteria in comparison to the compost with C/N ratio of 32.9:1, which had total coliform and fecal streptococci undetectable after 6 and 12 days of composting, respectively. For small-scale composting, it may not be possible to control C/N ratio of the compost mixture strictly within suggested limits. As a result, it would be difficult for the composting process to achieve time-temperature guidelines for pathogen inactivation. Also, if composting under

such conditions is allowed to continue then the composting process would take more time to mature due to an extended mesophilic and inadequate thermophilic phases (10, 13). However, the composting guidelines are not clear on such outcomes.

Historically it has been believed that microorganisms in the population follow a first-order kinetic of thermal inactivation with same probability of dying for all (25, 26). Microbial communities are heterogeneous in nature, therefore, a survival curve having a shoulder and tail configuration is observed, deviating from linearity (23). In the present study shape parameter p, which was > 1 for the majority of the conditions, highlighted the non-linear behavior of microbial populations in compost. Also, when microbial populations are exposed to stress, this characteristic further magnifies the variation in the populations (17).

In this study we used compost, which is complex substance that additionally contributes to the microbial community variability as the heating medium. Both δ_1 and δ_2 parameters of the model, which reflected sensitivity of the two populations in the microbial community to heat stress, were dependent on the physiological state of the microbial population and the physical state (percent moisture) of the compost. In a previous study (23), we also reported that microbial stress in the form of heat shock affected the values of these parameters. As discussed above, temperature is not the only factor that is important during composting. There are other factors that can affect the composting outcome and those factors also need to be taken into consideration while studying microbial behavior during the composting process. Mathematical models that incorporate most of the factors that microbial cells are exposed to during composting will

have high predictive strength (6). Based on our results, moisture was identified as one of the factors that affect pathogen survival during composting, with negative coefficients of correlation in the Weibull parameters δ_1 and δ_2 (Fig 3.1) ($P < 0.05$). This means that inactivation of the pathogen would be quicker (lower δ_1 and δ_2 values) with the initial optimal moisture content (50%) of the compost than in the compost with suboptimal levels (40%). Additionally, the come-up time (heating process) in which the compost heap initially heats up had a positive correlation with parameters δ_1, δ_2 and p values (Fig 3.1) ($P < 0.05$). This means that when the temperature rise is slow (5 days), then the inactivation of the pathogens is slow (higher $\delta_{1,}$ δ_2 and p) in comparison to that with optimal heating time (2 days). The parameter δ_2 was negatively correlated with temperature ($P < 0.05$), indicating that higher the inactivation temperature of the composting processes the quicker pathogen inactivation would be. Importantly, temperature is one of the critical factors during composting that adds selective pressure on microbial populations and differentiates the sensitive and resistant subpopulations. Clearly, in the present study, both moisture and come-up time contributed to the thermal resistance of the microbial population, in addition to temperature.

Although there was a correlation between the C/N ratio of the compost and the parameter α, the current experimental set-up could not explain this. A similar study performed under field conditions would give more valuable information about this factor in relation to Weibull parameters.

It is obvious from the Weibull parameters (Tables 3.4, 3.5 and 3.6) that 4 *D* values for pathogen inactivation after 5 days of come-up time were greater than those

after 2 days of come-up time for most of the treatments, suggesting the microbial population was more heat resistant when the temperature rise was slow. In the present study we found extensive tailing in the survival of *E. coli* O157:H7 during later stages of the inactivation study, indicating that some highly resistant subpopulations can survive the composting process. These surviving populations may later on regrow under suitable conditions (9, 15).

Predictive equations that were developed in the present study can be used in predicting composting outcomes, however, this needs further validation since it is based on the experimental outcomes obtained in lab studies and there are many other environmental factors, such as seasonality, UV exposure, and precipitation which may not be simulated correctly.

Conclusions

Our results clearly demonstrated that fresh dairy compost with 40% moisture supported better survival of *E. coli* O157 during active composting than the compost with 50% moisture. Come-up time was the most critical factor during our composting trials, with longer pathogen survival being observed for the composting condition which simulated a long mesophilic phase (5 days of come-up time) than for the one with a normal temperature rise (2 days of come-up time) regardless of the moisture level and C/N ratio. The thermal inactivation data fit well into the mixed Weibull model. Both experimental and modeling results suggest that microbial populations become adapted to composting temperatures when the temperature rise is slow, or the composting was

conducted under suboptimal conditions. Under certain conditions, e.g. dry and low C/N compost mix with a longer mesophilic phase, *E. coli* O157:H7 survival exceeded 3 days at 55°C, the composting time-temperature recommendation in EPA guidelines, suggesting inadequacy of the guidelines for composting. Therefore, in order to ensure the microbiological safety of the composting process, the composting guidelines need to be refined further by taking into consideration of suboptimal composting conditions.

Acknowledgements

This research was supported by a grant from the Center for Produce Safety, University of California at Davis.

References

1. Amner, W., A. J. McCarthy, and C. Edwards. 1991. Survival of a plasmid-bearing strain of *Bacillus subtilis* introduced into compost. J. Gen. Microbiol. 137:1931-1937.

2. Centers for Disease Control and Prevention (CDC). 2006. Ongoing multistate outbreak of *Escherichia coli* serotype O157:H7 infections associated with consumption of fresh spinach--United States, September 2006. MMWR Morb. Mortal. Wkly. Rep. 55:1045-1046.

3. Ceustermans, A., D. De Clercq, A. Aertsen, C. Michiels, A. Geeraerd, J. Van Impe, J. Coosemans, and J. Ryckeboer. 2007. Inactivation of *Salmonella* Senftenberg strain W 775 during composting of biowastes and garden wastes. J. Appl. Microbiol. 103:53-64.

4. Chapman, P. A., C. A. Siddons, J. Manning, and C. Cheetham. 1997. An outbreak of infection due to verocytotoxin-producing *Escherichia coli* O157 in four families: the influence of laboratory methods on the outcome of the investigation. Epidemiol. Infect. 119:113-119.

5. Cieslak, P. R., T. J. Barrett, P. M. Griffin, K. F. Gensheimer, G. Beckett, J. Buffington, and M. G. Smith. 1993. *Escherichia coli* O157:H7 infection from a manured garden. Lancet 342:367-367.

6. Coroller, L., I. Leguerinel, E. Mettler, N. Savy, and P. Mafart. 2006. General model, based on two mixed Weibull distributions of bacterial resistance, for describing various shapes of inactivation curves. Appl. Environ. Microbiol. 72:6493-6502.

7. Davis, C. L., S. A. Hinch, C. J. Donkin, and P. J. Germishuizen. 1992. Changes in microbial-population numbers during the composting of pine bark. Bioresour. Technol. 39:85-92.

8. Droffner, M. L., W. F. Brinton. 1995. Survival of *Escherichia coli* and *Salmonella* populations in aerobic thermophilic composts as measured with DNA gene probes. Zentralbl. Hyg. Umweltmed. 197:387-397.

9. Erickson, M. C., F. Critzer, and M. P. Doyle. 2010. Produce safety project. http://www.producesafetyproject.org/admin/assets/files/PEW-PSP-Composting-Manure-Narrative-v4-2.pdf (Accessed on July, 2010)

10. Erickson, M. C., J. Liao, L. Ma, X. Jiang, and M. P. Doyle. 2009. Inactivation of *Salmonella* spp. in cow manure composts formulated to different initial C:N ratios. Bioresour. Technol. 100:5898-5903.

11. Gotaas, H.R. 1956. Composting – Sanitary Disposal and Reclamation of Organic Wastes. World Health Organization Monograph Series Number 31. Geneva, Switzerland.

12. Haapapuro, E. R., N. D. Barnard, and M. Simon. 1997. Animal waste used as livestock feed: Dangers to human health - Review. Prev. Med. 26:599-602.

13. Huang, G. F., J. W. C. Wong, Q. T. Wu, and B. B. Nagar. 2004. Effect of C/N on composting of pig manure with sawdust. Waste Manage. 24:805-813.

14. Hutchison, M. L., L. D. Walters, S. M. Avery, and A. Moore. 2005. Decline of zoonotic agents in livestock waste and bedding heaps. J. Appl. Microbiol. 99:354-362.

15. Kim, J., F. Luo, and X. Jiang. 2009. Factors impacting the regrowth of *Escherichia coli* O157:H7 in dairy manure compost. J. Food Prot. 72:1576-1584.

16. Lafond, S., T. Pare, H. Dinel, M. Schnitzer, J. R. Chambers, and A. Jaouich. 2002. Composting duck excreta enriched wood shavings: C and N transformations and bacterial pathogen reductions. J. Environ Sci. and Health Part B-Pesticides Food Contaminants and Agric. Wastes 37:173-186.

17. Mangalassary, S., I. Han, J. Rieck, J. Acton, X. Jiang, B. Sheldon, and P. Dawson. 2007. Effect of combining nisin and/or lysozyme with in-package pasteurization on thermal inactivation of *Listeria monocytogenes* in ready-to-eat turkey bologna. J. Food Prot. 70:2503-2511.

18. Mead, P. S., L. Slutsker, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States - Reply. Emerg. Infect. Dis. 5:841-842.

19. Rynk, R. 1992. On-farm Composting Handbook. Northeast Regional Agricultural Engineering Service, Ithaca, NY.

20. Shepherd, M. W., Jr, P. Liang, X. Jiang, M. P. Doyle, and M. C. Erickson. 2007. Fate of *Escherichia coli* O157:H7 during on-farm dairy manure-based composting. J. Food Prot. 70:2708-2716. /topic/vermicomposting/pubs/ag593.pdf. (Accessed on June, 2010)

21. Shepherd, M. W., Jr, R. Singh, K. Jinkyung, and X. Jiang. 2010. Effect of heat-shock treatment on the survival of *Escherichia coli* O157:H7 and *Salmonella enterica* Typhimurium in dairy manure co-composted with vegetable wastes under field conditions Bioresour. Technol. 101:5407-5413.

22. Sherman, R. 2005. Large-scale organic materials composting. North Carolina Cooperative Extension Service. Pages 1 – 16. Available at: http://www.bae.ncsu.edu

23. Singh, R., X. Jiang, and F. Luo. 2010. Thermal inactivation of heat-shocked *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in dairy compost. J. Food Prot. 73:1633-1640.

24. U.S. Environmental Protection Agency. 1999. Standards for the Use or Disposal of Sewage Sludge (40 CFR Parts 403 and 503). Revised August 4, 1999. Available at: http://www.epa.state.oh.us/portals/35/sludge/503_08_04_99.pdf (Accessed June, 2010).

25. van Boekel, M. A. J. S. 2002. On the use of the Weibull model to describe thermal inactivation of microbial vegetative cells. Int. J. Food Microbiol. 74:139-159.

26. Virto, R., D. Sanz, I. Alvarez, S. Condon, and J. Raso. 2006. Application of the Weibull model to describe inactivation of *Listeria monocytogenes* and *Escherichia coli* by citric and lactic acid at different temperatures. J. Sci. Food Agric. 86:865-870.

Figure legend

Figure 3.1 Regression coefficients of four experimental factors, i.e. temperature, moisture, come-up time and C/N, for 4 different model parameters (α, top left panel; *δ1*, top right panel; *δ2*, bottom left panel; p, bottom right) in a mixed Weibull model.

Table 3.1 Thermal inactivation of *Escherichia coli* O157:H7 in fresh compost at 50°C under different conditions

C/N ratio	Come-up time (days)	MC (%)	Treatment[1]	log CFU/g at 50°C with heating time (h)														
				(-)72	(-) 24	0	2	4	6	8	24	72	96	120	144	168	240	288
25:1	2	40	YC	NA[2]	6.09±0.12A[3]X[4]	a[5]2.9±0.5AX	a2.09±0.14AX	a2.09±0.18AX	a2.07±0.34AX	a1.56±0.30AX	+[6]	+	-[7]	-				
			LC	NA	5.5±0.31Y	2.09±0.19AY	1.95±0.24Y	1.98±0.28X	1.99±0.31X	1.59±0.33X	+	+	-	-				
		50	YC	NA	5.94±0.11BX	a2.52±0.41AX	a1.98±0.53AX	a1.98±0.45AX	a1.8±0.22AX	a1.56±0.19AX	+	+	-	-				
			LC	NA	5.18±0.17Y	2.17±0.56X	1.94±0.32X	1.97±0.34X	1.7±0.19X	+Y	+	+	-	-				
	5	40	YC	6.92±1.58A	NS[8]	b2.24±0.32A	a2.07±0.31A	a1.96±0.25A	a1.96±0.28A	a1.64±0.41A	+	+	+	+	+	-		
		50	YC	6.57±1.26A	NS	b1.49±0.22B	b+B	b+B	b+B	b+B	+	+	+	-	-			
16:1	2	40	YC	NA	6.37±0.15A	a5.22±0.07A	a5.09±0.20A	a4.97±0.08A	a4.67±0.31A	a4.58±0.23A	a3.71±0.11A	a2.77±0.18A	a2.21±0.15A	+	+	+	+	+
		50	YC	NA	6.01±0.30B	a3.91±0.05B	a3.75±0.42B	a3.44±0.18B	a3.17±0.34B	a2.79±0.30B	a2.5±0.18B	a1.85±0.23B	+B	+	+	+	+	-
	5	40	YC	6.3±0.07A	NA	b4.67±0.24A	b4.6±0.17A	b4.41±0.21A	a4.41±0.16A	b4.29±0.03A	b3.54±0.18A	a2.77±0.47A	a2.19±0.11A	+	+	+	+	+
		50	YC	6±0.36B	NA	b4.18±0.28B	a3.45±0.39B	a3.35±0.16B	a3.21±0.23B	a2.88±0.36B	a2.56±0.22B	a2.11±0.40B	+B	+	+	+	+	+

[1]YC, Young, LC, Low nutrient adapted culture, [2]NA sample not available

[3]Mean log CFU± SD with different upper case letter differs significantly (P<0.05) between two moisture level with same C/N ratio & come-up time within column for the same culture.

[4]Mean log CFU± SD with different upper case letter differs significantly (P<0.05) between two different treatments within same moisture, C/N ratio & come-up time within column.

[5]Mean log CFU± SD with different lower case letter differs significantly (P<0.05) between two different come-up time within same moisture and C/N ratio within column for the same culture.

[6] + Positive by enrichment, [7]-, not detected, [8]NS, not sampled,

Table 3.2 Parameters of mixed Weibull distribution of *E. coli* O157:H7 inactivation curves at 50°C

C/N ratio	MC (%)	Come-up time (days)	Mixed Weibull model parameters						
			Treatment[1]	α	δ_1(h)	δ_2 (h)	p	4*D*- (h)	(R^2)
25:1	40	2	YC	5.39±0.30[2]	24.92±4.15	127.01±16.42	2.23±0.47	47.52[3]	0.986[4]
			LC	5.13±0.31	18.75±3.78	112.31±18.59	1.70±0.32	43.20	0.984
		5	YC	5.68±0.20	78.61±10.8	278.06±15.8	3.75±1.12	115.20	0.990
	50	2	YC	5.40±0.27	22.67±3.62	124.50±15.5	2.06±0.38	44.64	0.989
			LC	5.24±0.34	15.95±3.57	116.12±26.03	1.46±0.26	41.76	0.984
		5	YC	5.87±0.32	51.80±7.36	246.41±20.7	3.42±1.49	77.76	0.989
16:1	40	2	YC	5.40±0.55	17.22±3.78	449.53±441.05	0.81±0.08	97.44	0.987
		5	YC	4.78±0.26	21.79±3.82	188.32±25.86	1.56±0.26	57.76	0.980
	50	2	YC	5.36±0.46	52.16±6.93	580.21±396.52	1.18±0.11	171.36	0.989
		5	YC	5.41±0.56	32.03±9.78	572.02±506.07	1.01±0.19	130.56	0.969

[1] YC, Young culture; LC, Low nutrient-adapted culture
[2] '±', indicated standard deviation
[3] 4 *D*-(h), time in hour required to reduce 4 logs of *E. coli* O157 population at 50°C
[4] adjusted R^2 value

Table 3.3 Thermal inactivation of *Escherichia coli* O157:H7 in fresh compost at 55°C under different conditions

C/N ratio	Come-up time (days)	MC (%)	Treatment[1]	log CFU/g at 55°C with heating time (h)													
				(-)72	(-) 24	0	1	2	4	8	12	24	48	72	96	120	144
25:1	2	40	YC	NA[2]	6.00±0.02A[3]X[4]	a[5]2.23±0.62AX	a1.93±0.53AX	a1.81±0.42AX	a1.61±0.36AX	+[6]	+	+	+	-[7]			
			LC	NA	5.92±0.15X	2.03±0.58X	1.79±0.53X	1.61±0.36X	1.59±0.37X	+	+	+	+	-			
		50	YC	NA	6.02±0.04AX	a+B	+B	+B	a+B	+	+	+	-	-			
			LC	NA	5.95±0.12X	+	+	+	+	+	+	+	-	-			
	5	40	YC	6.32±1.38A	NS[8]	a2.29±0.57A	a2.03±0.55A	a1.70±0.29A	a1.72±0.32A	+	+	+	+	+	+	+	-
		50	YC	5.87±1.24A	NS	b1.64±0.11B	+B	+B	a1.41±0.16B	+	+	+	+	-	-	-	-
16:1	2	40	YC	NA	6.14±0.17A	a4.23±0.05A	a4.08±0.07A	a3.97±0.05A	a3.78±0.12A	a3.64±0.07A	a3.43±0.11A	+	+	+	-	-	-
		50	YC	NA	5.94±0.34A	a3.52±0.43B	a3.18±0.24B	a3.02±0.23B	a2.81±0.13B	a2.57±0.17B	a1.77±0.19B	+	+	+	-	-	-
	5	40	YC	4.57±0.21A	NS	b3.69±0.23A	b3.52±0.27A	b3.29±0.37A	b3.04±0.46A	b2.36±0.51A	b1.64±0.41A	+	+	+	+	+	-
		50	YC	4.69±0.33A	NS	b2.80±0.61B	b2.37±0.99B	b2.04±0.81B	b1.94±0.70B	b1.80±0.57A	b1.49±0.22A	+	+	+	+	-	-

[1]YC, Young, LC, Low nutrient adapted culture, [2]NA sample not available

[3]Mean log CFU± SD with different upper case letter differs significantly (P<0.05) between two moisture level with same C/N ratio & come-up time within column for the same culture.

[4]Mean log CFU± SD with different upper case letter differs significantly (P<0.05) between two different treatment within same moisture, C/N ratio & come-up time within column.

[5]Mean log CFU± SD with different lower case letter differs significantly (P<0.05) between two different come-up time within same moisture and C/N ratio within column for the same culture.

[6] + Positive by enrichment, [7]-, not detected, [8]NS, not sampled,

Table 3.4 Parameters of mixed Weibull distribution of *E. coli* O157:H7 inactivation curves at 55°C

C/N ratio	MC (%)	Come-up time (days)	Mixed Weibull model parameters						
			Treatment[1]	α	δ_1(h)	δ_2 (h)	p	4*D*- (h)	(R^2)
25:1	40	2	YC	5.48±0.10[2]	23.55±1.32	97.94±3.98	2.28±0.16	44.4[3]	0.998[4]
			LC	5.40±0.09	23.25±1.18	97.62±3.52	2.28±0.14	43.2	0.998
		5	YC	5.30±0.21	93.04±9.64	1260.60±10.55	6.00±2.31	118.8	0.985
	50	2	YC	5.61±0.33	22.20±2.11	78.62±10.91	2.65±1.12	38.4	0.993
			LC	5.50±0.32	22.56±2.14	78.90±10.44	2.68±1.1	38.40	0.993
		5	YC	5.43±0.35	37.95±7.8	163.30±29.55	1.53±0.25	95.04	0.983
16:1	40	2	YC	5.21±0.55	30.76±4.65	124.07±29.06	2.15±0.44	59.04	0.976
		5	YC	-[5]	-	-	-	-	-
	50	2	YC	5.25±0.2	21.91±2.4	102.87±9.91	1.69±0.16	43.20	0.995
		5	YC	-	-	-	-	-	-

[1] YC, Young culture; LC, Low nutrient-adapted culture
[2] '±', indicated standard deviation
[3] 4 *D*-(h), time in hour required to reduce 4 logs of *E. coli* O157 population at 55°C
[4] adjusted R^2 value
[5] data could not be fit to the mixed Weibull model

Table 3.5 Thermal inactivation of *Escherichia coli* O157:H7 in fresh compost at 60°C under different conditions

C/N ratio	Come-up time (days)	MC (%)	Treatment[1]	log CFU/g at 60°C with heating time (h)												
				(-)72	(-) 24	0	0.5	1	1.5	3	4	8	24	48	72	96
25:1	2	40	YC	NA[2]	5.89±0.54A[3]X[4]	a[5]3.18±0.70AX	a3.02±0.71AX	a2.85±0.78AX	a2.78±0.77AX	a2.64±0.86AX	a2.20±0.76AX	+[6]	+	-[7]	-	-
			LC	NA	5.88±0.55X	2.30±0.9Y	2.14±0.91Y	2.03±0.74Y	1.98±0.65Y	1.90±0.59Y	1.52±0.39Y	+	+	-	-	-
		50	YC	NA	5.83±0.51AX	a1.70±0.43BX	a1.59±0.34BX	a1.50±0.30BX	a1.48±0.27BX	+B	+B	+	+	-	-	-
			LC	NA	5.53±0.16X	+Y	+Y	1.44±0.28X	+X	+	+	+	+	-	-	-
	5	40	YC	5.74±0.25A	NS[8]	b1.62±0.27A	b1.53±0.14A	b1.49±0.22A	b+	b+	b+	+	+	+	+	-
		50	YC	5.71±0.22A	NS	b+B	b+B	a+B	a+	+	+	+	+	+	-	-
16:1	2	40	YC	NA	6.09±0.27A	a3.79±0.26A	a3.58±0.25A	a3.43±0.24A	a3.24±0.25A	a3.13±0.28A	a2.99±0.34A	a2.69±0.58A	+	+	-	-
		50	YC	NA	5.42±0.6B	a2.61±0.55B	a2.52±0.55B	a2.34±0.57B	a1.97±0.40B	a1.70±0.29B	a1.49±0.22B	+B	+	-	-	-
	5	40	YC	4.18±0.11A	NS	b2.25±0.49A	b2.10±0.33A	b1.59±0.24A	b+	b+	b+	b+	+	+	+	-
		50	YC	4.28±0.14A	NS	b1.71±0.16B	b+B	b+B	b+	b+	b+	+	+	-	-	-

[1]YC, Young, LC, Low nutrient adapted culture, [2]NA sample not available

[3]Mean log CFU± SD with different upper case letter differs significantly (P<0.05) between two moisture level with same C/N ratio & come-up time within column for the same culture.

[4]Mean log CFU± SD with different upper case letter differs significantly (P<0.05) between two different treatment within same moisture, C/N ratio & come-up time within column.

[5]Mean log CFU± SD with different lower case letter differs significantly (P<0.05) between two different come-up time within same moisture and C/N ratio within column for the same culture.

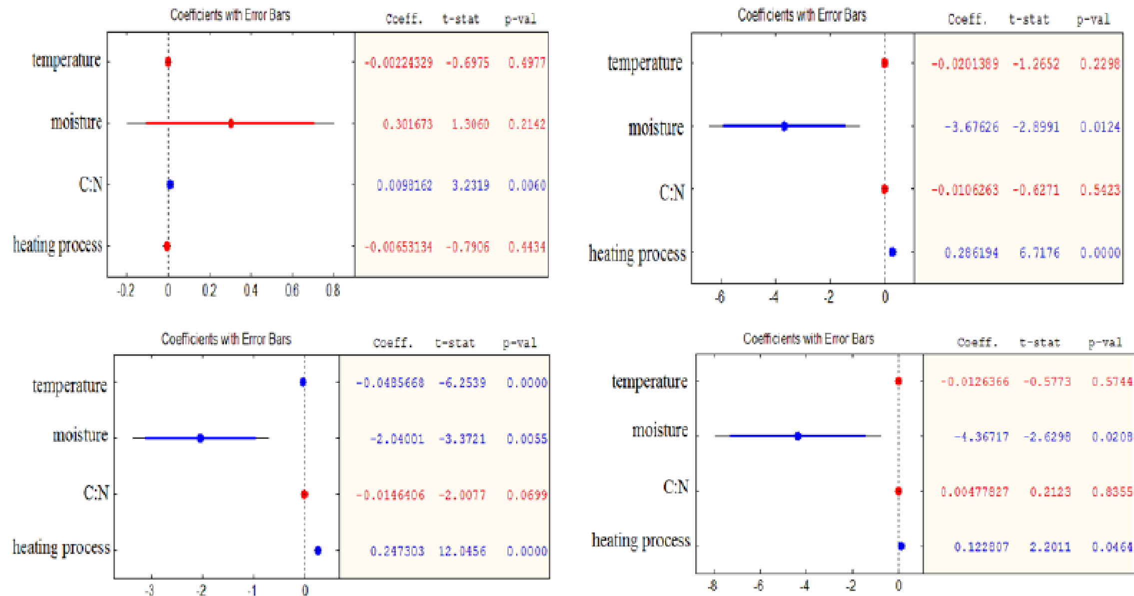
[6] + Positive by enrichment, [7]-, not detected, [8]NS, not sampled,

Table 3.6 Parameters of mixed Weibull distribution of *E. coli* O157:H7 inactivation curves at 60°C

C/N ratio	MC (%)	Come-up time (days)	Mixed Weibull model parameters						
			Treatment[1]	α	δ_1(h)	δ_2 (h)	p	4*D*- (h)	(R^2)
25:1	40	2	YC	4.97±0.30[2]	27.36±3.34	74.51±8.72	2.41±0.43	48.96[3]	0.997[4]
			LC	4.95±0.18	23.65±2.15	71.67±5.77	2.24±0.25	44.16	0.996
		5	YC	5.58±0.16	44.03±1.52	193.50±8.63	3.98±1.06	62.64	0.998
	50	2	YC	5.33±0.21	21.41±2.23	71.67±6.56	2.14±0.25	41.28	0.993
			LC	5.28±0.21	19.32±2.23	69.64±6.8	2.06±0.33	38.40	0.993
		5	YC	5.15±0.80	34.02±7.44	123.08±59.91	1.66±0.93	79.92	0.994
16:1	40	2	YC	4.63±0.25	22.82±2.27	71.69±7.43	1.70±0.18	52.8	0.994
		5	YC	-[5]	-	-	-	-	-
	50	2	YC	5.20±0.36	18.48±3.62	77.06±14.09	1.65±0.29	43.2	0.985
		5	YC	-	-	-	-	-	-

[1] YC, Young culture; LC, Low nutrient-adapted culture
[2] '±', indicated standard deviation
[3] 4 *D*-(h), time in hour required to reduce 4 logs of *E. coli* O157 population at 60°C
[4] adjusted R^2 value
[5] data could not be fit to mixed Weibull model

Figure 3.1 Regression coefficients of four factors, i.e. temperature, moisture, come-up time and C/N, for 4 different model parameters (α, top left panel; *δ1*, top right panel; *δ2*, bottom left panel; p, bottom right) in a mixed Waybill model.



CHAPTER FOUR

HEAT INACTIVATION OF *SALMONELLA* SPP. IN FRESH POULTRY COMPOST BY SIMULATING EARLY PHASE OF COMPOSTING PROCESS

Abstract

The purpose of this study was to determine the effect of moisture on thermal inactivation of *Salmonella* spp. in poultry litter under optimal composting conditions. Thermal inactivation of *Salmonella* was studied in fresh poultry compost by simulating early phase of composting process. A mixture of three *Salmonella* serotypes grown in Cryptic soy broth with revamping (TSB-R) was inoculated in fresh compost with 40 or 50% moisture at a final concentration of ca. 10^7 CFU g^{-1}. The inoculated compost was kept in an environmental chamber which was programmed to rise from room temperature to target composting temperatures in 2 d. In poultry compost with optimal moisture content (50%), *Salmonella* spp. survived for 96, 72, and 24 h at 50, 55, and 60°C, respectively, as compared with 264, 144, and 72 h at 50, 55, and 60°C, respectively, in compost with suboptimal moisture (40%). Pathogen decline was faster during the come-up time due to higher ammonia volatilization. Our results demonstrated that *Salmonella* spp. survived longer in fresh poultry compost with suboptimal moisture of 40% than in compost with optimal moisture (50%) during thermophilic composting. High nitrogen content of the poultry compost is an additional factor contributing to *Salmonella* inactivation through ammonia volatilization during thermal exposure. This research provides information regarding the effectiveness of the current composting guidelines while simulating poultry composting under controlled laboratory conditions. Initial

moisture level and controlled ammonia volatilization are important for ensuring the microbiological safety and quality of compost product.

Introduction

In the United States ca.1.3 billion tones and 54 million tones of livestock and poultry wastes, respectively, are produced annually (Boersma and Murarka, 1995; Bassis, 2011). Animal manure as fertilizer has been used effectively for centuries. Among animal wastes, poultry waste is considered as the most desirable of the natural fertilizers because of its high nitrogen content (Sloan et al., 2011). However, poultry litter is also source of some major human pathogens that have potential to cause food safety concern, such as *Salmonella*, *Staphylococcus*, *Campylobacter* (Montrose et al., 1985; Martin et al., 1998; Terzich et al., 2000). Although foodborne illnesses reported so far have rarely been associated with poultry litter, the heightened consumer awareness has made management of poultry litter all the more important. According to the Centre for Disease Control and Prevention (CDC), in comparison to 2004-2006 there was no improvement in the incidence of *Salmonella* in 2007 in relation to other foodborne illnesses, indicating the urgent need for efficient *Salmonella* control in food supply chain (CDC, 2008).

Recently, Alali et al., (2010) reported *Salmonella* prevalence of 5.6 and 38.8 % in the fecal samples collected from organic and conventional poultry farms, respectively. Application of such waste directly to agricultural land could spread this pathogen to the crops growing on the field. In one such study it was found that *Salmonella* Typhimurium persisted for 161 to 231 d in soil fertilized with poultry litter, and survived for 63 and 231

d on lettuce and parsley, respectively, grown in that soil (Islam et al., 2004). It is therefore important to treat poultry litter properly before being used for agricultural applications.

Composting of waste material including animal manure has been recognized as the important way of treating and managing ever increasing waste problem (Tiquia and Tam, 2002). Metabolic activities of the indigenous microflora of composting material generate heat that brings about inactivation of pathogens that may be present in the composting material (Erickson et al., 2009, 2010). Properly composted waste is rich in nutrients which can then be used as an important soil amendment on agricultural field, home garden etc.

Ammonia volatilization and higher temperature reached during composting of poultry litter create a hostile environment for the persistence of human pathogens (Wilkinson et al., 2010). However, persistence of pathogens in compost has still been reported. Shepherd et al. (2010) detected 26 and 6.1% of the surface and internal samples from poultry compost heaps positive for *Salmonella* during initial phase of composting. In another study compost temperature of 55°C was unable to inactivate *Salmonella*, *Escherichia coli* O157:H7 and *Listeria* for more than 8 d in poultry compost (Hutchison et al., 2005). All these are indicative of complex nature of microbial inactivation during composting process.

There have been a lot of studies on the fate of pathogens in dairy manure-based compost and only few on poultry litter compost (shepherd et al., 2007, 2009; Erickson et

al., 2009; Wilkinson et al., 2010; Singh et al., 2011). In a recently concluded study on dairy manure composting, Singh et al., (2011) reported the extended survival of *E. coli* O157:H7, beyond the recommended temperature-time guideline of the United States Environmental Protection Agency (USEPA) in compost mix with sub-optimal moisture and C:N (carbon:nitrogen) ratio of 40% and 16:1, respectively, in comparison to the optimal conditions of moisture and C:N ratio of 50% and 25:1, respectively. In that same study it was observed that the extended mesophilic phase of the composting process could prolong pathogen survival due to heat-adaption. Therefore the objective of this study was to investigate the effect of moisture on thermal inactivation of *Salmonella* spp. in poultry litter under optimal composting conditions.

Materials and Methods

Compost preparation: Fresh poultry compost was prepared by mixing fresh poultry litter from layers (collected from Charles Lee Morgan Poultry farm, Clemson University) with saw dust. The ingredients were mixed thoroughly in a way to get C:N ratio of 25:1. The C:N ratio of the duplicate compost samples were analyzed by the Agricultural Service Laboratory (Clemson University, Clemson, SC). The compost was stored under refrigeration until used. Prior to experiment compost was split into two and dried under fume hood (Kewaunee Scientific Equipment corp., Michigan) to reduce the moisture to ca. 50 % (for optimal composting condition) and 40 % (for suboptimal composting condition). The compost moisture was analyzed by a Moisture Analyzer IR-35 (Denver Instrument, Germany).

Culture preparation: Three serotype mixture of rifampin resistant (Rif^r) *Salmonella* spp. serotype Typhimurium 8243 (Dr. Roy Curtis III, Washington University), Enteritidis H2292 (Dr. Mike Doyle, University of Georgia, Griffin) and Heidelburg 21380 (Dr. Hua Zhao, Food Drug and Administration) were used in this study. Each serotype of *Salmonella* spp. was grown separately overnight at 37°C in 10 ml of cryptic soy broth (Difco™, Becton & Dickinson, MD) containing 100 µg ml^{-1} of rifampin (TSB-R). A loopful of each culture was further propagated overnight at 37°C in 30 ml of TSB-R. The grown cultures were washed twice in sterile 0.85% saline by centrifuging at 5,000 x *g* for 10 min and then resuspended in sterile saline to an optical density at 600 nm (OD_{600}) of ca. 0.6 to 0.7 (ca. 10^9 CFU ml^{-1}). Above three washed *Salmonella* cultures were then mixed in equal volume as inocula for the following experiments.

Inoculation of poultry compost: Mixed inocula of three *Salmonella* serotypes were inoculated into 2.2 kg of fresh poultry compost with 40 or 50% moisture at a final concentration of ca. 10^7 CFU g-1 in sterile polypropylene tray. Culture was inoculated with spray nozzle which was sanitized with 70% ethanol. Inoculated compost was thoroughly mixed by hands wearing sterile gloves. The enumeration of inoculated levels of the pathogens in compost was done by plating serial dilutions of the compost on tryptic soy agar (BD, MD) containing 100 µg ml^{-1} of rifampin (TSA-R). The initial levels of mesophilic background microflora and total enterobacteriacea were enumerated by plating serial dilutions on TSA and by pour-plating on violet red bile glucose agar (Difco, BD, MD), respectively.

Thermal inactivation study: Inoculated compost (100 g) with 40 or 50% moisture was packed in duplicate Tyvek pouch (size 5.25 in. by 10 in., SPS Medical, Rush, NY) for each treatment and spread evenly into thin layer (ca. 1 cm in depth). Tyvek pouches were then kept in a layer on shelf of an environmental chamber (model no. EC2047N**,** Thermo Scientific, Barnstead International, Dubuque, IA) with humidity set at ca. 70% to mimic inside condition of the composting heap. The temperature of the environmental chamber was programmed to ramp step-wise from room temperature (ca. 26 °C) to target temperature in 2 d (representing normal temperature rise during composting process). Target temperatures of 50, 55, and 60°C were used in the study. Temperature was monitored constantly using type-T thermocouples (DCC Corporation, NJ) with one cord inserted inside the compost pouch and other was kept in the chamber. After temperature of the compost reached target temperature, duplicate sample bags were removed at pre-determined sampling intervals and cooled in ice water bath to bring down the temperature of the compost for enumeration. A 25 g of compost sample was weighed from each bags and homogenized in a stomacher bag (Lansing, MI). Serial dilutions of the homogenate were made and plated in duplicate on TSA-R to analyze for the surviving population. The detection limit for direct plating was 25 CFU g^{-1}. The samples which were negative by direct plating were pre-enriched in universal preenrichment broth (UPB) (Acumedia, Neogen Corp. MI) followed by enrichment in Rappaport-Vassiliadis medium broth with 100 µg ml^{-1} of rifampin (RV-R) (USFDA, 2001). Enriched samples were plated on Xylose Lysine Tergitol Agar (XLT-4) (Difco, Sparks, MD) with rifampin

(XLT-4-R). Detection limit for enrichment was 0.02 CFU g^{-1}. Two or three trials were conducted for each experiment.

Ammonia analysis: Uninoculated poultry compost (10 g) with 40 or 50% moisture packed in duplicate Tyvek bag (size 3.5 in. by 5.25 in.) for each sampling interval was also put in the environmental chamber along with the inoculated compost. Samples were taken at predetermined intervals to analyze for ammonia. Ammonia concentration in poultry compost was analyzed based on Weatherburn method (1967). Both reagent A (5 g of phenol and 25 mg of sodium nitroprusside in 500 ml of distilled water) and reagent B (2.5 g of sodium hydroxide and 0.2 ml of 5% chlorine in 500 ml distilled water) were stored in amber color bottle in refrigerator and were used within 1 month. Ammonia standard stock solution was prepared using ammonium sulfate containing 6.0 g ammonia 100^{-1} ml of distilled water along with the adjustment for sulfate in the solution.

In order to extract ammonia from the compost, 2.5 g of compost was weighed into 50 ml of centrifuge tube to which 15 ml of 2 M KCl was added. Tube was vortexed for 30 s and pH of the solution was adjusted to 7.0 either with HCl or NaOH. The tube was then centrifuged at 6,000 rpm for 1 min at 21°C, and 20 µl of the compost supernatant was taken and added to 15 ml screw cap test tube. To the same test tube 5 ml of reagent A was added and the tube was vortexed for 10 s, followed by addition of 5 ml of reagent B to the tube. The tube was incubated at 37°C for 20 min, and absorbance of the solution was read at OD_{625}. Ammonia solution of 4, 8, 12, 16, and 20 µl from the ammonia standard stock solution was also subjected to same procedure as compost supernatant.

The amount of ammonia (μg) in standard ammonia solutions corresponded to 1.2, 2.4, 3.6, 4.8, and 6 μg, respectively. Standard curve was drawn between ammonia amounts (μg) in standards and corresponding absorbance. Regression equation was set using standard curve which was used to determine quantity of ammonia in 20 μl of compost sample extract.

Statistical analysis: Plate count data of different treatments were compared by converting surviving bacterial population into $\log_{10}$ values, and subjected to analysis of variance with a test criterion (F statistic) and type I error controlled at $P = 0.05$. Amount of ammonia between different treatments was also subjected to similar statistical analysis as above. The Tukey's multiple comparison procedure of the Statistical Analysis System (2001, SAS, Cary, N.C.) was used.

Results

Salmonella survival in compost

Thermal inactivation of *Salmonella* spp. was studied in fresh poultry compost by simulating early phase of composting process in an environmental chamber. The inactivation process was compared between different moisture content, i.e. 50% (optimal) and 40% (suboptimal) in compost mix with C:N ratio of 25:1 (optimal).

The initial levels of mesophilic background and enterobacteriacea microflora were ca. 9.12±0.05 and 6.23±0.21 log CFU g^{-1}, respectively, and the initial pH of the compost was 8.87±0.01. For compost mix with 50% moisture, the levels of *Salmonella* spp. achieved after inoculation for thermal inactivation study at 50, 55, and 60°C were ca.

7.23±0.05, 7.20±0.09 and 7.21±0.1 log CFU g^{-1}, respectively, as enumerated by plating on TSA-R. Similarly, the levels in compost mix with 40% moisture were ca. 7.17±0.06, 7.09±0.05 and 7.16±0.06 log CFU g^{-1}, respectively.

Salmonella spp. population was reduced rapidly during the come-up time before temperature of the environmental chamber reached the set target levels of 50, 55, and 60°C. The compost mix with 50% moisture had ca. 4.39, 4.51, and 4.82 log reduction in pathogen levels, respectively, at 24 h before the temperature reached the target composting temperatures of 50, 55, and 60°C, whereas compost mix with 40% moisture had ca. 3.49, 3.50, and 3.71 log CFU g^{-1} reduction of *Salmonella* spp., respectively, before temperature reached the set target levels (Fig. 4.1a-c). The difference in the survival of *Salmonella* population at 24 h before the temperature reached the set level was significantly ($P < 0.05$) different between compost with 50 and 40% moisture.

The reduction in the *Salmonella* population was still higher in compost with 50% moisture than in compost with 40% initial moisture when temperature of the compost reached the set level (0 time interval). The total reduction of *Salmonella* populations in compost with 50% moisture was ca. 4.50, 5.03, and 5.26 log CFU g^{-1}, respectively, when temperature reached the target levels of 50, 55, and 60°C, whereas the compost with 40% moisture, at same temperatures had ca. 4.24, 4.33, and 4.56 log CFU g^{-1} reduction in *Salmonella* population, respectively, when temperature reached the set levels. The difference in the survival of pathogens was still significantly ($P < 0.05$) different between compost with 50 and 40% moisture.

The decline in the pathogen levels was gradual once the temperature inside the compost reached the designated level in both type of compost mix. However, the length of *Salmonella* survival in compost with optimal moisture (50%) was much shorter than in the compost with suboptimal moisture (40%). The duration of *Salmonella* survival in compost mix with optimal moisture (50%) at 50, 55, and 60°C was 96, 72, and 24 h, respectively, whereas in compost with suboptimal moisture (40%) this duration was 264, 144, and 72 h, respectively, as detected by enrichment (Fig. 4.1a-c).

Salmonella survival in the compost with optimal and suboptimal moisture levels was significantly ($P < 0.05$) different at most of the sampling intervals until 12 h at 50 and 55°C (Fig. 4.1a, 4.1b). At 60°C the difference in the survival of pathogen was significantly ($P < 0.05$) different at all the sampling times until 8 h of the sampling interval (Fig. 4.1c).

<u>Ammonia loss in poultry compost during thermal inactivation</u>

The amounts of ammonia detected in two types of compost mix are presented in Table 4.1. The initial levels of ammonia varied from 615 to1018 μg g^{-1} compost (- 48 h) as the amount of ammonia was higher ($P < 0.05$) in compost mix with optimal moisture (50%) than in compost with suboptimal moisture (40%). Loss of ammonia due to volatilization was high in compost mix with 50% than with 40% moisture at 24 h before the temperature reached the designated temperature. The loss of ammonia in compost with 50% moisture at 50, 55, and 60°C was 352.1, 430.6, and 343.3 μg g^{-1} of compost,

respectively (Table 4.1). The loss in compost with 40% moisture content was 53.2, 151.8, and 169.8 μg g^{-1} of compost at 50, 55, and 60°C, respectively.

The ammonia loss was gradual once temperature ramped to the designated set level. However, ammonia loss was still significant ($P < 0.05$) between compost mix of 50 and 40% moisture at sampling intervals of 0, 12, 48, and 288 h at 50°C, 0, 2, 8, and 48 h at 60°C and most of sampling intervals at 55°C (Table 4.1). The total loss of ammonia due to volatilization in compost with optimal moisture (50%) content at the end of experiment at 50, 55, and 60°C, was 677.8, 815.5, and 725.6 μg g^{-1} of compost, respectively, as compared with ammonia loss in compost with 40% moisture was 329.8, 540.1, and 617.8 μg g^{-1} of compost at 50, 55, and 60°C, respectively. Overall, loss of ammonia from the poultry compost mix was more in compost with optimal moisture (50%) than in compost with suboptimal moisture (40%). Also, ammonia volatilization was higher when poultry compost with the same moisture level was exposed to higher temperature of 60°C than to 50 or 55°C with the exception of compost with 50% moisture at 55°C. The highest initial level of ammonia in that compost mix can be attributed to this discrepancy.

Discussion

Pathogen inactivation during composting is function of rate and level of heat generated by microbial metabolism which in turn is influenced by several factors. In this study we investigated the effect of initial moisture and ammonia levels on the survival of *Salmonella* spp. in poultry compost mix with optimal C:N ratio of 25:1 at several

composting temperatures by simulating early phase of optimal composting process (2 d of come-up time) under controlled environment. Our results indicate that inactivation of *Salmonella* spp. was prolonged in compost with suboptimal (40%) moisture than in compost with optimal (50%) moisture. In a similar study, Ceustermans et al. (2007) demonstrated the increase in survival rate of *Salmonella* strain W 775 by 0.5 log h^{-1}, when the moisture content of the compost was decreased by 5% from 65 to 60%. In a similar study of fresh dairy compost, we found that the compost with initial suboptimal moisture level (40%) supported extended survival of *E. coli* O157:H7 than the compost with initial optimal moisture level (50%) (Singh et al., 2011).

Initial moisture content of compost mix is one of the critical factors that can determine composting outcome. According to the current composting guidelines, moisture range of 50 to 60% is preferred, however, a range of 40 to 65% is acceptable (Sherman, 2005). Compost mix with too high initial moisture content can limit oxygen diffusion into the composting mix and create anaerobic conditions due to water logging in the pore spaces causing slow rise of temperature (extended mesophilic phase), resulting in prolonged pathogen survival. On the other hand very low initial moisture of the compost mix is also believed to extend pathogen survival as less microbial activities can occur thereby having extended duration of sub-lethal temperature range. Simulation of extended mesophilic phase (5 d of come-up time) of the composting process in our recently concluded study showed that compost mix with sub-optimal moisture level (40%) supported extended survival of the *E. coli* O157:H7 than the composting process that simulated normal mesophilic phase (2 d of come-up time) (Singh et al., 2011).

The time-temperature guidelines under current composting condition consider temperature of 55°C or above for 3 d in static aerated piles to be sufficient enough to get rid of pathogens from compost (USEPA, 1999). However, in current study *Salmonella* spp. still survived in compost with 40% moisture for 11, 6, and 4 d at 50, 55, and 60°C, respectively. As a comparison, *E. coli* O157:H7 survived in fresh dairy compost with 40% moisture for 12, 3, and 2 d at 50, 55, and 60°C, respectively (Singh et al., 2011). The longer survival of *Salmonella* spp. in present study could be due to higher resistance of *Salmonella* to heat inactivation in poultry compost than *E. coli* O157:H7 in dairy compost (Osaili et al., 2006, 2007). Apparently, subjecting the results from the present study to current composting guideline may not be sufficient to ensure microbiological safety of the compost. Additionally, composting process under field condition is subjected to many environmental influences, including rainfall, UV radiation and wide temperature fluctuation which can further amplify these variations. Therefore, there is a need to redefine acceptable moisture range for composting process as it can affect temperature distribution within the compost heap thereby affecting pathogen inactivation (Goltaas 1956; Shepherd et al., 2007).

Ammonia released during composting process by indigenous microorganism and through volatilization can cause significant reduction in pathogens present in manure (Himathongkham and Riemann, 1999). Ammonia volatilization is affected by temperature, moisture content, initial ammonia concentration, aeration and pH of poultry litter (Himathongkham and Riemann, 1999; Meisinger and Jokela, 2000; Ogunwande, et al., 2008). Association of ammonia loss with volatilization at higher temperature and

moisture content has been well-documented (Himathongkham and Riemann, 1999; Meisinger and Jokela, 2000, Ogunwande, et al., 2008). In present study initial reduction of *Salmonella* spp. in compost with two levels of moisture content varied from ca 3.3 to 4.6 log CFU g^{-1} 24 h before temperature reached the target composting temperatures of 50, 55, and 60°C. However, in a similar study but in fresh dairy compost initial decline of *E. coli* O157:H7 was less and varied ca 0.9 to1.2 log CFUg^{-1} at 50, 55, and 60°C with the same optimal and suboptimal moisture contents (Singh et al., 2011). The high initial *Salmonella* reduction in poultry compost is probably attributed to high ammonia volatilization during come-up time of composting process since poultry manure contains higher nitrogen content than in dairy manure (Martins and Dewes, 1992). Our data also revealed that initial pathogen decline was higher in compost mix with optimal moisture (50%) and higher temperature. Himathongkham et al. (2000) and Himathongkham and Riemann (1999) have also reported bactericidal effect of ammonia on pathogen during composting. Ammonia exerts bactericidal effect by increasing the pH of the media and inside the cells (Turnbull and Snoeyenbos 1972). Himathongkham et al. (2000) in their study found that *S*. Typhimurium and *E. coli* O157:H7 had decimal reduction time of 12 h in poultry manure at 37°C and 1 to 2 weeks at 4°C. This pathogen reduction also correlated with ammonia loss which was higher in manure held at 37°C than at 20 or 4°C**.** Furthermore, Himathongkham and Riemann (1999) observed ca. 3 and 4 log CFU g^{-1} reduction in *S*. Typhimurium and *E. coli* O157, respectively, in dried poultry manure followed by gassing with ammonia during initial period of 24 h. Aeration (turning frequency) has also been reported to affect nitrogen loss as ammonia (Ogunwande, et al.,

2008). More turning frequency (aeration) increases ammonia loss. In our present study, high initial ammonia loss can also be partially correlated to aeration of the compost by the fan inside the environmental chamber meant for uniform circulation of air.

Conclusions

Overall, our results demonstrated that *Salmonella* spp. survived longer in fresh poultry compost with sub-optimal moisture of 40% than in compost with optimal moisture (50%) during thermophilic composting. High nitrogen content of the poultry compost is an additional factor that contributed to *Salmonella* inactivation through ammonia volatilization during thermal inactivation study. However, this loss of nitrogen as ammonia reduces the value of compost as fertilizer and constitutes an important economic loss. Therefore, it is important to strike a balance between the quality and microbiological safety of compost products by controlling the rate of ammonia volatilization during composting.

Acknowledgments

This research was supported by a grant from the Center for Produce Safety, University of California at Davis.

References

1. Alali, W. Q., Thakur, S., Berghaus, R. D., Martin, M. P. and Gebreyes, W. A. (2010) Prevalence and distribution of *Salmonella* in organic and conventional broiler poultry farms. *Foodborne Pathog. and Dis.* 7, 1363-1371.

2. Bassis, L. (2011) Waste disposal. http://www.umich.edu/~gs265/society/wastedisposal.htm (accessed 29 January 2011)

3. Boersma, L. L. and Murarka, P. I. (1995) Poultry-farm waste management and utilization. In. Waste management and utilization in food production and processing. Task Force Report. Council for Agriculture Science and Technology.

4. Centers for Disease Control and Prevention (CDC) (2010) Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food - 10 states, 2007. *MMWR Morb. Mortal. Wkly. Rep.* 57, 366-370.

5. Ceustermans, A., De Clercq, D., Aertsen, A., Michiels, C., Geeraerd, A., Van Impe, J., Coosemans, J. and Ryckeboer. J. (2007) Inactivation of *Salmonella* Senftenberg strain W 775 during composting of biowastes and garden wastes. *J. Appl. Microbiol.* 103, 53-64.

6. Erickson, M. C., Critzer, F. and Doyle. M. P. (2010) Produce safety project. http://www.producesafetyproject.org/admin/assets/files/PEW-PSP-Composting-Manure-Narrative-v4-2.pdf (accessed 26 June 2011)

7. Erickson, M. C., Liao, J., Ma, L., Jiang, X. and Doyle. M. P. (2009) Inactivation of *Salmonella* spp. in cow manure composts formulated to different initial C:N ratios. *Bioresour. Technol.* 100, 5898-5903.

8. Gotaas, H.R. (1956) Composting – sanitary disposal and reclamation of organic wastes. World Health Organization Monograph Series Number 31. p. 20. Geneva.

9. Himathongkham, S. and Riemann, H. (1999) Destruction of *Salmonella* Typhimurium, *Escherichia coli* O157: H7 and *Listeria monocytogenes* in chicken manure by drying and/or gassing with ammonia. *FEMS Microbiol. Lett.* 171, 179-182.

10. Himathongkham, S., Riemann, H., Bahari, S., Nuanualsuwan, S., Kass, P. and Cliver. D. O. (2000) Survival of *Salmonella* Typhimurium and *Escherichia coli* O157: H7 in poultry manure and manure slurry at sublethal temperatures. *Avian Dis.* 44, 853-860.

11. Hutchison, M. L., Walters, L. D., Avery, S. M. and Moore. A. (2005) Decline of zoonotic agents in livestock waste and bedding heaps. *J. Appl. Microbiol.* 99, 354-362.

12. Islam, M., Doyle, M. P., Phatak, S. C., Millner, P. and Jiang. X. (2004) Persistence of enterohemorrhagic *Escherichia coli* O157:H7 in soil and on leaf lettuce and parsley grown in fields treated with contaminated manure composts or irrigation water. *J Food Prot* 67, 1365-1370.

13. Martin, S. A., McCann, M. A. and Waltman. W. D. (1998) Microbiological survey of Georgia poultry litter. *J. Appl. Poult. Res.* 7, 90-98.

14. Martins, O. and Dewes. T. (1992) Loss of nitrogenous compounds during composting of animal wastes. *Bioresour. Technol.* 42, 103-111.

15. Montrose, M. S., Shane, S. M. and Harrington. K. S. (1985) Role of litter in the transmission of *Campylobacter jejuni*. *Avian Dis.* 29, 392-399.

16. Ogunwande, G. A., Osunade, J. A., Adekalu, K. O. and Ogunjimi. L. A. O. (2008) Nitrogen loss in chicken litter compost as affected by carbon to nitrogen ratio and turning frequency. *Bioresour. Technol.* 99, 7495-7503.

17. Osaili, T., Griffis, C. L., Martin, E. M., Beard, B. L., Keener, A. and Marcy. J. A. (2006) Thermal inactivation studies of *Escherichia coli* O157: H7, *Salmonella*, and *Listeria monocytogenes* in ready-to-eat chicken-fried beef patties. *J Food Prot* 69, 1080-1086.

18. Osaili, T. M., Griffis, C. L., Martin, E. M., Beard, B. L., Keener, A. E. and Marcy. J. A. (2007) Thermal inactivation of *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in breaded pork patties. *J. Food Sci.* 72, 56-61.

19. Sherman, R. (2005) Large-scale organic materials composting. North Carolina Cooperative Extension Service. Pages 1 – 16. Available at: http://www.bae.ncsu.edu/topic/vermicomposting/pubs/ag593.pdf. (accessed 26 June 2011)

20. Shepherd, M. W., Liang, P., Jiang, X., Doyle, M. P. and Erickson. M. C. (2010) Microbiological analysis of composts produced on South Carolina poultry farms. *J. Appl. Microbiol.* 108:2067-2076.

21. Shepherd, M. W., Liang, P., Jiang, X., Doyle, M. P. and Erickson. M. C. (2007) Fate of *Escherichia coli* O157:H7 during on-farm dairy manure-based composting. *J Food Prot* 70, 2708-2716.

22. Singh, R., Kim, J., Shepherd Jr., M. W., Luo, F. and Jiang. X. (2011) Determining thermal inactivation of *Escherichia coli* O157:H7 in fresh compost by simulating early phases of the composting process. *Appl. Environ. Microbiol.* 77, 4126-4135.

23. Sloan, D. R., Kidder, G. and Jacobs. R.D. (2011) Poultry manure as a fertilizer. Institute of Food and Agricultural Sciences, University of Florida, Gainesville http://edis.ifas.ufl.edu/aa205 (accessed 29 January 2011)

24. Terzich, M., Pope, M. J., Cherry, T. E. and Hollinger. J. (2000) Survey of pathogens in poultry litter in the United States. *J. Appl. Poult. Res.* 9, 287-291.

25. Tiquia, S. M. and Tam. N. F. Y. (2002) Characterization and composting of poultry litter in forced-aeration piles. *Process Biochem.* 37, 869-880.

26. Turnbull, P. C. and Snoeyenb.G. H. (1973) Roles of ammonia, water activity, and pH in salmonellacidal effect of long-used poultry litter. *Avian Dis.* 17, 72-86.

27. United States Environmental Protection Agency. (1999) Standards for the use or disposal of sewage sludge (40 CFR Parts 403 and 503). Revised August 4, 1999. Available at: http://www.epa.state.oh.us/portals/35/sludge/503_08_04_99.pdf (accessed 26 June 2011).

28. U.S. Food and Drug Administration. (2001) Bacteriological analytical manual. Available at: http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm063568.htm. (Accessed 15 January 2011).

29. Weatherburn M. W. (1967) Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.* 39, 971-974.

30. Wilkinson, K. G., Tee, E., Tomkins, R. B., Hepworth, G. and Premier. R. (2011) Effect of heating and aging of poultry litter on the persistence of enteric bacteria. *Poult. Sci.* 90, 10-18.

Figure legend

Figure 4.1 Thermal inactivation of *Salmonella* spp. in fresh poultry compost at 50 (a), 55 (b), 60°C (c) with different moisture content and 2 days of come-up time.

40% (——□——), 50% (- - - -○- - - -), Target temperature (————),

Temperature-ramp (··········×··········)

Table 4.1 Changes of ammonia concentration in poultry compost with different moisture content during early phase of composting

Temperature (°C)	Moisture Content (%)	Ammonia concentration (μg/g compost) at following sampling times (h)											
		(-) 48	(-) 24	0	2	4	8	12	24	48	96	168	288
50	40	615.55 ±21.13A[1]	562.34 ±5.40A	450.50 ±10.38A	407.41 ±51.60A	326.55 ±5.77A	ND[2]	416.91 ±5.50A	441.43 ±5.96A	357.33 ±2.49A	ND	ND	285.76 ±7.59A
	50	911.33 ±16.97B	559.27 ±2.92A	530.93 ±37.49B	385.92 ±3.56A	353.04 ±36.69A	ND	453.56 ±7.61B	447.70 ±13.11A	373.65 ±8.22B	ND	ND	233.49 ±7.72B
55	40	754.13 ±69.50A	602.38 ±8.18A	536.49 ±15.85A	473.71 ±7.65A	435.14 ±11.12A	442.43 ±22.58A	ND	443.76 ±14.09A	363.73 ±5.63A	ND	214.03 ±4.85A	ND
	50	1018.86 ±30.14B	588.22 ±13.39A	511.26 ±6.77B	477.03 ±7.46A	403.52 ±5.39B	405.51 ±7.76B	ND	412.08 ±5.93B	337.76 ±8.45B	ND	203.41 ±3.05B	ND
60	40	796.09 ±65.53A	626.32 ±18.09A	570.66 ±10.51A	558.17 ±10.72A	401.78 ±7.22A	409.12 ±4.68A	ND	367.53 ±8.18A	331.88 ±31.93A	178.31 ±7.46A	ND	ND
	50	911.43 ±43.48B	568.09 ±17.81B	532.22 ±17.24B	354.69 ±13.16B	389.89 ±7.34A	376.59 ±6.00B	ND	391.28 ±20.55A	281.66 ±6.24B	185.83 ±5.92A	ND	ND

[1]Mean ammonia amount ± SD with different upper case letter differs significantly ($P < 0.05$) within different moisture content with same temperature.
[2]Not detected.

Figure 4.1a **50°C**

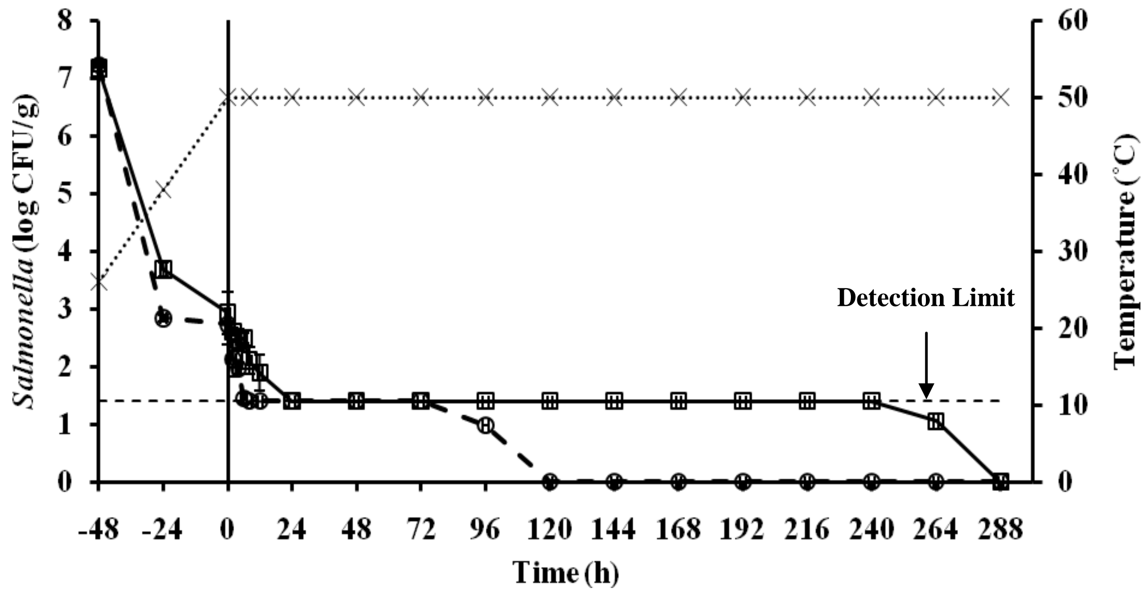


Figure 4.1b **55°C**

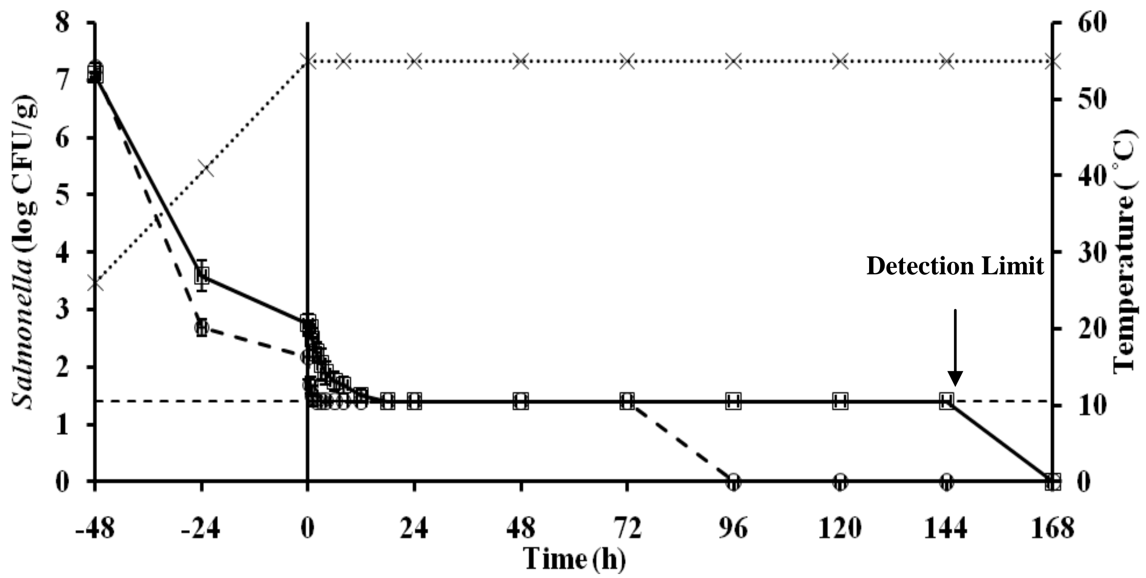
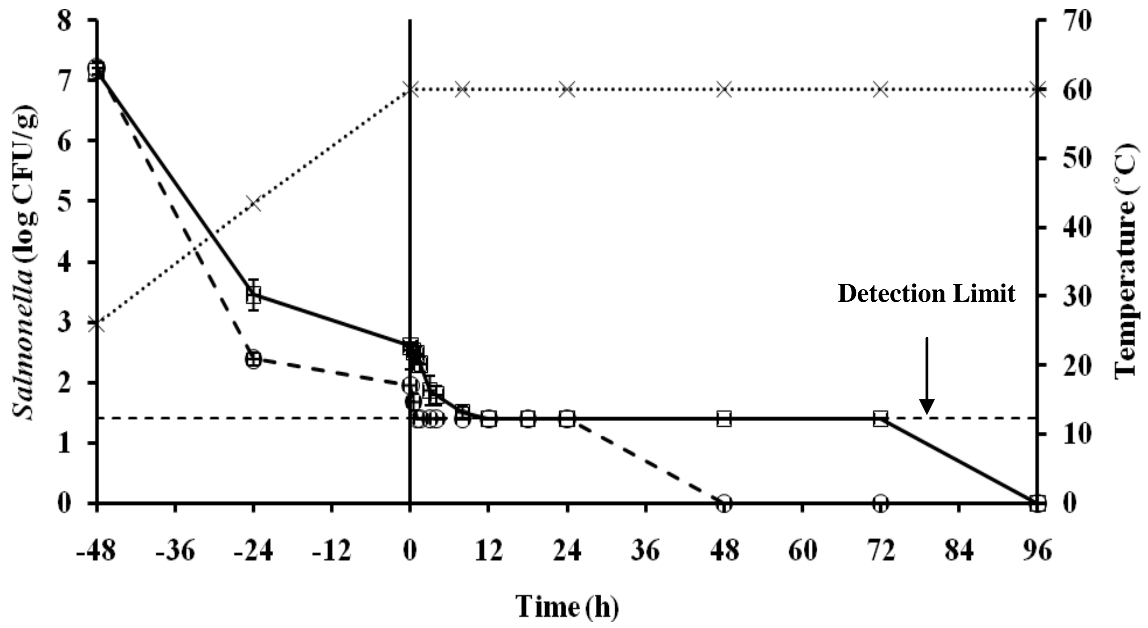


Figure 4.1c **60°C**



CHAPTER FIVE

THERMAL INACTIVATION OF ACID-ADAPTED *ESCHERICHIA COLI* O157:H7 IN FRESH DAIRY COMPOST

Abstract

Three-strain mixture of *Escherichia coli* O157:H7, which was acid-adapted in tryptic soya broth without dextrose (TSB w/o D)(pH 5.0), was inoculated into fresh dairy compost to a final concentration of ca. 10^7 CFU/g. *E. coli* O157 grown in TSB w/o D with neutral pH served as control. The inoculated compost was kept in an environmental chamber which was programmed to raise temperature from room to target temperatures in 2 days, simulating early phase of composting. After temperature reached the target temperatures (50, 55 and 60°C) samples were taken out and analyzed for surviving population by direct plating or enrichment. In fresh dairy compost with 2 days of come-up time, acid-adapted and control *E. coli* O157:H7 survived for 19 and 17 days at 50°C, respectively, and 6 and 4 days for both types of culture at 55 and 60°C, respectively. Overall, pathogen survival was non-significant ($P > 0.05$) between control and acid-adapted cultures at all sampling intervals at all tested temperatures. In finished compost (Black Kow®), the survival of *E. coli* O157 was also non-significant ($P > 0.05$) at most of the sampling times between control and acid-adapted cultures at 55°C. However, the duration of survival for both cultures was short in comparison to that in fresh compost. In fresh compost with short come-up time (15 min) acid-adaptation provided *E. coli* O157 some cross-protection to heat at 55°C upto 30 min of exposure. In saline, acid-adapted *E. coli* O157 was inactivated at 55°C significantly slow as compared to control culture with

short come-up time at 0.5 and 1 h of heat exposure. Our results revealed that cross-protection against heat in *E. coli* O157:H7 due to acid adaptation was demonstrated in saline but lost in fresh dairy compost with 2 days of come-up time during composting. Additionally, type of compost and heating medium can influence the rate of pathogen inactivation at composting temperatures.

Introduction

Composting is an important process for recycling large amount of organic wastes. During composting wastes, such as animal manures and other agricultural wastes, are converted into a stable organic substance through the activity of microorganisms indigenous to the material, creating a product of high nutritive value for crop growth (Sherman, 2005).

Animal waste such as manure is one of the important raw ingredients for composting as the waste treatment method. However, manure has been known to be source of some zoonotic pathogens (Sobsey et al., 2006). High temperature generated during composting process due to metabolic activities of indigenous microflora is critical for pathogen inactivation. According to the United States Environmental Protection Agency (EPA) composting guidelines, a minimum temperature of 55°C for 3 days in aerated static piles or in-vessel systems, or 15 days with 5 turnings in windrow systems is sufficient to sanitize biosolids of pathogens when composted (USEPA, 1999). However, extended survival of pathogens in compost has been reported despite high temperatures are reached during the composting process (Droffner and Brinton 1995; Hutchison et al., 2005). Droffner and Brinton (1995) reported *E. coli* B survival as long as 9 days at 60-70°C in a biowaste (food waste) compost or a waste water sludge compost, and

Salmonella Typhimurium Q survival for at least 9 and 5 days over 60°C in the food biowaste compost and the waste water sludge compost, respectively, in a bench-scale trials. Under field conditions *Salmonella*, *E. coli* O157:H7, and *Listeria* survived for more than 8 days in poultry manure-based compost piles with temperature above 55°C (Hutchison et al., 2005). On the basis of above studies it was suggested that induction of bacterial stress mechanism could be the reason for the extended survival of pathogens beyond recommended time-temperature guidelines. Therefore, inactivation of pathogens during composting process is not as straight forward as it seems to be. A bench-scale study on thermal inactivation of *E. coli* O157:H7, *Salmonella* and *Listeria monocytogenes* in finished dairy compost showed extended survival of the heat-shocked pathogens at 55 and 60°C in comparison to non-stressed cultures (Singh et al., 2010). Besides heat-shock response other stresses such as acid or low nutrients (starvation) are also known to provide protection to microorganisms through cross-protection mechanisms (Buchanan and Edelson, 1999; Rowe and Kirk, 2000). These protection mechanisms may extend pathogen survival in compost, thereby leading to contamination of crop and produce later on.

E. coli O157:H7, one of the residents of cattle gastrointestinal tract has to pass through varying physiological conditions of their host gastric system, which are often governed by animal feeding regimen. The impact of grain feeding on fecal shedding of *E. coli* O157:H7 is another important factor that is under intense scientific discussion. Diez-Gonzalez et al. (1998) reported that grain feeding of ruminants created an acidic environment in the gut of the cattle which in turn leads to selection of acid tolerant

microorganism including *E. coli* O157, which are then shed in animal feces. Presence of volatile fatty acids (VFA) such as lactic, acetic and butyric acid in rumen and inorganic acid (HCl) in abomasums can also induce acid-adaptation of pathogens (Booth et al., 1999 and Rasmussen et al., 1999). Due to the concern of cross protection, acid-adapted pathogen may extend their survival to thermal inactivation. However, no study has been reported in that so far. Therefore, the objective of this study was to investigate the effect of acid-adaption on thermal inativation of *E. coli* O157:H7 in fresh dairy compost by simulating early phases of composting process under controlled environmental conditions.

Materials and Methods

Compost preparation. Fresh dairy compost was made by mixing manure (collected from LaMaster dairy farm, Clemson University) with saw dust and dried hay in a ratio to give compost mixture with optimal carbon to nitrogen (C:N) ratio as 24:1. The freshly made compost was analyzed by the Agricultural Service Laboratory (Clemson University, Clemson, SC) for the C:N ratio. The compost mix thus made was stored under refrigeration condition and used within 2 weeks.

One day before the experiment, the moisture content of the compost was adjusted to ca. 50 % by drying under an air flow supreme fume hood (Kewaunee Scientific Equipment corp., Michigan). The moisture content of the compost was measured by a Moisture Analyzer IR-35 (Denver Instrument, Germany).

Culture preparation. *E. coli* O157:H7 strains F06M-0923-21 (spinach outbreak strain from California Dept. of Health), F07M-020-1(Taco John's outbreak strain from

California Dept. of Health) and avirulent strain B6914 (Dr. Pina Fratamico, Eastern Regional Research Center, USDA-ARS) were used in the study. All the strains were induced to rifampin resistance as described previously (Singh et al., 2010)

Three *E. coli* O157:H7 strains were grown separately in 10 ml tryptic soy broth (Difco, Becton, Dickinson & company, Sparks, MD) containing 100 µg/ml of rifampin (TSB-R) at 37°C. The overnight grown culture were then propagated in 20 ml each of TSB-R without Dextrose (TSB-R w/o D) (pH 7.0) (Difco, Becton, Dickinson & company, Sparks, MD) by inoculating a loopful of culture and incubated overnight at 37°C (control culture). For acid-adaptation each strains was grown at 37°C in TSB-R w/o D with pH 5.0 (adjusted with 1 N HCl). After overnight incubation cultures were sedimented by centrifugation at 5,000 x g for 10 min, washed twice with sterile 0.85% saline and resuspended to an optical density (OD_{600}) of ca. 0.6 to 0.7 (ca. 10^9 CFU/ml). Equal volume of three *E. coli* O157:H7 cultures were mixed as inocula for the following experiments.

Inoculation of fresh dairy compost. Both control and acid-adapted cultures of *E. coli* O157:H7 were prepared as described above, and inoculated into 2.8 kg of fresh compost with 50% moisture at a final concentration of ca. 10^7 CFU/g separately, with a spray nozzle sanitized with 75% ethanol and rinsed with sterile distilled water. The inoculated compost was mixed continuously for 10 min on sterile polypropylene trays by hand wearing sterile gloves. The inoculated levels of the pathogens in compost were enumerated by plating serial dilutions of the compost in 0.85% saline on tryptic soy agar (BD, MD) containing 100 µg/ml of rifampin (TSA-R). The level of background

microflora in fresh dairy compost was also enumerated by plating serially diluted compost on TSA.

Thermal inactivation study. The inoculated fresh compost (ca. 100 g) was packed in a Tyvek pouch (size 5.25 in by 10 in., SPS Medical, Rush, NY) and spread evenly into a thin layer (ca. 1 cm in depth). The pouches with compost were then kept in a single layer on the shelf of an environmental chamber (Model No. EC2047N**,** Thermo Scientific, Barnstead International, Dubuque, IA) with the humidity set at ca. 70% to mimic conditions inside of the composting heap. The temperatures of 50, 55 and 60°C were used for the thermal inactivation study, which were monitored constantly using type-T thermocouples (DCC Corporation, NJ) with one cord inserted inside the compost pouch and others were kept in the chamber. For the inactivation study the temperature rise of the environmental chamber was programmed to ramp step-wise from room temperature (ca. 26°C) to the target temperature in 2 days (representing normal temperature rise during composting process) and then maintained at that temperature.

The sample bags were removed at predetermined time intervals and cooled immediately in an ice water bath after the temperature of the compost inside the bag reached the target temperature. A compost sample of 25 g was taken and mixed with 225 ml of universal pre-enrichment broth (UPB; Acumedia manufacturers Inc., Lansing, MI) and homogenized in a stomacher bag. The homogenized samples were serially diluted in sterile 0.85% saline and plated in duplicate on TSA-R to analyze the surviving population of *E. coli* O157. A 250 µl of sample was spiroplated (Autoplate® 4000, Spiral Biotech, MD) to increase the detection limit to 10 CFU/g.

The samples, negative for *E. coli* O157:H7 after direct-plating, were pre-enriched in UPB followed by overnight selective enrichment in TSB-R at 37°C, and then streaked on TSA-R and Sorbitol MacConkey agar with rifampin (SMAC-R). The detection limit for enrichment was 0.02 CFU/g. Duplicate or triplicate trials were conducted for each experiment.

The presumptive colonies of the *E. coli* O157:H7 on the plates were confirmed using an immuno-latex agglutination test (Oxoid, Hampshire, UK).

Similar thermal inactivation study only at 55°C with 2 days of come-up time was also conducted with commercial dairy compost (Black Kow, Black Gold Compost Co., Oxford, Fla.) purchased from a local store with moisture content adjusted to 50% for comparing effect of different types of compost. Another trial at 55°C was also done to compare the effect of short come-up time (15 min) on thermal inactivation of acid-adapted and control cultures in the fresh dairy compost as per method discussed elsewhere (Singh et al., 2010). In order to verify the influence of heating medium on the thermal inactivation of acid-adapted and control cultures, the individual strains of both types of cultures were thermally inactivated in sterile 0.85% saline at 55°C in circulating water bath (HAAKE, Type 003-3332, Germany) in glass test tube and then plated on TSA-R after serial dilution in saline.

Statistical analysis. To compare the survival difference in bacterial populations with or without acid-adaptation plate count data were converted to $\log_{10}$ values, and subjected to analysis of variance with a test criterion (F statistic) and type I error

controlled at $P = 0.05$. The Tukey's multiple comparison procedure of the Statistical Analysis System (2001, SAS, Cary, N.C.) was used.

Results

The effect of acid-adaption on thermal resistance of *E. coli* O157:H7 was studied in fresh dairy compost and compared under laboratory conditions. The thermal inactivation studies were performed inside an environmental chamber by simulating the early phase of optimal composting process (2 days of come-up time). The level of background microflora in fresh dairy compost was 9.28±0.06 CFU/g.

Cross-protection to heat due to acid-adaptation in saline

To determine if acid- adaption in *E. coli* O157:H7 results in cross protection against heat, three strains of both control and acid-adapted cultures were suspended in 0.85% of saline and exposed to temperature of 55°C for 1 h. The acid-adapted cultures of all the three strains survived significantly better ($P < 0.05$) than the control culture at almost all sampling times (Table 5.1).

Thermal inactivation in fresh dairy compost

At 50°C, acid-adaptation of *E. coli* O157:H7 cultures survived slightly longer than the control culture in dairy compost when inactivated in environmental chamber (Table 5.2). The surviving population of control and acid-adapted cultures could be detected by direct plating until 24 h and after that samples were positive by enrichment

only until 17 and19 days, respectively. The decline in the population of both types of culture was gradual at 24 h before the temperature reached the target level with ca. 1.47 log reductions in control and acid-adapted cultures. There were ca. 4.21 and 4.44 log reductions in control and acid-adapted cultures, respectively, when the temperature in the compost reached the set level (0 h). The decline after the temperature inside the compost reached the target was gradual in both control and acid-adapted cultures between times 0 -12 h of the sampling (Table 5.2). The pathogen survival was not significantly different ($P > 0.05$) at most of the sampling time between control and acid-adapted cultures.

At 55°C, both control and acid-adapted cultures survived for 144 h (6 days) as detected by enrichment (Table 5.3). The decline in the population of control and acid-adapted cultures were ca. 4.65 and 5.24 log reductions when temperature inside the compost reached the target level (0 h). The decline in the population of *E. coli* O157 in control and acid-adapted cultures were gradual once the temperature reached the set level. The surviving population of *E. coli* O157 in control and acid-adapted cultures were detected by plating until 12 and 24 h, respectively, followed by detection by enrichment after that. The survival difference between control and acid-adapted cultures were not significantly different ($P > 0.05$) at almost all the sampling time.

At higher temperature of 60°C, inactivation in the population of *E. coli* O157 was quicker in comparison to 50 and 55°C with populations of control and acid-adapted cultures surviving for 96 h as detected by enrichment (Table 5.4). The decline in the population of control and acid-adapted cultures were ca. 5.03 and 5.10 log reductions, respectively, when temperature of the compost reached set value (0 h). Although decline

in control and acid-adapted cultures were maximum during 2 days of come-up time but after that decline was slow and gradual. The surviving population of *E. coli* O157 in control and acid-adapted cultures were detected by plating until 6 h of the sampling time followed by detection by enrichment. The difference in the survival of control and acid-adapted cultures were not significantly different ($P > 0.05$) at most of the sampling time.

Thermal inactivation with short come-up time

In order to compare the effect of come-up time on thermal inactivation of acid-adapted *E. coli* O157:H7 during composting. A trial with short come-up time (15 min) was conducted at 55°C in fresh dairy compost. The control and acid-adapted cultures survived for 6 and 2 h, respectively (Table 5.5). During come-up time of 15 min the population of control and acid-adapted cultures were reduced ca. 0.73 and 0.69 logs, respectively. At 55°C, acid-adapted culture was inactivated slower ($P < 0.05$) at sampling times of 0.25 and 0.5 h than the control. However, after 1 h at 55°C, acid-adapted culture was inactivated more rapidly ($P < 0.05$) than the control.

Thermal inactivation in finished compost

In order to compare above results from fresh compost another similar trial was performed at 55°C only in finished dairy compost (purchased from local store) with 2 days of come-up time. The pathogen in the finished compost survived for only 24 h in both control and acid-adapted cultures as detected by enrichment (Table 5.6). During 2 days of come-up time there was a slight decline in the surviving population of both

control and acid-adapted cultures, within 24 h, followed by a rapid decrease of ca. 5.53 and 5.14 logs of control and acid-adapted culture populations, respectively, when the temperature reached the set value. At target temperature of 55°C, there was a gradual reduction in control and acid-adapted cultures. The reduction was not significantly different ($P > 0.05$) at most of the sampling times.

Moisture changes in compost during thermal inactivation study

In this study moisture loss from dairy compost with 2 days of come-up time at all the three temperatures were ca. 35.5 to 39.9 % when compost temperature reached the set value (0 h) for both types of treatment (Table 5.7). The loss was slightly more at higher temperature than at the lower temperature. In fresh compost with short come-up time (15 min) the moisture loss was comparatively slow within 24 h of the sampling with ca. loss of 17.3 and 18.6 % in control and acid-adapted cultures, respectively. The loss of moisture in commercial finished compost was even higher than the fresh compost with control and acid-adapted cultures losing ca. 41.6 and 42.6 %, respectively when temperature inside the compost reached 55°C.

Discussion

Pathogen inactivation during composting process is the major concern for the microbial safety of the compost. High temperatures reached during composting process are critical to pathogen inactivation. However, microbial cell has ability to adapt to a stress that may provide protection (cross-protection) to an unrelated stress (Rodriguez-

Romo and Yousef, 2005). This adaptive mechanism in microorganisms, especially, pathogens, can influence composting process outcome as well. Thus, microbial populations developing cross protection to high temperature due to acid-adaption is of practical importance for composting process. There is sufficient information available in scientific literature on induction of cross protective mechanism against heat stress in pathogens and other microorganisms in response to acid-adaption in culture media and food (Ryu and Beuchart, 1998, 1999).

In this study we investigated effect of cross protection on thermal inactivation of acid-adapted *E. coli* O157 in saline or in compost by simulating early phase of composting process. Our results indicated that in saline, acid-adaptation resulted in extended survival ($P < 0.05$) of *E. coli* O157:H7 at 55°C for 1 h, however in fresh compost, this cross protection was not observed at higher composting temperatures of 55 and 60°C. Similar to these results, Singh et al. (2006) also reported lower cross protection in acid-adapted *E. coli* O157:H7 to heat at 62 and 65°C in ground beef. On the contrary acid-adapted *E. coli* O157:H7 exhibited higher *D*-values than non-adapted cultures at 52°C in fruit juice (Ryu and Beuchat, 1998, 1999). Buchanan and Edelson (1999) also reported higher cross protection in acid-adapted *E. coli* O157:H7 when thermally inactivated at 58°C in lab media, milk, chicken broth, and apple juice. Based on their study and findings reported elsewhere, Singh et al. (2006) concluded that acid-adaption might increase cross protection to heat in liquid food, but the same effect is lost in solid food matrix, which is in agreement with our results in saline and in compost matrix.

Microorganisms adaptation to stress is a mechanism under genetic control which is activated on exposure to sub-lethal stresses, such as heat, acid, starvation etc (Chung et al., 2006). Stress-adapted microorganisms retain this stress-induced mechanisms for countering further lethal stresses for a period of time until they return to their normal physiological state, which depends upon their growth conditions and storage after stress or may even vary from strain to strain (Leenanon and Drake 2001; Mckellar and Knight 1999; Murano and Pierson, 1992; Rowe and Kirk, 2000). Lin et al. (1996) found that acid-adapted mechanism in enterohaemorrhagic *E. coli* persisted for 1 month under refrigeration conditions. In another study acquired resistance (thermotolerance) of *L. monocytogenes* was lost within 1 h when stressed cells were immediately returned to the growth temperature of 35°C, suggesting that induction of stress was not long-lived (Bunning et al., 1990). In their study, Singh et al. (2006) stored ground beef inoculated with non adapted and acid-adapted *E. coli* O157:H7 at 4 and -20°C for 28 and 120 days, respectively, before subjecting them to thermal inactivation. Therefore, loss of cross protection in *E. coli* O157:H7 to heat in their study could also be due to prolong storage of acid-adapted culture at low temperature resulting in loss of acid-adaptation response. In our study we did see that acid-adaptation of *E. coli* O157 was able to provide protection against heat in saline solution, and in fresh compost when inactivated immediately (short come-up time) but the protection was for a short period of time which lasted for 30 min. However, acid-adaption of *E. coli* O157:H7 was unable to cross protect them against heat during composting with 2 days of come-up time suggesting loss of acid-adaptation response during long come-up time. Therefore, based on these

observations we can conclude that cross protection due to acid-adaptation of *E. coli* O157:H7 in dairy compost was induced but was for short duration of time.

In addition to induction of acid-adaptation system in *E. coli* O157, other virotypes of *E. coli* such as enteroinvasive and enteropathogenic *E. coli* have been found to be naturally more acid adaptive than nonpathogenic strains of *E. coli* indicating natural acid-adaptation may minimize the initial artificial acid-adaptation (Gorden and Small, 1993). This property, particularly in *E. coli* O157 is of great importance because of its low infective dose, which means that mere survival of the pathogen during composting process may be all that is needed to cause further contamination of produce or water.

It is well established that induction of heat-adaption or heat-shock in microorganism provides protection against lethal temperature (Bunning et al., 1990; Murano and Pierson, 1992; Singh et al., 2010). In present study acid-adaptation does not seem to provide additional protection to the acid-adapted culture during composting with 2 days of come-up time. However, both control and acid-adapted cultures survived for long duration at all the three temperatures used in the study. This longer survival of the pathogen especially when temperature rise of the composting process was gradual (2 days) could possibly be due to induction of heat-shock response (Singh et al., 2011). In real-world composting process under field conditions the dynamics of temperature rise in compost heap is similar to what we have simulated in lab. So, these results again underline the importance of temperature evolution during composting process and the possibilities of microorganism surviving longer due to heat-adaptation. These results also points out to the fact that previous acid-adaptation of microbial communities does not

have much impact on their inactivation by heat as those stresses are either lost during the mesophilic phase of the composting process during come-up time or induction of heat-shock response over power any other previous stress adaptations.

Besides temperature, composting process is also affected by various other factors such as moisture content, carbon: nitrogen ratio (C:N), pH, aeration and heap size (Sherman 2005). All these factors make pathogen inactivation during compost a very complex process. Initial moisture content of the compost mix is one of the important factors that can affect the rate of pathogen inactivation during composting process by affecting the metabolic activity of the microbial community present in the compost heap (Singh et al., 2011). In present study, moisture loss during the 2 days of come-up time would have been additional contributing factor for stress adaption of the pathogen during the trial. Which can be supported by the fact that *E. coli* O157:H7 was inactivated faster in dairy compost with short come-up time. Singh et al (2011) in their study also found that compost samples at the end of the long mesophilic phase (5 days of come-up time) lost more moisture than the compost samples with optimal mesophilic phase (2 days of come-up time) leading to prolonged survival of *E. coli* O157 in compost with long mesophilic phase than optimal mesophilic phase of composting.

Conclusions

Our results clearly demonstrated that in saline solution, acid-adaptation of *E. coli* O157:H7 provide cross protection to thermal inactivation, however, there were no difference in the survival of control and acid-adapted pathogen in fresh dairy compost

that simulated optimal mesophilic phase (2 days of come-up time) of the composting process. Thus, the acid-adaptation of pathogen only provides short term cross-protection when they were exposed to heat immediately (short come-up time) depending upon the type of heating medium. The acid-adaptation of the pathogen before composting plays minimal role in providing cross-protection to thermal inactivation and was lost during 2 days of come-up time.

Acknowledgements

This research was supported by a grant from the Center for Produce Safety, University of California at Davis.

References

1. Booth, I. R., F. Thomson-Carter, P. Carter, S. Jordan, S. Park, L. Malcolm and J. Glover. 1999. Acid tolerance of *Escherichia coli*-the sting in the tail? p. 27-38. *In:* Stewart, C. S. and H. J. Flint (ed.), *Escherichia coli* O157 in farm animals. CABI Publishing, Wallingford, UK.

2. Buchanan, R., S. Edelson. 1999. Effect of pH-dependent, stationary phase acid resistance on the thermal tolerance of *Escherichia coli* O157:H7. *Food Microbiol.* 16:447-458.

3. Bunning, V. K., R. G. Crawford, J. T. Tierney, and J. T. Peeler. 1990. Thermotolerance of *Listeria monocytogenes* and *Salmonella* Typhimurium after sublethal heat shock. *Appl. Environ. Microbiol.* 56:3216-3219.

4. Chung, H. J., W. Bang, and M. A. Drake. 2006. Stress Response of *Escherichia coli*. *Compr. Rev. Food Sci. Food Safety* 5:52-64.

5. Diez-Gonzalez, F., T. R. Callaway, M. G. Kizoulis, and J. B. Russell. 1998. Grain feeding and the dissemination of acid-resistant *Escherichia coli* from cattle. *Science* 281:1666-1668.

6. Droffner, M. L., W. F. Brinton. 1995. Survival of *E. coli* and *Salmonella* populations in aerobic thermophilic composts as measured with DNA gene probes. *Zentralbl. Hyg. Umweltmed.* 197:387-397. [Abstract Pubmed]

7. Gorden, J., P. L. C. Small. 1993. Acid resistance in enteric bacteria. *Infect. Immun.* 61:364-367.

8. Hutchison, M. L., L. D. Walters, S. M. Avery, and A. Moore. 2005. Decline of zoonotic agents in livestock waste and bedding heaps. *J. Appl. Microbiol.* 99:354-362.

9. Leenanon, B., D. Elhanafi, and M. A. Drake. 2003. Acid adaptation and starvation effects on shiga toxin production by *Escherichia coli* O157:H7. *J. Food Prot.* 66:970-977.

10. Lin, J. S., M. P. Smith, K. C. Chapin, H. S. Baik, G. N. Bennett, and J. W. Foster. 1996. Mechanisms of acid resistance in enterohemorrhagic *Escherichia coli*. *Appl. Environ. Microbiol.* 62:3094-3100.

11. McKellar, R. C., K. P. Knight. 1999. Growth and survival of various strains of enterohemorrhagic *Escherichia coli* in hydrochloric and acetic acid. *J. Food Prot.* 62:1466-1469.

12. Murano, E. A., M. D. Pierson. 1992. Effect of heat-shock and growth atmosphere on the heat-resistance of *Escherichia coli* O157:H7. *J. Food Prot.* 55:171-175.

13. Rasmussen,M.A., T. L. Wickman, W. C. Cray, Jr and T. A. Casey. 1999. *Escherichia coli* O157:H7 and the rumen environment, p. 39-50. *In:* Stewart, C. S. and H. J. Flint (ed.), *Eshcherichia coli* O157 in farm animals. CABI Publishing, Wallingford, UK.

14. Rodriguez-Romo, L and A. Yousef. 2005. Cross –protective effect of bacterial stress, p. 128-151. *In* M**.** Griffiths (ed), Understanding pathogen behaviour: virulence, stress response and resistance. CRC Press, Washington D.C.

15. Rowe, M. T., R. B. Kirk. 2000. Effect of nutrient starvation on the resistance of *Escherichia coli* O157:H7 to subsequent heat stress. *J. Food Prot.* 63:1745-1748.

16. Ryu, J. H., L. R. Beuchat. 1998. Influence of acid tolerance responses on survival, growth, and thermal cross-protection of *Escherichia coli* O157:H7 in acidified media and fruit juices. *Int. J. Food Microbiol.* 45:185-193.

17. Ryu, J. H., L. R. Beuchat. 1999. Changes in heat tolerance of *Escherichia coli* O157:H7 after exposure to acidic environments. *Food Microbiol.* 16:317-324.

18. Sherman, R. 2005. Large-scale organic materials composting. North Carolina Cooperative Extension Service. Pg.1 – 16. Available at: http://www.bae.ncsu.edu/topic/vermicomposting/pubs/ag593.pdf. (Accessed April 2011)

19. Singh, M., S. M. Simpson, H. R. Mullins, and J. S. Dickson. 2006. Thermal tolerance of acid-adapted and non-adapted *Escherichia coli* O157:H7 and *Salmonella* in ground beef during storage. *Foodborne Pathog. Dis.* 3:439-446.

20. Singh, R., J. Kim, M. W. Shepherd Jr., F. Luo and X. Jiang. 2011. Determining thermal inactivation of *Escherichia coli* O157:H7 in fresh compost by simulating early phases of the composting process. *Appl. Environ. Microbiol*. 77:4126-4135.

21. Singh, R., X. Jiang, and F. Luo. 2010. Thermal inactivation of heat-shocked *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in dairy compost. J. Food Prot. 73:1633-1640.

22. Sobsey, M. D., L. A. Khatib, V. R. Hill, E. Alocilja, and S. Pillain. 2006. Pathogens in animal wastes and the impacts of waste management practices on their survival, transport and fate, p. 609-666. *In:* J. M. Rice, D. F. Caldwell and F. J. Humenik (ed.), Animal agriculture and the environment: National Center for Manure and Animal Waste Management White Papers. st. Joseph, Michigan.

23. United States Environmental Protection Agency. 1999. Standards for the use or disposal of sewage sludge (40 CFR Parts 403 and 503). Revised August 4, 1999. Available at: http://www.epa.gov/EPAWATER/1999/August/Day-04/w18604.htm> (Accessed June, 2010).

Table 5.1 Thermal inactivation of acid-adapted *E. coli* O157:H7 at 55°C in saline

		E. coli O157:H7 Strains		
Treatment	**Time (h)**	**B6914**	**F06M-0923-21**	**F07M-020-1**
Control	0	6.95±0.12A[1]	6.97±0.23A	7.01±0.13A
	0.25	5.06±0.83A[2]	4.76±1.11A	5.84±0.22A
	0.5	1.12±1.29A	1.95±0.06A	2.73±0.62A
	1	0.38±0.45A	NDA[2]	NDA
Acid-adapted	0	7.11±0.08A	7.08±0.20A	7.23±0.15A
	0.25	6.14±0.42A	6.62±0.12B	6.61±0.54B
	0.5	4.09±0.21B	5.68±0.15B	5.98±0.48B
	1	3.24±0.07B	2.65±0.08B	3.27±0.19B

[1]Bacterial population (CFU/ml); Mean log CFU± SD with different upper case letter within column differs significantly ($P < 0.05$) between two treatments at the same heat exposure time for each strain.
[2] ND; Not detected by direct plating.

Table 5.2 Thermal inactivation of acid-adapted (AA) *Escherichia coli* O157:H7 in fresh dairy compost at 50°C.

Treatment	*E. coli* O157:H7 population (log CFU/g) at 50°C with heating time (h)													
	(-) 48	(-) 24	0	2	4	6	8	12	24	168	264	312	408	456	504
Control	7.14±0.08A[1]	5.67±0.09A	2.93±0.31A	2.75±0.27A	2.41±0.41A	2.18±0.42A	2.49±0.13A	1.43±0.50A	1.30±0.46A	+[2]	+	+	+	-[3]	-
AA	7.17±0.05A	5.70±0.09A	2.73±0.46A	2.35±0.87A	2.50±0.27A	2.23±0.54A	2.62±0.36A	1.23±0.31A	1.26±0.38A	+	+	+	+	+	-

[1]Mean log CFU± SD with different upper case letter differs significantly ($P<0.05$) between two treatments at that sampling interval.

[2]Positive by enrichment.

[3] Negative by enrichment.

Table 5.3 Thermal inactivation of acid-adapted (AA) *Escherichia coli* O157:H7 in fresh dairy compost at 55°C.

Treatment	*E. coli* O157:H7 population (log CFU/g) at 55°C with heating time (h)													
	(-) 48	(-) 24	0	0.5	1	2	3	4	6	12	24	96	144	168
Control	7.07±0.07A[1]	5.18±0.65A	2.42±0.36A	2.43±0.24A	1.67±0.61A	1.96±0.68A	2.04±0.32A	1.83±0.67A	1.61±0.43A	1.11±0.32A	+[2]	+	+	-[3]
AA	7.17±0.05B	5.48±0.41A	1.93±0.63A	2.38±0.31A	2.10±0.54A	1.94±0.70A	2.14±0.19A	1.95±0.57A	1.73±0.41A	1.40±0.45A	1.39±0.72	+	+	-

[1]Mean log CFU± SD with different upper case letter differs significantly ($P<0.05$) between two treatments at that sampling interval.

[2]Positive by enrichment.

[3] Negative by enrichment.

Table 5.4 Thermal inactivation of acid-adapted (AA) *Escherichia coli* O157:H7 in fresh dairy compost at 60°C.

	***E. coli* O157:H7 population (log CFU/g) at 60°C with heating time (h)**													
Treatment	**(-) 48 h**	**(-) 24 h**	**0**	**0.5**	**1**	**1.5**	**3**	**4**	**6**	**12**	**24**	**72**	**96**	**120**
Control	7.08±0.10A[1]	4.69±0.44A	2.05±0.32A	1.85±0.74A	1.87±0.52A	1.76±0.51A	1.83±0.70A	1.66±0.71A	1.29±0.42A	+[2]	+	+	+	-[3]
AA	7.06±0.11A	4.7±0.46A	1.96±0.30A	1.65±0.63A	2.03±0.38A	1.72±0.36A	1.82±0.45A	1.80±0.39A	1.19±0.36A	+	+	+	+	-

[1]Mean log CFU± SD with different upper case letter differs significantly (P<0.05) between two treatments at that sampling interval.

[2]Positive by enrichment.

[3] Negative by enrichmen.

Table 5.5 Thermal inactivation of acid-adapted (AA) *Escherichia coli* O157:H7 in fresh dairy compost at 55°C with short come-up time.

	***E. coli* O157:H7 population (log CFU/g) at 55°C with heating time (h)**									
Treatment	**(-0.25)**	**0**	**0.25**	**0.5**	**1**	**2**	**3**	**4**	**6**	**12**
Control	7.2±0.09A[1]	6.47±0.06A	5.37±0.05A	3.39±0.30A	2.97±0.51A	+[2]	+	+	+	-[3]
AA	7.17±0.09A	6.48±0.08A	5.87±0.13B	4.23±0.41B	1.92±0.65B	+	-	-	-	-

[1]Mean log CFU± SD with different upper case letter differs significantly (P<0.05) between two treatments at that sampling interval.

[2]Positive by enrichment.

[3] Negative by enrichment.

Table 5.6 Thermal inactivation of acid-adapted (AA) *Escherichia coli* O157:H7 in finished commercial dairy compost at 55°C.

	***E. coli* O157:H7 population (log CFU/g) at 55°C with heating time (h)**											
Treatment	**(-) 48**	**(-) 24**	**0**	**0.5**	**1**	**2**	**3**	**4**	**6**	**12**	**24**	**72**
Control	7.27±0.03A[1]	7.14±0.07A	1.74±0.34A	2.25±0.63A	1.15±0.28A	2.09±0.68A	1.88±0.95A	1.49±0.45A	+A	1.42±0.59A	+[2]	-[3]
AA	7.29±0.09A	7.14±0.07A	2.15±0.79A	2.40±0.70A	1.67±0.68A	1.93±0.70A	2.10±1.03A	1.29±0.42A	1.62±0.69B	1.08±0.21A	+	-

[1]Mean log CFU± SD with different upper case letter differs significantly ($P<0.05$) between two treatments at that sampling interval.

[2]Positive by enrichment.

[3] Negative by enrichment.

Table 5.7 Moisture contents of the compost mix for different trials.

	Fresh dairy compost exposed to temperature (°C)								Finished dairy compost	
	50		55		60		55[1]		55	
Time (h)	**Control**	**AA**	**Control**	**AA**	**Control**	**AA**	**Control**	**AA**	**Control**	**AA**
-48	50.64±0.32	50.62±0.31	50.2±0.10	50.12±0.09	50.5±0.53	50.8±0.60	-[2]	-	50.68±0.31	51.05±0.34
-24	44.08±0.65	43.54±0.49	44.25±2.57	42.22±0.88	40.87±0.59	40.84±0.56	-	-	40.72±2.25	39.02±1.33
0	15.15±2.81	13.07±0.94	12.61±0.86	12.91±0.48	11.09±0.39	10.91±0.30	50.81±0.36	50.77±0.56	9.07±0.94	8.47±1.00
4	25.87±2.59	22.08±1.16	11.71±1.02	11.745±0.68	9.37±0.98	8.61±0.25	46.32±2.46	45.23±2.44	7.21±0.67	7.43±1.57
24	6.89±0.45	6.87±0.14	10.58±1.76	8.15±1.37	6.79±0.40	6.67±0.27	33.52±2.03	32.19±2.14	4.39±0.86	3.74±0.84
End[3]	6.66±0.34	6.91±1.31	7.39±0.37	6.92±0.36	5.98±0.11	5.85±0.24	-	-	3.72±0.54	3.67±0.45

[1] Trial with short come-up time (15 min), initial moisture in Control and AA was 51.05±0.23 and 50.71±0.56, respectively.

[2] No sample available at that time for the experiment.

[3] Last sample for the experiment when the detection was negative by enrichment.

CHAPTER SIX

EPRESSION OF STRESS AND VIRULENCE GENES IN *ESCHERICHIA COLI* O157:H7 IN FRESH DAIRY COMPOST

Abstract

This study was to understand the mechanisms for pathogen survival during composting. A two- step real-time PCR assay was used to evaluate expression of stress and virulent genes in *E. coli* O157:H7 heat-shocked in compost. *E. coli* O157:H7 (strain F07-020-1) was inoculated in autoclaved fresh dairy compost which was heat-shocked at 47.5°C for 10 min in water bath. To serve as medium control, heat-shock of the pathogen was also conducted in tryptic soy broth (TSB). In compost heat-shock genes, *clpB*, *dnaK, groEL*, and alternative sigma factor (*rpoH*) were all up regulated significantly ($P < 0.05$). There was no significant ($P > 0.05$) difference in the expression of trehalose synthesis genes. Virulent genes such as *stx1* and *fliC* were up-regulated while rest of the genes was down-regulated with no significant difference ($P > 0.05$). In toxin-antitoxin system, toxin genes, *mazF*, *hipA*, and *yafQ* were up-regulated with no significant ($P > 0.05$) difference, whereas antitoxin gene *dinJ* was up-regulated with level of expression significantly ($P < 0.05$) different. Most of the other antitoxin genes were down regulated. In broth as the heat-shock medium, all heat-shock genes were up-regulated with relative fold change significantly ($P < 0.05$) different. There was no significant ($P > 0.05$) change in trehalose synthesis genes in broth medium either. Except *eaeA*, rest of the virulent genes was down regulated with no significant ($P > 0.05$) change. Majority of the toxin-antitoxin genes were down-regulated with relative fold change in toxin gene *hipA* and *chpB* only

significantly ($P < 0.05$) different. Our results suggest that induction of heat-shock response in pathogens play important role in providing protection to pathogens against lethal temperature during composting. Activation of toxin-antitoxin (TA) system in addition to heat-shock response may also be supporting pathogen survival in compost as parallel mechanism.

Introduction

In the United States composting of animal waste on farm is often and increasingly being used as a method to reduce environmental pollution and promote sustainable agriculture practices (Kashmanian and Rynk, 1996; USDA 2009). Although, elevated temperatures during composting due to metabolic activity of the microorganisms is not the sole factor but is considered as the most impactful factor that brings about inactivation of pathogens that may be present in animal waste (Shepherd 2007). However, improperly composted manure is a potential source of pathogens when used as a soil amendment. Several foodborne illnesses have been associated with produce contaminated with improperly treated waste (CDC 2006, Chapman et al., 1996). There has also been report showing extended survival of pathogens during composting despite high temperatures reached during the process (Hutchison et al., 2005; Singh et al., 2011). For example our recent study reported *E. coli* O157:H7 survival at 55°C for 5 days in optimal and sub-optimal compost mixes which exceeded the EPA requirement of composting at 55°C for 3 days in aerated static pile. Hutchison's field study demonstrated, *Salmonella*, *E. coli* O157:H7, and *Listeria* survived for more than 8 days in poultry manure based compost pile with temperature of the composting process above

55°C. These results suggest other factors that may contribute to extended survival of pathogens during composting process.

Heat generated during composting process is the primary factors responsible for microbial inactivation, however, heat-adaptation of pathogen during the mesophilic phase of the composting process can induces stress response, thereby extending their survival (Singh et al., 2010). Recently, Singh et al. (2010) reported that heat-shocked *E. coli* O157:H7, *Salmonella* and *L. monocytogenes* survived better than non heat-shocked cultures at 50, 55 and 60°C. Physiologically, microbial cells have ability to respond to different types of stress such as heat, acid, starvation etc. by mounting a stress response which provides protection against the same or different stress (Murano et al., 1992; Rowe and Kirk, 2000; Singh et al., 2006). Besides, heat-shock response in microbial communities during composting, influence of other mechanisms cannot be ruled out either (Christensen et al., 2003; Pedersen et al., 2002). Bacterial toxin-antitoxin (TA) systems have also been hypothesized to play role in general stress response (lewis, 2010).

Microbial cells that enter dormant and non dividing state are termed as persister cells. These cells represent small subpopulation of cells and arise spontaneously (Lewis, 2010). Also, these cells are just the phenotypic variant of the wild type and not mutant since upon reinoculation produce a culture similar to that of wild type (Keren et al, 2004). TA systems are believed to induce a temporary and reversible reduction or cessation of growth (Lewis, 2010). Toxin-antitoxin module present on the chromosome is believed to be the genetic element behind this phenomenon (Magnuson, 2007; Lewis, 2010). There are several TA systems that have been identified so far in the *Escherichia coli* K-12

(MG1655) chromosome: *mazEF*, *relBE*, *yefM-yoeB*, *chpB*, and *dinJ-yafQ* (Tsilibaris et al., 2007). These systems consist of a toxic gene that encodes stable toxic proteins that target an essential cellular function (transcription and translation) and an antitoxin gene encoding unstable antitoxin that binds and inhibits toxin. Differential stability of toxin-antitoxin gene regulates toxin activity (Lewis, 2010). Therefore, we hypothesize that TA modules may play additional role in longer survival of pathogens during composting process by generating persisters. To our knowledge, persister phenomenon has been investigated only for antimicrobial therapy and microbes exposure to heat in minimal media (Hansen et al., 2008; Kolodkin-Gal and Engelberg-Kulka, 2006). Therefore, role of TA system in microbes' under-going composting process needs to be elucidated. Additionally, there are many other genes including virulent genes can be affected when bacterial cells are exposed to a complex environment, such as compost system. There are studies where researchers have looked into stress and virulence genes expression in *E. coli* O157:H7 in food such as lettuce and apple juice (Cary et al., 2009; Bergholz et al., 2009). However, there is a lack of information on response of these genes in *E. coli* O157:H7 during composting. The objective of this study was to study expression of various stress and virulent genes in *E. coli* O157:H7 heat-shocked in compost and in broth as well.

Material and Methods

Culture preparation: *E. coli* O157:H7 strain F07M-020-1(Taco John's outbreak strain from California Dept. of Health) was used in this study. *E. coli* O157:H7 was grown

overnight at 37°C in tryptic soy broth (TSB) (Difco, BD, MD). After two transfers a loopful of freshly cultured *E. coli* O157:H7 was inoculated in separate 10 ml TSB tubes and were incubated for 8 h at 37°C to obtain mid log phase of the growth. The grown cultures were washed twice in TSB by centrifuging at 5,000 x g for 10 min and then resuspended to an optical density (OD_{600}) of ca. 0.6 to 0.7 (ca. 10^9 CFU/ml).

For preparing culture for gene expression experiment 100 µl of above grown mid log phase culture was inoculated into 500 ml of TSB and incubated at 37°C for 8 h in shaker incubator (Series C25, New Brunswick Scientific, Edison, NJ) for mid log phase culture. The grown culture was washed twice in phosphate buffer saline (PBS) by centrifugation and resuspended to OD_{600} of 0.6 to 0.7. The resuspended culture was further concentrated 100 times (ca. 10^{11} CFU/ml) by centrifuging.

Heat-shock optimatization: The one set of washed culture (2 ml each, ca 10^9 CFU/ml) was dispensed into 4 tubes which were heat-shocked for duration of 10, 15, 30 and 60 min in a water bath (Precision shaking water bath, Precision Scientific Inc., Chicago, IL) at each temperature of 42, 45 and 47.5°C. The non-heat-shocked cultures (control) were kept at room temperature for the same duration of time at each temperature mentioned above.

Immediately after heat-shock treatment, 100 µl of the heat-shocked and control cultures were transferred to 9.9 ml sterile saline tube (ca. 10^7 CFU/g) in duplicate. These tubes were then transferred to water bath for thermal inactivation study at 55°C for 20 min. The temperature rise inside the tube was monitored using type-T thermocouples (DCC Corporation, NJ) with one cord put inside an un-inoculated saline tube and the

other inside the water bath. Immediately after 20 min of inactivation, tubes were removed from the water bath and cooled in ice water bath (5 min) to bring down the temperature. Enumeration of surviving population of *E. coli* O157:H7 was done by plating serial dilution on tryptic soy agar (TSA). Based on the results (Table 1) of the experiment temperature-time combination of 47.5°C for 10 min was chosen for heat-shock treatment for the rest of following studies.

Preparation of fresh dairy compost: Fresh dairy compost with carbon to nitrogen (C:N) ratio of 25:1 was prepared as mentioned previously (Singh et al., 2011). The compost was stored at refrigeration temperature until used.

Thermal inactivation of heat-shocked *E. coli* O157:H7 in fresh dairy compost: The freshly grown *E. coli* O157:H7 in log phase was washed twice in PBS and then resuspended to an OD_{600} of 0.6 to 0.7. Washed culture was then inoculated into (100 g) autoclaved (121°C for 15 min) fresh dairy compost in a ratio of 1:100 at a final conc. of ca. 10^7 CFU/g. The inoculated compost was divided into two halves. One half (ca. 50 g) was put in Tyvek pouch (size 5.25 in. by 10 in., SPS Medical, NY), spread evenly, and then heat-shocked in circulating water bath at 47.5°C for 10 min. The rest (50 g) was kept at room temperature as control. Immediately after 10 min of heat-shock treatment pouch was removed and cooled in ice water bath for 5 min to reduce the temperature. The number of surviving *E. coli* O157:H7 in control and after 10 min of heat-shock were enumerated by plating serial dilution on TSA. Each of compost sample (15 g) with either heat-shocked or control culture was put in duplicate Tyvek pouches and spread evenly, which were then submerged in water bath for thermal inactivation study at 55°C

for 5 min only to see the protective effect of heat-shock in compost. The temperature rise within compost was monitored with type-T thermocouples as mentioned before. After thermal inactivation at 55°C, 10 g of compost was weighed and homogenized in a stomacher bag (Lansing, MI). Serial dilution of the homogenate was made in saline and plated on TSA for enumerating surviving population of *E. coli* O157 in heat-shocked and control compost.

Heat-shock treatment of inoculated compost for gene expression study: To provide sufficient amount of RNA for gene expression study a large inoculum was used for autoclaved fresh dairy compost. The compost (50 g) was inoculated with concentrated culture (ca. 10^{11} CFU/ml) in the ratio of 1:10 (ca. 10^{10} CFU/g), and mixed thoroughly on sterile polypropylene tray by hand wearing gloves. The level of inoculation achieved in the compost after inoculation was enumerated by plating on TSA. The inoculated compost ca. 25 g each was put in two Tyvek pouches. One of the pouches was heat-shocked at 47.5°C for 10 min in water bath as mentioned above and the other (control) was kept at room temperature. Immediately after heat-shock treatment pouch was cooled in ice water bath for 15 sec. The level of surviving *E. coli* O157:H7 in control and heat-shocked compost was enumerated by plating on TSA.

For comparing expression of genes in *E. coli* O157 heat-shocked in compost, *E. coli* O157 heat-shocked in broth was used as control. *E. coli* O157:H7 grown to mid log phase in TSB was washed and heat-shocked at 47.5°C for 10 min, whereas control was kept at room temperature for same amount of time. After heat-shock tube was put in ice water bath of 10 sec to bring down the temperature. Followed by immediate transfer of

control and heat-shocked culture (200 µl) to 1.5 ml of centrifuge tube containing 1 ml of RNA protect reagent, that was incubated at room temperature for 5 min. This was followed by centrifugation as mention above. The centrifuged culture was then processed for extraction of total RNA directly as mentioned below without using Dynabeads.

Immuno-magnetic bead separation of *E. coli* O157:H7 from compost: The inoculated compost (10 g) from both control and heat-shocked treatment was mixed with 50 ml of PBS in filtered stomacher bag (7.5 in. by 12 in.) (Whirl-Pak, Nasco, Fort Atkinson WI). The sample was homogenized thoroughly and transferred to 50 ml centrifuge tube (Greiner-Bio-one, Germany). The tube was centrifuged for 1 min at 1,500 x g to remove any larger compost particles. Compost supernatant (5 ml) was then transferred to 5 ml of sterile glass tube (13 by 100 mm) with cap and 100 µl of anti-*E. coli* O157 magnetic beads (Dynabeads anti-*E. coli* O157, Invitrogen, Dynal A.S, Oslo, Norway) were added to the glass tube. The tube was vortexed for 5 sec and then put on Dynal sample mixer (Dynabeads, Invitrogen, Dynal A.S, Oslo, Norway) for 2 min at maximum speed. After concentration of the beads using magnetic particle concentrator (Dynal MPC-L, Invitrogen, Dynal A.S, Oslo, Norway) compost homogenate was removed carefully with sterile disposable pipette. The beads were then washed with 5 ml of PBS, and about 1 ml of RNAProtect Bacteria (Qiagen Sciences, Maryland) was added. The tube was then allowed to remain at room temperature for 5 min, and entire bead homogenate was centrifuged at 5,000 x g for 10 min. The washed beads with bacteria were immediately processed for total RNA extraction. This experiment was performed twice.

Total RNA extraction: The RNAeasy Mini kit (Qiagen, Maryland) was used to isolate total RNA from the bacteria following manufacturer's instructions. To each RNAProtect Bacteria treated tubes 200 µl of lysozyme (10 mg/ml in TE buffer, pH 7.4) and 15 µl of proteinase K (20 mg/ml) was added and incubated at room temperature for 10 min with frequent vortexing every 2 min, followed by vigorous vortexing in 700 µl of buffer RLT containing 1% β-mercaptoethanol. The lysate was centrifuged for 5 min at 5,000 x g to remove beads. The lysate supernatant was transferred to fresh tube to which 500 µl of ethanol (100 %) was added and mixed by pipetting. The lysate was then passed through RNeasy mini column. Followed by wash with 700 µl of buffer RW1, the column with lysate was then washed twice with buffer RPE. The residual RPE was removed by centrifuging the column at 8,000 x g or above with open lid for 5 min. The total RNA was eluted with 50 µl of RNase-free water. The eluted total RNA was again passed through the same column to further increase the concentration of total RNA.

Contaminating genomic DNA from the total RNA extract was removed by using rigorous method of the Turbo DNA-free kit (Ambion, Austin, TX) according to the manufacturer's instructions. The total RNA concentration was determined spectrophotometrically using NanoDrop-2000 (Thermos Scientific, Wilmington, DE). The quality of total RNA with the $A_{260/280}$ ratio between 1.90 and 2.10 (Jain et al., 2006) was considered acceptable.

cDNA synthesis: RNA was reverse-transcribed (RT) using iScript-cDNA synthesis kit (BioRad, CA) as per manufacturer's instructions. Two µl (ca. 400 ng) of RNA was reverse transcribed in 20 µl total reaction volume, containing 1 µl of iScript reverse

transcriptase, 4 µl of 5x iScript reaction mix and 13 µl of nuclease-free water. A no reverse transcriptase control (NRTC) was also run to check for any DNA contamination. cDNA synthesis was done in thermocycler (iCycler iQ, BioRad, CA) with following reaction conditions: 25°C for 5 min, 42°C for 30 min and finally 80°C for 5min. Cycle threshold (C_t) value of 10 or higher between RT sample and NRTC was consider acceptable for contaminating genomic DNA (Applied Biosystems, 2008)

Gene expression study by real- time PCR: Expression of stress and virulence genes in *E. coli* O157:H7 in response to heat-shock in compost was studed using comparative real-time PCR. Each reaction volume of 25 µl contained 1 µl of transcribed cDNA, 1 µl (0.4 µM) of each primer, 12.5 µl of iQ SYBR green supermix (BioRad, CA) and the rest as nuclease-free water. The sequence of all the primers used to examine expression of stress and virulence genes in present study are listed in Table 2. The primers sets listed in Table 2 were designed using online software from PrimerQuest (Integrated DNA Technologies, USA) or OligoPerfect designer (Invitrogen, Life technologies, USA). The specificity of each primer was determined by analyzing melt curve analysis after real-time PCR. Efficiency (E) of each primer was determined by running real time PCR of 2 fold serial dilution of total RNA from bacteria in duplicate. Efficiency of the target or reference gene was then calculated by plotting the log of the RNA template dilution versus C_t value using following equation:

$$E = 10^{-(1/s)} - 1$$

Where‘s’ represent the slope of the standard curve. Efficiency of different genes used in the study is presented in Table 2.

Amplification and quantification of target genes were carried out in iCycler iQ (BioRad, CA) with an initial denaturation at 95°C for 5 min, followed by 40 cycles of amplification at 95°C for 1 min, 57.5°C for 30 sec and 72°C for 90 sec. This was subsequently followed by melt curve analysis involving heating of the product to 95°C from 55°C with temperature increment of 0.5°C every 10 sec, for 80 cycles. No template controls (NTC) and NRTC were included with each assay.

Pfaffl method (2001) was used to determine relative fold change of mRNA for the gene of interest in control and treatment sample. Fold change in different genes were calculated by following equation.

$$\text{Fold change} = \frac{(1+E_{\text{Target}})^{\Delta Ct,\ \text{Target}}}{(1+E_{\text{Reference}})^{\Delta Ct,\ \text{Reference}}}$$

Where, $\Delta C_{t,\ \text{Target}} = (C_{t,\ \text{Target, Control}} - C_{t,\ \text{Target, Heat-shock}})$

$\Delta C_{t,\ \text{Reference (16S rRNA)}} = (C_{t,\ \text{16S rRNA, Control}} - C_{t,\ \text{16S rRNA, Heat-shock}})$

The heat-shocked and control samples were amplified in separate tubes in duplicate. The 16S rRNA gene was used to normalize input amount of RNA. Two independent experiments were done for both compost and broth study.

Statistical analysis: Plate count data for heat-shock optimization of *E. coli* O157:H7 in broth and saline was converted to $\log_{10}$ values, and subjected to mixed procedure of analysis of variance with F - statistic. Plate count data of *E. coli* O157:H7 survival in compost was also converted to $\log_{10}$ values and subjected to analysis of variance with F-

statistic using Tukey's multiple comparison procedure of the Statistical Analysis System (2001, SAS, Cary, NC).

Statistical analysis of gene expression study was done on normalized value of each gene. Normalization was done by dividing the C_t values of gene of interest to that of C_t value of reference gene (16S rRNA) of the sample. The normalized values were then subjected to ANOVA using test criterion (F-statistic) and type I error controlled at $P = 0.05$, with the Statistical Analysis System (2001, SAS, Cary, NC).

Results

Expression of various genes in *E. coli* O157:H7 in response to heat-shock in both broth and fresh dairy compost was studied using real-time PCR. The groups of genes that were included in the study were stress, virulence, toxin-antitoxin, and trehalose synthesis genes. Expression of genes in compost was also compared with that in broth medium. Efficiency of the genes used in the study varied from 88 to 110 % (Table 6.1)

Optimization of heat-shock treatment

The results of thermal inactivation of *E. coli* O157:H7 heat-shocked at different temperatures such as 42, 45 and 47.5°C for 10, 15, 30, and 60 min is presented in Table 2. When exposed at 55°C for 20 min there were ca. 2.21, 1.05, and 0.46 log reductions in *E. coli* O157 heat-shocked for 10 min, at 42, 45, and 47.5°C, respectively. Similarly there were ca. 1.74, 1.12, and 0.52 log reductions after heat-shocked for 15 min, at 42, 45, and 47.5°C, respectively, ca. 1.30, 1.16, and 0.55 log reductions after heat-shocked for 30

min, at 42, 45, and 47.5°C, respectively, and ca. 1.31, 1.64, and 0.69 log reductions after heat-shocked for 60 min, at 42, 45, and 47.5°C, respectively. In control culture (not heat-shocked) there were ca. 4.10, 4.10, and 2.43 log reductions in *E. coli* O157 when thermally inactivated at 55°C for 20 min. Surviving population of *E. coli* O157:H7 was significantly ($P < 0.05$) different at all the sampling points within the same heat-shock temperature but with different heat-shock duration time. The surviving population was also significantly ($P < 0.05$) different at all the sampling points within the same duration of heat-shock time but between different heat-shock temperatures. Based on above results heat-shock temperature of 47.5°C for duration of 10 min was selected for subsequent experiments.

Survival of *E. coli* O157:H7 heat-shocked in dairy compost

Ability of *E. coli* O157:H7 to resist lethal temperature after they have been acclimatized/heat-shocked in compost matrix was studied. The levels of *E. coli* O157:H7 achieved in compost after inoculation, in control culture and after heat-shock at 47.5°C for 10 min were ca. log 7.32± 0.07, 7.31± 0.07, 7.19± 0.05 CFU/g, respectively. When the control and heat-shocked compost was thermally inactivated at 55°C for 5 min, heat-shocked *E. coli* O157 in compost was inactivated slower ($P < 0.05$) than the control culture at 5 min. As shown in Fig. 6.1, there was ca. 1.57 and 2.63 log reduction within 5 min of exposure at 55°C in heat-shocked and control culture, respectively.

Expression of stress-related genes in *E. coli* O157:H7 after heat-shock in compost and broth

The levels of *E. coli* O157:H7 enumerated in compost for gene expression study after inoculation, in control compost, and after 10 min of heat-shock were ca. 10.42± 0.11, 10.41± 0.12, 10.12±0.08 log CFU/g, respectively, (data not shown).

Relative changes in the expression of stress genes in *E. coli* O157:H7 in response to heat-shock in compost and in broth have been shown in Fig. 2A & B, respectively**.** Exposure of *E. coli* O157 to heat-shock in compost induced expression of four genes, *rpoH*, *clpB*, *dnaK*, and *groEL*, which were up-regulated by 3.3, 32, 27.7 and 17.8 fold, respectively (Fig 6.2A) . Similarly, when *E. coli* O157:H7 was heat-shocked in broth same genes were up-regulated by 3.7, 34.6, 39.6, and 24.2 fold, respectively (Fig 6.2B). However, gene *sodB* was down-regulated by 1.6 fold when *E. coli* O157 was exposed to stress in compost and up-regulated by 1.2 fold when exposed to heat-shock in broth. But, the relative change in the expression of *sodB* in compost and in broth was not significantly ($P > 0.05$) different in relation to their non heat-shocked counterpart in compost and broth. However, relative change in genes *rpoH*, *clpB*, *dnaK*, and *groEL* was significantly ($P < 0.05$) different in relation to their non heat-shocked treatment.

Expression of trehalose synthesis genes after heat-shock in compost and broth

Effect of heat stress on expression of trehalose synthesis genes (*otsA* and *otsB*) is shown in Fig 6.3A &B. There was no change in the expression of the *otsA* gene on subjecting to heat-shock in both compost and liquid (broth) medium (Fig 6.3A & B).

However expression of gene *otsB* showed slight tendency towards up-regulation with relative change of 1.2 fold in compost and tendency towards down-regulation in broth with relative change of 1.4 fold. The relative expression of both genes in compost and broth was not significantly ($P > 0.05$) different.

<u>Expression of virulent genes in *E. coli* O157:H7 after heat-shock in compost and broth</u>

Fold change in the expression of virulent genes in *E. coli* O157:H7 in response to heat stress in compost and broth is shown in Fig. 6.4A & B, respectively. Virulent genes *stx1* and *fliC* were up-regulated with 2.5 and 1.4 fold change in their relative expression in compost (Fig 6.4A) as compared with genes *stx2*, *eaeA* and *hlyA* which were down-regulated with relative fold change of 5, 1.1, and 2.5, respectively. On the other hand expression of the virulent genes in liquid medium (broth) was different from that observed in compost matrix. Here, virulent gene *eaeA* had 1.2 fold relative up-regulations in relation to non heat-shocked treatment. Whereas, virulent genes such as *stx1*, *stx2*, *hlyA*, and *fliC* were down-regulated with 2, 5, 10, and 1.6 fold change in their relative expression (Fig 6.4B). Expression of virulent genes *stx2* and *hlyA* of heat-shocked culture in liquid medium (broth) were significantly ($P < 0.05$) different in relation to their expression in control treatment, however, expression of *stx1* and *fliC* was not ($P > 0.05$).

<u>Expression of toxin-antitoxin (TA) genes in *E. coli* O157:H7 after heat-shock in compost and broth</u>

Influence of heat-shock on the expression of TA genes of *E. coli* O157:H7 when heat-shocked in compost and broth is represented in Fig 6.5A & B, respectively. These TA genes exist in pair in *E. coli* O157 genome. In compost, the antitoxin genes *chpS* and *dinJ* were up-regulated with relative fold change of 1.8 and 5.7, respectively, whereas antitoxin genes *mazE* and *hipB* were down-regulated with fold change of 1.3 and 1.7 fold, respectively (Fig 6.5A). Comparatively, there was difference in the response of antitoxin genes when *E. coli* O157:H7 was subjected to heat-shock in broth. Most of the antitoxin genes, *mazE*, *hipB*, and *dinJ* were down-regulated with relative fold change of 1.4, 10, 1.3, respectively, whereas, antitoxin gene *chpS* was slightly up-regulated with 1.1 fold change (Fig 6.5B). The expression of antitoxin gene *dinJ* in compost was significantly ($P < 0.05$) different in relation to their change in the control group.

Expression profile of toxin genes in *E. coli* O157 heat-shocked in compost showed up-regulation in *mazF*, *hipA*, and *yafQ,* and with relative fold change of 1.2, 2.8, and 3.7, respectively, whereas, toxin gene *chpB* was down-regulated with 1.7 fold relative change in its expression (Fig 6.5A). Expression of toxin genes in broth showed different expression with all the genes *mazF*, *hipA, yafQ, and* chpB being down-regulated with relative change of 2, 2.5, 3.3, and 10 fold, respectively (Fig 6.5B). Expression analysis of toxin genes *hipA* and *chpB*, heat-shocked in broth medium, was significantly ($P < 0.05$) different, whereas, expression of all the toxin genes heat-shocked in compost was not significantly ($P > 0.05$) different.

Discussion

To calculate relative change in the level of target genes, Pfaffl (2001) method was used as it takes into account efficiency of target and reference gene to estimate fold change. Delta-delta cycle threshold ($2^{\Delta\Delta Ct}$) is another method used to calculate relative change in gene of interest, however this method is based on the assumption that efficiency of both target and reference gene is 100%. This is not always possible as efficiency of the target gene depends on quality of mRNA and the reaction mix. Therefore, there is overestimation of fold change in gene of interest based on $2^{\Delta\Delta Ct}$ method (Hunt, 2010). Efficiency of genes used in present study varied from 88 to 110 %. Similarly, Carey et al. (2009) in their study reported efficiency of 85 % or above for the genes they studied. However, they used $2^{\Delta\Delta Ct}$ method to calculate the fold change.

Expression of stress genes

Stress genes present in microorganism provide them with resistance to a number of stresses such as heat, acid, osmotic and starvation (Wesche et al., 2009). Stress, responses especially in the form of heat-shock response, is universally conserved among microorganisms and help to remedy damage induced by heat or other stresses (El-Samad et al., 2005). In order to understand mechanisms of *E. coli* O157:H7 adaptability and response to temperature during composting process. In this study the effect of *E. coli* O157:H7 response to heat-shock response in compost environment on stress and virulent gene expression was studied at the genetic level. Heat-shock proteins in *E. coli* O157:H7 are induced immediately following exposure to high temperature and is mediated by an

alternative sigma factor - σ^{32} (encoded by *rpoH*) (El-Samad et al., 2005). Many heat-shock proteins are chaperones such as DnaK, GroEL and ClpB which are encoded by genes *dnaK*, *groEL,* and *clpB*, respectively, which are under the control of *rpoH* (Rasouly and Ron, 2009; El-Samad et al., 2005).

In present study all the four genes (*rpoH, clpB, dnaK, and groEL*) were up-regulated in *E. coli* O157:H7 on exposure to heat-shock in compost. This response was independent of heating medium as all these genes were also expressed in *E. coli* O157:H7 heat-shocked in broth. Based on the above results we can say that when *E. coli* O157:H7 was exposed to sub lethal stress in the form of temperature heat shock genes were up-regulated to counter act the stress. The response of the genes was better in terms of fold change when *E. coli* O157 was heat-shocked in broth medium than in compost. This difference in expression could be due to difference in level and composition of nutrients in broth and compost. Additionally, heat transfer in liquid is much better than in solid medium.

Induction of heat-shock response in microorganism in response to sub-lethal temperature is very effective in extending their survival on subsequent exposure to lethal temperatures (Murano and Pierson, 1992; Carruthers and Minion, 2009; Singh et al., 2010). Carruthers and Minion (2009) in their study on *E. coli* O157:H7 reported up-regulation of heat shock genes *rpoH*, *dnak*, *groEL* in response to heat stress at 50°C for 10 min in broth as measured by microarray and real time PCR. However, up-regulation of gene *clpB* was only reported when measured by real time PCR, emphasizing the sensitivity of real time PCR- based estimation methods over microarray. In that study

fold change observed in the genes *rpoH*, *clpB*, *dnaK*, and *groEL* were 5.2, 5.7, 20.3, and 12.8, respectively. Our results revealed ca. 3 to 32 fold increases of these heat-shock genes, suggesting up-regulation of these genes in compost plays a definitive role in extending survival of microorganisms during composting process. In our previous study we have reported extended survival of heat-shocked *E. coli* O157:H7 in finished dairy compost when exposed to composting temperatures inside a humidity chamber (Singh et al 2010). Shepherd et al. (2010) also reported better survival of heat-shocked *E. coli* O157:H7 and *Salmonella* under field conditions.

Gradual rise of temperature during initial phase (mesophilic) of composting process is believed to be responsible for induction of heat-shock response in microorganism thereby giving them opportunity to survive longer. In our previous study where composting under controlled condition was done by simulating early phase of composting process showed extended survival of *E. coli* O157:H7 in fresh dairy compost indicating induction of heat-shock response (Singh et al., 2011). In this study, our results clearly revealed the genetic mechanism underlying the thermal résistance of *E. coli* O157:H7 during composting. Since heat-shock response is transient mechanism, its induction during composting process make this phenomenon all the more important as composting process under real-time field conditions is subjected to temperature variation due to several environmental conditions such as rain fall and seasonality (Shepherd et al., 2010). These variations additionally contribute to the extended survival of pathogens. Heat-shock response is not limited to *E. coli* O157:H7 alone but is conserved in all

microorganisms and provide protection when induced due to stress (van der Veen et al., 2007; Lund 2001, Sirsat et al., 2011).

Superoxide dismutase plays important role in oxidative stress (Touati, 1997). Privalle and Fridovich (1987) reported expression of superoxide dismutase when exposed to heat stress. However, in our study *sodB* gene was down-regulated in compost and slightly up-regulated in broth when heat-shocked. Expression of this gene in both matrix was not significantly ($P > 0.05$) different, indicating the limited role of this gene in heat-shock response.

<u>Expression of trehalose synthesis genes</u>

One of the mechanisms for microorganisms to adapt adverse environment is to accumulate protective compounds that protect macromolecules and membranes (Csonka, 1989; Kempf and Bremer, 1998; Leslie et al., 1994). Endogenous production of trehalose (a non-reducing disaccharide) by stressed cells is one such example for microorganisms to be protected against variety of lethal environmental stresses, including heat, dessication, high osmotic strength and cold (Kandror et al., 2002; Attfield, 1987; Giaever et al., 1988). In this study we evaluated expression of trehalose synthesis gene on exposure to heat shock in compost matrix and in broth medium. In compost with moisture content of ca. 55%, there was no change in the expression of gene *otsA* while gene *otsB* was slightly up-regulated with 1.2 fold change. However, expression of both genes in heat-shocked *E. coli* O157 was down regulated in broth medium. It has been observed that disaccharide trehalose play important role in maintaining viability of

microbial cells under dry condition and would eventually extend their survival (Leslie et al., 1995). In our previous study we observed extended survival of a very small population of *E. coli* O157:H7 in fresh dairy compost (Singh et al., 2011). In another study, extended survival of *E. coli* O157:H7 and *Salmonella* was reported on the surface of compost heaps under field conditions (Shepherd et al., 2007). Beside heat-shock response, possible protective role of endogenously produced disaccharides in extended survival of *E. coli* O157 cannot be ruled out. However, in our current study we did not see any appreciable change in the expression of trehalose synthesis genes. This could be high moisture content of compost used in heat-shock treatment was not enough to induce dessication as well. Additionally, osmotic strength of the compost mix was not known, as osmotic stress is believed to be primary inducer for the synthesis of trehalose. In a study on *Saccharomyces cerevisiae* heat-shock treatment induced production of trehalose and peak level was achieved by 90 min of treatment (Attfield, 1987). Down regulation of trehalose synthesis genes in *E. coli* O157 on heat-shock in broth is not unexpected as cells were heat-shocked in fresh TSB after washing with PBS. Water play critical role in structural and functional relationships of proteins, and certain sugars also contribute to this process. Under conditions of moderate stress compatible solutes appear to play important function in this mechanism. However, under conditions of extreme dessication, disaccharide trehalose and sucrose seems to afford protection (Potts, 1994).

Expression of virulent genes

Virulent genes of the microbial pathogens are responsible for infection and disease and contribute to the fitness of the pathogen within the host but not involved in general housekeeping functions (Mekalanos, 1992; Heithoff et al., 2000). Expression of virulent genes of pathogens is also considered as an adaptive response to stress encountered in a host (Imlay 2003). Many stresses that a pathogen is subjected to on exposure to host's defense system are similar to those encountered in the natural environment. Exposure of pathogens to stresses in natural and food processing environments act as a signal for the expression of virulence factors/genes (Luo and Yousef, 1997). Purpose of evaluating expression of virulent genes in *E. coli* O157:H7 was to understand changes in bacterial pathogenicity during composting, thereby predicting risk of this pathogen transmitted to produce and other food supplies.

In this study, heat-shocked *E. coli* O157 demonstrated variable expression of virulent genes. Gene *stx1* was up-regulated while *stx2* was down-regulated in compost, but in broth, both these genes were down-regulated. However expression of all these genes was not statistically significant. Carey et al. (2009) also reported variability in the expression of shiga toxin genes in their study done on Romaine lettuce stored at 4 and 15°C, where these genes (*stx1* and *stx2*) were up-regulated during prolong storage. Up-regulation of virulence genes was also reported in another study in which gene expression profile of *E. coli* O157:H7 inoculated in sterile soil stored at 15°C was studied, however, virulent genes evaluated in that study were different from our study (Duffitt et al., 2011). Allen et al. (2008) reported nutrients replenishment of *E. coli* O157:H7 in TSB was

another inducer for the expression of several virulent genes (*stx1*, *eaeA*, *hlyA*), while cold-shock accounted for expression of gene *stx1* only. Most of the other studies reported to evaluate expression of virulent genes of *E. coli* O157:H7 have been to study interaction of this organism with human and animal host defense system to elucidate its pathogenicity (Berlutti et al., 1998; Rashid et al., 2006). Rashid et al. (2006) reported higher expression of hemolysin (*hlyA*) in feces from human infection cases. In our study *hlyA* was down-regulated when heat-shocked at a temperature of 47.5°C for 10 min both in compost and in broth.

Virulence factor, intimin (encoded by gene *eaeA*) is involved in the formation of attaching and effacing lesion by enterohemorrhagic *E. coli* and enteropathogenic *E. coli* in the intestine. This gene is located on locus of enterocyte effacement (LEE) of chromosome. Regulation of this gene is complex. LEE-encoded regulator and quorum sensing are believed to be one of the factors that control its expression (Kanamaru et al., 2000; Elliott et al., 2000). In this study gene *eaeA* was slightly down-regulated in compost and was up-regulated 1.2 fold in broth media with no statistical significance. Again higher expression of this gene has been reported in feces of human infection and bovine carrier (Rashid et al., 2006). Carey et al. (2009) in their study on Romaine lettuce observed down-regulation of this gene at 15°C, but up-regulated at 4°C. So, there is variation in the expression of this gene based on the temperature, environment and the host. There is a lack of information available on the behavior of this gene under heat-shock conditions. Flagellin, encoded by *fliC* plays important role in motility and chemotaxis of microorganisms. In this study flagellin gene was slightly (1.4 fold) up-

regulated when heat-shocked in compost and 1.6 fold down-regulated when heat-shocked in broth with no statistical significance. Carey et al. (2009) observed higher expression of gene *fliC* at 15°C, while slight change was reported at lower temperature of 4°C in their study on leafy vegetable. Expression profile data on some other microorganisms have shown inhibition of expression of this gene at temperature of 37°C or above (Kapatral et al. 1996). Higher temperature used in this study could be one reason for the non significant expression of this gene.

Most of the virulent genes evaluated in this study were either down- regulated or slightly up-regulated with no significant difference. This is true with proved hypothesis that virulence associated genes are differentially expressed on host-specific manner with chances of expression more in non-host individual (Rashid et al. 2006). Our results suggest that during composting process *E. coli* O157:H7 are not able to maintain their virulence as most of the virulent genes were down-regulated.

<u>Expression of toxin-antitoxin system</u>

Toxin-antitoxin systems are ubiquitous in microbial genome and have been proposed to play important role in many cellular functions, such as generation of persisters (Magnuson, 2007).These modules are genetic element present on the chromosome (Magnuson, 2007; Lewis, 2010).

In our previous study we observed extended survival of *E. coli* O157:H7 in fresh dairy compost. Based on the thermal inactivation curves, a very small population of cells persisted for at least 12, 3, and 2 days as detected by enrichment at 50, 55, and 60°C,

respectively (Singh et al., 2011). In this study we examined expression of some known TA module of *E. coli* O157:H7 in compost and broth medium for their possible role in generation of persister under heat stress condition. Our results in this study showed that toxin gene *mazF*, *hipA*, of TA modules, *mazEF*, *hipAB*, respectively, was up-regulated. Toxin genes *yafQ* in *dinJ-yafQ* module was also up-regulated, however, expression of its counterpart antitoxin gene was also up-regulated more than its toxin pair. In broth only antitoxin *chpS* of module *chpBS* was up-regulated slightly, while rest of the genes in toxin-antitoxin system was-down-regulated. Difference in heat transfer through the two heating medium and difference in nutrients could be the reason for variable expression of TA module in compost and in broth.

Most of the studies done so far have proposed role of TA system in the generation of persister cells in response to antibiotic stress leading to multi drug tolerance (Keren e al., 2004, Hansen et al., 2008). Overexpression of *mazF* and *hipA* has been reported for increased antibiotic tolerance in microbes hence persistence of microorganisms (Vazquez-Laslop et al., 2006). Toxins are believed to be responsible for inhibition of protein synthesis by different mechanisms (Zhang et al., 2005; Schumacher et al., 2009). The role of TA system in persister formation is still under debate with some report proposing them to be responsible for programmed cell death in response to stress of high temperature, antibiotic stress and starvation (Kolodkin-Gal and Engelberg-Kulka, 2006). Hansen et al. (2008) suggested high degree of redundancy in persister genes as they identified several mutants showing 10-fold decrease in persister formation, these mutants were deficient in either of the global regulator genes such as *dnaKJ*, *dkaA*, *hupAB*, and

IhAB. Interestingly in our study also one such global regulator (*dnaK*) was highly expressed on account of heat-shock in compost and could be one possible reason of tailing observed in thermal inactivation curves in composting (Singh et al., 2011). We also observed increased expression of three toxin genes *mazF*, *yafQ*, *hipA,* although not statistically significant but may be other parallel and independent mechanisms operating in the tailing. Most of the studies on persister genes have been done in *E. coli*, where as genetic makeup of *E. coli* O157:H7 genome is more versatile with virulence related plasmid and chromosomal pathogenicity island (Nataro and Kaper, 1998), which could also account for difference in expression. Overall, better understanding the mechanism of persister cell formation and their behavior in composting process would require design or formulation of effective strategies for ensuring compost safety from persister pathogens.

Conclusions

Overall, our results show that induction of heat-shock response during mesophilic phase of the composting process may cause surviving pathogens to become more resistant to further stress (lethal temperature) during thermophilic phase. Several heat-shock genes such as *rpoH*, *dnaK*, *clpB*, and *groEL* were up-regulated in *E. coli* O157:H7 when heat-shocked in compost. Although, induction of TA system in present study was not significantly different, but this system may also be having complimentary role in extended survival of pathogen during composting. Type of heating medium also had influence on difference in gene expression. Additionally, virulence in gene expression of the pathogen is either unchanged or suppressed during composting. Therefore, sub-lethal

heat exposure leading to induction of heat-shock genes in pathogens during early phase of composting may results in persistence of pathogens in the finished compost products.

Acknowledgments

This research was supported by a grant from the Center for Produce Safety, University of California at Davis. We would like to thank Dr. Jeremy Tzeng for allowing us to use his instrument for some of our gene expression experiment.

References

1. Allen, K. J., D. Lepp, R. C. McKellar and M. W. Griffiths. 2008. Examination of stress and virulence gene expression in *Escherichia coli* O157: H7 using targeted microarray analysis. Foodborne Pathog Dis. 5:437-447.

2. Applied Biosystems. 2008. http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042380.pdf (accessed October 2011)

3. Attfield, P. 1987. Trehalose accumulates in *Saccharomyces cerevisiae* during exposure to agents that induce heat-shock response. FEBS Lett. 225:259-263.

4. Bergholz, T. M., S. K. Vanaja and T. S. Whittam. 2009. Gene expression induced in *Escherichia coli* O157:H7 upon exposure to model apple juice. Appl. Environ. Microbiol. 75:3542-3553.

5. Berlutti, F., M. Casalino, C. Zagaglia, P. Fradiani, P. Visca and M. Nicoletti. 1998. Expression of the virulence plasmid-carried apyrase gene (*apy*) of enteroinvasive *Escherichia coli* and *Shigella flexneri* is under the control of H-NS and the VirF and VirB regulatory cascade. Infect. Immun. 66:4957-4964.

6. Carey, C. M., M. Kostrzynska and S. Thompson. 2009. *Escherichia coli* O157:H7 stress and virulence gene expression on Romaine lettuce using comparative real-time PCR. J. Microbiol. Methods. 77:235-242.

7. Carruthers, M. D. and C. Minion. 2009. Transcriptome analysis of *Escherichia coli* O157:H7 EDL933 during heat shock. FEMS Microbiol. Lett. 295:96-102.

8. Centers for Disease Control and Prevention (CDC). 2006. Ongoing multistate outbreak of *Escherichia coli* serotype O157:H7 infections associated with consumption of fresh spinach--United States, September 2006. MMWR Morb. Mortal. Wkly. Rep. 55:1045-1046.

9. Chapman, P. A., C. A. Siddons, J. Manning and C. Cheetham. 1997. An outbreak of infection due to verocytotoxin-producing *Escherichia coli* O157 in four families: the influence of laboratory methods on the outcome of the investigation. Epidemiol. Infect. 119:113-119.

10. Christensen, S. K., K. Pedersen, F. G. Hansen and K. Gerdes. 2003. Toxin-antitoxin loci as stress-response-elements: ChpAK/MazF and ChpBK cleave translated RNAs and are counteracted by tmRNA. J. Mol. Biol. 332:809-819.

11. Csonka, L. 1989. Physiological and genetic responses of bacteria to osmotic-stress. Microbiol. Rev. 53:121-147.

12. Duffitt, A. D., R. T. Reber, A. Whipple and C. Chauret. 2011. Gene expression during survival of *Escherichia coli* O157:H7 in soil and water. Int. J. Microbiol. 2011:1-12

13. Elliott, S., V. Sperandio, J. Giron, S. Shin, J. Mellies, L. Wainwright, S. Hutcheson, T. McDaniel and J. Kaper. 2000. The locus of enterocyte effacement (LEE)-encoded regulator controls expression of both LEE- and non-LEE-encoded virulence factors in enteropathogenic and enterohemorrhagic *Escherichia coli*. Infect. Immun. 68:6115-6126.

14. El-Samad, H., H. Kurata, J. Doyle, C. Gross and M. Khammash. 2005. Surviving heat shock: control strategies for robustness and performance. Proc. Natl. Acad. Sci. U.S.A. 102:2736-2741.

15. Giaever, H., O. Styrvold, I. Kaasen and A. Strom. 1988. Biochemical and genetic-characterization of osmoregulatory trehalose synthesis in *Escherichia coli*. J. Bacteriol. 170:2841-2849.

16. Hansen, S., K. Lewis and M. Vulic. 2008. Role of global regulators and nucleotide metabolism in antibiotic tolerance in *Escherichia coli*. Antimicrob. Agents Chemother. 52:2718-2726.

17. Heithoff, D., R. Sinsheimer, D. Low and M. Mahan. 2000. *In vivo* gene expression and the adaptive response: from pathogenesis to vaccines and antimicrobials. 355:633-642.

18. Hunt, M. 2010. Real time PCR. http://pathmicro.med.sc.edu/pcr/realtime-home.htm (accessed November 2011)

19. Hutchison, M. L., L. D. Walters, S. M. Avery and A. Moore. 2005. Decline of zoonotic agents in livestock waste and bedding heaps. J. Appl. Microbiol. 99:354-362.

20. Imlay, J. 2003. Pathways of oxidative damage. Annu. Rev. Microbiol. 57:395-418.

21. Jain, M., A. Nijhawan, A. K. Tyagi and J. P. Khurana. 2006. Validation of housekeeping genes as internal control for studying gene expression in rice by quantitative real-time PCR. Biochem. Biophys. Res. Commun. 345:646-651.

22. Kanamaru, K., K. Kanamaru, I. Tatsuno, T. Tobe and C. Sasakawa. 2000. SdiA, an *Escherichia coli* homologue of quorum-sensing regulators, controls the expression of virulence factors in enterohaemorrhagic *Escherichia coli* O157: H7. Mol. Microbiol. 38:805-816.

23. Kandror, O., A. DeLeon and A. Goldberg. 2002. Trehalose synthesis is induced upon exposure of *Escherichia coli* to cold and is essential for viability at low temperatures. Proc. Natl. Acad. Sci. U.S.A. 99:9727-9732.

24. Kapatral, V., J. Olson, J. Pepe, V. Miller and S. Minnich. 1996. Temperature-dependent regulation of *Yersinia enterocolitica* class III flagellar genes. Mol. Microbiol. 19:1061-1071.

25. Kashmanian, R. and R. Rynk. 1996. Agricultural composting in the United States: Trends and driving forces. J. Soil Water Conserv. 51:194-201.

26. Kempf, B. and E. Bremer. 1998. Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. Arch. Microbiol. 170:319-330.

27. Keren, I., D. Shah, A. Spoering, N. Kaldalu and K. Lewis. 2004. Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. J. Bacteriol. 186:8172-8180.

28. Kolodkin-Gal, I. and H. Engelberg-Kulka. 2006. Induction of *Escherichia coli* chromosomal mazEF by stressful conditions causes an irreversible loss of viability. J. Bacteriol. 188:3420-3423.

29. Leslie, S., E. Israeli, B. Lighthart, J. Crowe and L. Crowe. 1995. Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. Appl. Environ. Microbiol. 61:3592-3597.

30. Leslie, S., S. Teter, L. Crowe and J. Crowe. 1994. Trehalose lowers membrane phase-transitions in dry yeast-cells. 1192:7-13.

31. Lewis, K. 2010. Persister cells. Annu. Rev. Microbiol. 64:357-372.

32. Lou, Y. and A. Yousef. 1997. Adaptation to sublethal environmental stresses protects *Listeria monocytogenes* against lethal preservation factors. Appl. Environ. Microbiol. 63:1252-1255.

33. Lund, P. 2001. Microbial molecular chaperones. Adv. Microb. Physiol. 44:93-140.

34. Magnuson, R. D. 2007. Hypothetical functions of toxin-antitoxin systems. J. Bacteriol. 189:6089-6092.

35. Mekalanos, J. 1992. Environmental signals controlling expression of virulence determinants in bacteria. J. Bacteriol. 174:1-7.

36. Murano, E. A. and M. D. Pierson. 1992. Effect of heat-shock and growth atmosphere on the heat-resistance of *Escherichia coli* O157:H7. J. Food Prot. 55:171-175.

37. Nataro, J. and J. Kaper. 1998. Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. 11:142-201.

38. Pedersen, K., S. K. Christensen and K. Gerdes. 2002. Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. Mol. Microbiol. 45:501-510.

39. Pfaffl, M. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29:e45.

40. Potts, M. 1994. Desiccation tolerance of prokaryotes. Microbiol. Rev. 58:755-805.

41. Privalle, C. and I. Fridovich. 1987. Induction of superoxide-dismutase in *Escherichia coli* by heat-shock. Proc. Natl. Acad. Sci. U.S.A. 84:2723-2726.

42. Rashid, R. A., T. A. Tabata, M. J. Oatley, T. E. Besser, P. I. Tarr and S. L. Moseley. 2006. Expression of putative virulence factors of *Escherichia coli* O157: H7 differs in bovine and human infections RID A-4655-2011. Infect. Immun. 74:4142-4148.

43. Rasouly, A. and E. Z. Ron. 2009. Interplay between the heat shock response and translation in *Escherichia coli*. Res. Microbiol. 160:288-296.

44. Rowe, M. T. and R. B. Kirk. 2000. Effect of nutrient starvation on the resistance of *Escherichia coli* O157: H7 to subsequent heat stress. J. Food Prot. 63:1745-1748.

45. Schumacher, M. A., K. M. Piro, W. Xu, S. Hansen, K. Lewis and R. G. Brennan. 2009. Molecular mechanisms of HipA-mediated multidrug tolerance and its neutralization by HipB. Science. 323:396-401.

46. Shepherd, M. W.,Jr, P. Liang, X. Jiang, M. P. Doyle and M. C. Erickson. 2007. Fate of *Escherichia coli* O157:H7 during on-farm dairy manure-based composting. J. Food Prot. 70:2708-2716.

47. Shepherd, M. W., Jr., R. Singh, J. Kim and X. Jiang. 2010. Effect of heat-shock treatment on the survival of *Escherichia coli* O157:H7 and *Salmonella* enterica Typhimurium in dairy manure co-composted with vegetable wastes under field conditions. Bioresour. Technol. 101:5407-5413.

48. Singh, M., S. M. Simpson, H. R. Mullins and J. S. Dickson. 2006. Thermal tolerance of acid-adapted and non-adapted *Escherichia coli* O157:H7 and *Salmonella* in ground beef during storage. Foodborne Pathog. Dis. 3:439-446.

49. Singh, R., X. Jiang and F. Luo. 2010. Thermal inactivation of heat-shocked *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in dairy Compost. J. Food Prot. 73:1633-1640.

50. Singh, R., J. Kim, M. W. Shepherd Jr., F. Luo and X. Jiang. 2011. Determining thermal inactivation of *Escherichia coli* O157:H7 in fresh compost by simulating early phases of the composting process. Appl. Environ. Microbiol. 77:4126-4135.

51. Sirsat, S. A., K. M. Burkholder, A. Muthaiyan, S. E. Dowd, A. K. Bhunia and S. C. Ricke. 2011. Effect of sublethal heat stress on *Salmonella* Typhimurium virulence. J. Appl. Microbiol. 110:813-822.

52. Touati, D. 1997. Superoxidase dismutases in bacteria and pathogen protists. Cold Spring Harbour Laboratory, Cold spring harbor, NY. Pp.447-493.

53. Tsilibaris, V., G. Maenhaut-Michel, N. Mine and L. Van Melderen. 2007. What is the benefit to *Escherichia coli* of having multiple toxin-antitoxin systems in its genome? J. Bacteriol. 189:6101-6108.

54. United States Department of Agriculture (USDA). 2009. Manure use for fertilizer and for energy- Report to Congress. http://www.ers.usda.gov/publications/ap/ap037/ap037.pdf (accessed October 2011)

55. van der Veen, S., T. Hain, J. A. Wouters, H. Hossain, W. M. de Vos, T. Abee, T. Chakraborty and M. H. J. Wells-Bennik. 2007. The heat-shock response of *Listeria monocytogenes* comprises genes involved in heat shock, cell division, cell wall synthesis, and the SOS response. 153:3593-3607.

56. Vazquez-Laslop, N., H. Lee and A. Neyfakh. 2006. Increased persistence in *Escherichia coli* caused by controlled expression of toxins or other unrelated proteins. J. Bacteriol. 188:3494-3497.

57. Wesche, A. M., J. B. Gurtler, B. P. Marks and E. T. Ryser. 2009. Stress, sublethal injury, resuscitation, and virulence of bacterial foodborne pathogens. J. Food Prot. 72:1121-1138.

58. Zhang, Y., J. Zhang, H. Hara, I. Kato and M. Inouye. 2005. Insights into the mRNA cleavage mechanism by MazF, an mRNA interferase. J. Biol. Chem. 280:3143-3150.

Figure legend

Figure 6.1 Thermal inactivation of *E. coli* O157:H7 heat-shocked in autoclaved fresh dairy compost

Control ( ), Heat-shock ( )

Figure 6.2 Expression of stress genes in *E. coli* O157:H7 heat-shocked in autoclaved fresh dairy compost (A) and in broth (B)

rpoH ( ), *clpB* ( ), *dnaK* ( ), *groEL* ( ), *sodB* ( )

Figure 6.3 Expression of trehalose synthesis genes in *E. coli* O157:H7 heat-shocked in autoclaved fresh dairy compost (A) and in broth (B)

otsA ( ), *otsB* ( )

Figure 6.4 Expression of virulent genes in *E. coli* O157:H7 heat-shocked in autoclaved fresh dairy compost (A) and in broth (B)

stx1( ), *stx2* ( ), *eaeA* ( ), *hlyA* ( ), *fliC* ( )

Figure 6.5 Expression of toxin-antitoxin genes in *E. coli* O157:H7 heat-shocked in autoclaved fresh dairy compost (A) and in broth (B)

Toxin genes; *mazF* ( ), *hipA* ( ), *yafQ* ( ), *chpB* ( )

Antitoxin genes; *mazE* ( ), *hipB* ( ), *dinJ* ( ), *chpS* ( )

Table 6.1 Primers used in real-time PCR expression assays along with their efficiency

No.	Gene name	Primer sequence (5' - 3')	AmpliconSize (bp)	Primer efficiency (%)	R^2
1	Alternative Sigma factor - σ^H (*rpoH*)	F: CGC AAA CTG TTC TTC AAC CTG CGT R: AAT CGT CGT CGG AAG ACA GGT CAA	172	100	0.998
2	Heat-shock protein gene (*dnaK*)	F: GCG AAA CTG GAA AGC CTG GTT GAA R: TTG GCA TAC GAG TCT GAC CAC CAA	136	103	0.995
3	Heat-shock protein gene (*groEL*)	F: TTC TGG AAG CTG TTG CCA AAG CAG R: TGC TTT AAC CGC AGC GAC TTT CAC	128	100	0.998
4	Heat-shock protein gene (*clpB*)	F: GGT GCG CGT TCT TAA TCT TTG CGA R: CCT CCA CGC ATT TGT TCA ATC GCT	177	105	0.994
5	Superoxide dismutase (*sodB*)	F: GCGATCAAAAACTTTGGTT R: CCAGAAGTGCTCAAGAT	201	105	0.998
6	Shiga toxin 1 (*stx1*)	F: GAC TGC AAA GAC GTA TGT AGA TTC G R: ATC TAT CCC TCT GAC ATC AAC TGC	150	96	0.997
7	Shiga toxin 2 (*stx2*)	F: ATT AAC CAC ACC CCA CCG R: GTC ATG GAA ACC GTT GTC AC	200	89	0.995
8	Intamin (*eaeA*)	F: GTA AGT TAC ACT ATA AAA GCA CCG TCG R: TCT GTG TGG ATG GTA ATA AAT TTT TG	106	95	0.994
9	Hemolysin (*hlyA*)	F: ACG ATG TGG TTT ATT CTG GA R: CTT CAC GTC ACC ATA CAT AT	166	88	0.998
10	Flagellin (*fliC*)	F: TTCGCAGCATCACTGGATTC R: CATCGCAAAAGCAACTCCTG	201	105	0.998
11	Antitoxin (*mazE*)	F: GCT ACG TTA ATG CAG GCG CTC AAT R: TCA GCA AGC GTA AAT ACG GGC TCT	116	100	0.999
12	Toxin (*mazF*)	F: TCC AGC TGT TGT CCT GAG TCC TTT R: TCC TTT CTT CGT TGC TCC TCT TGC	190	102	0.988
13	Antitoxin (*hipB*)	F: ATG CAA TGA AAC TGG TTC GCC AGC R: CGA AAT TGG AAA TCG TCG CCT GCT	93	95	0.970
14	Toxin (*hipA*)	F: GCT GGC GAA AGA ACT TGG GTT GAA R: ATC CTC CTG TGG CAA GCG AAG TAA	139	89	1.000
15	Antitoxin (*dinJ*)	F: AGA TCT GAA GAA TCA GGC AGC GGA R: TCA TCG GCG TCT TTG GCC TTA TGA	198	101	0.997
16	Toxin (*yafQ*)	F: CGC TGC AAG GTT CAT GGA AAG GTT R: CGC GTG AGT TCC AGT TCT CTC AAA	113	109	0.993
17	Antitoxin (*chpS*)	F: GAA GTG CAG GTG AGC AAC AA R: CAT CCT GCT CGC TAA GTT CC	115	110	0.991
18	Toxin (*chpB*)	F: TGT TGT CCT GAG TCC GTT CA R: CGT TCC TTT CTT CGT TGC TC	187	95	0.970
19	Trehalose-6 phosphate synthase gene (*otsA*)	F: GTT TGC CAG AGC GTT TTC TC R: CAT TTT CGA GCT GAT GAC GA	144	108	0.991
20	Trehalose phosphatase gene (*otsB*)	F: GTC GAG ATC AAA CCG AGA GG R: GAC TGC GAA GCC AGA TTC AT	129	110	0.996
21	16S rRNA gene	F: GGC TGA AAA GCT GCA TTA CC R: CAT CAG GCC GAT GTT ACC TT	151	103	0.998

All primers were designed from gene bank accession number NC_002655, except for genes *sodB* and *fliC* (Carey et al., 2009)

Table 6.2. Heat-shock optimization of *E. coli* O157:H7in saline

Heat-shock temperature (°C)	log CFU/ml at 55°C with heating time of 20 (min)				
	heat-shock duration (min)				
	0[1]	10	15	30	60
42	2.90±0.50[2] A[3]a[4]	4.79±0.20Ba	5.26±0.26Ba	5.70±0.32Ba	5.69±0.22Ba
45	2.90±0.88Ab	5.95±0.31Bb	5.88±0.35Bb	5.84±0.24Bb	5.36±0.18Bb
47.5	4.57±1.19Ac	6.54±0.30Bc	6.48±0.34Bc	6.45±0.35Bc	6.31±0.16Bc

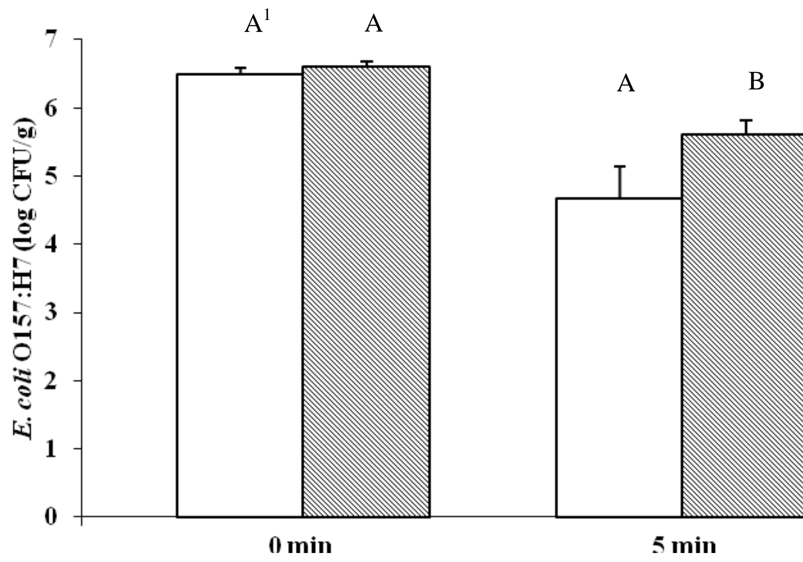
[1]Control culture (not heat-shocked).

[2]Initial inoculum level was ca. 7 log CFU/ml.

[3]Mean log CFU±SD with upper case letter differs significantly ($P < 0.05$) with respect to control treatment within rows at same heat-shock temperature.

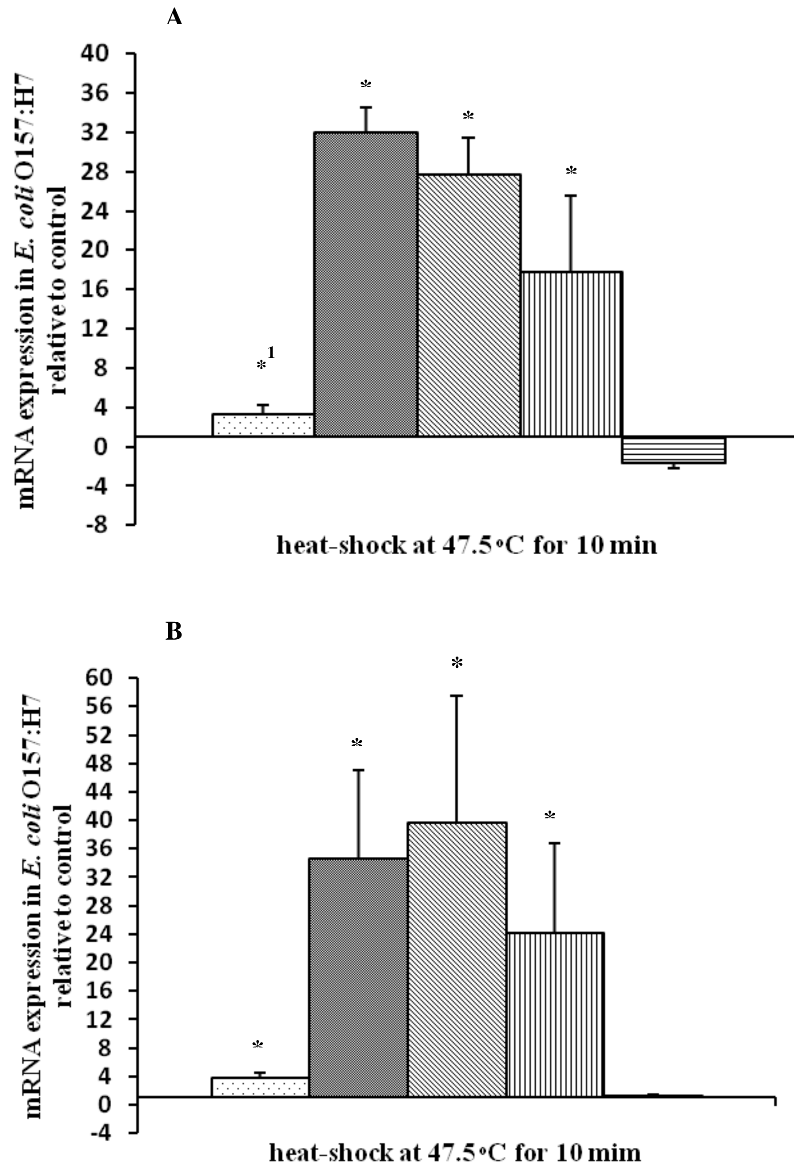
[4]Mean log CFU±SD with lower case letter differ significantly ($P < 0.05$) within columns with same treatment time.

Figure 6.1



Surviving *E. coli* O157: H7 (CFU/g) with upper case letter between two columns with same sampling time differ significantly (P < 0.05)

Figure 6.2



[1]* Relative fold change in mRNA level was significantly ($P < 0.05$) different in comparison to control treatment.

Figure 6.3

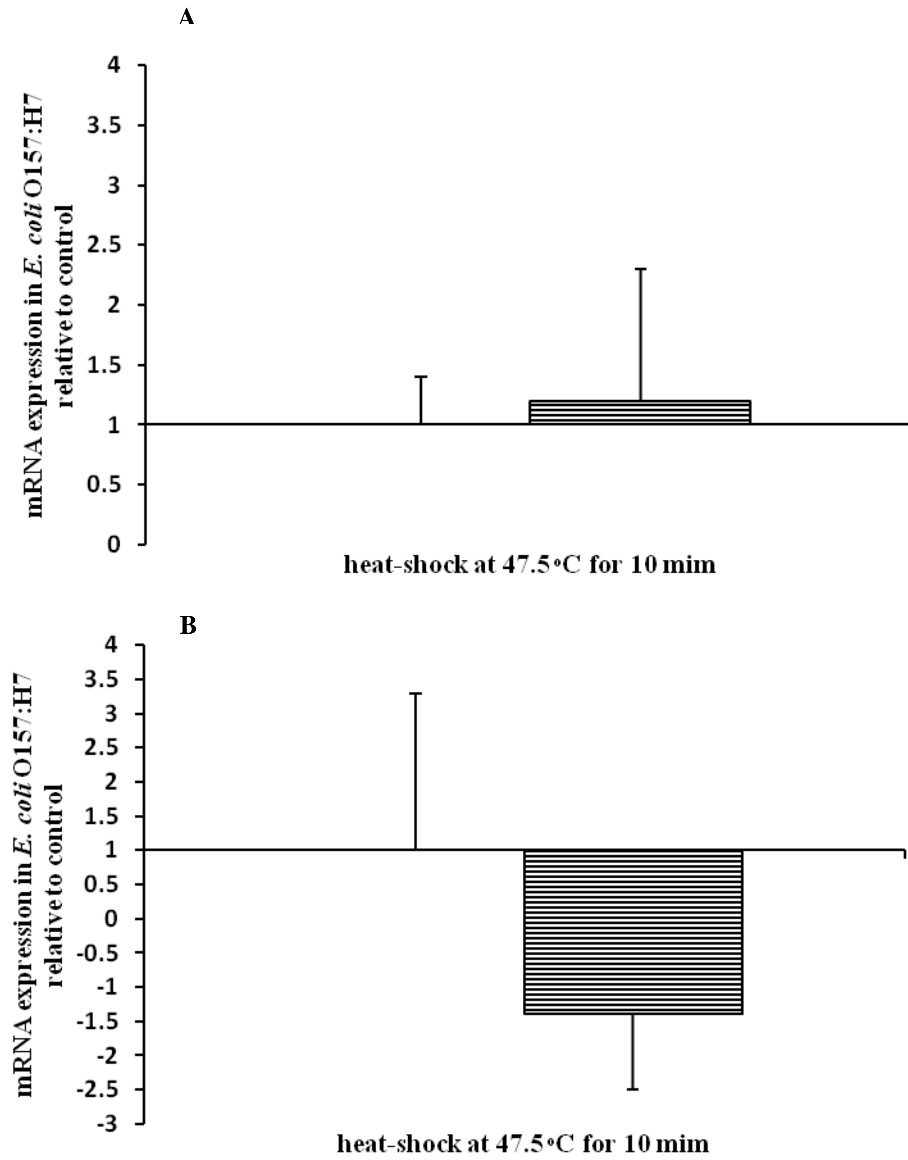
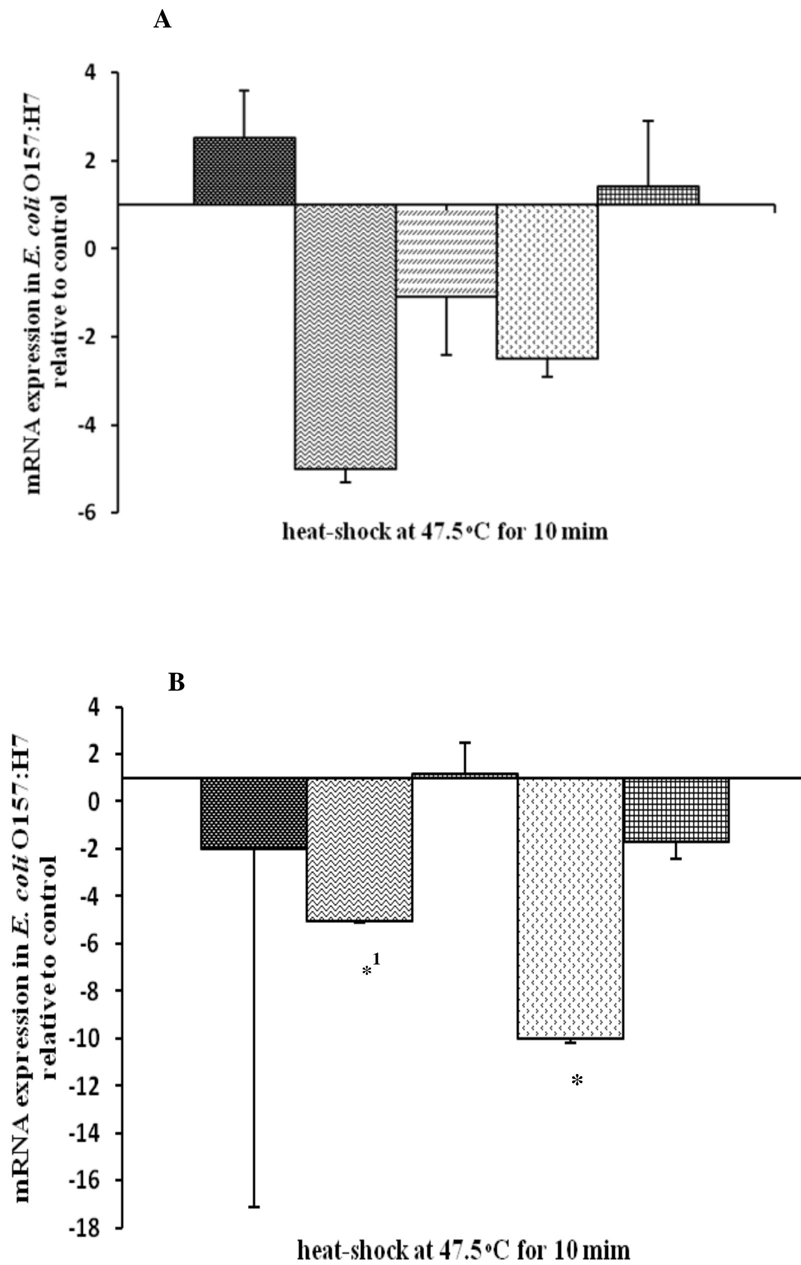
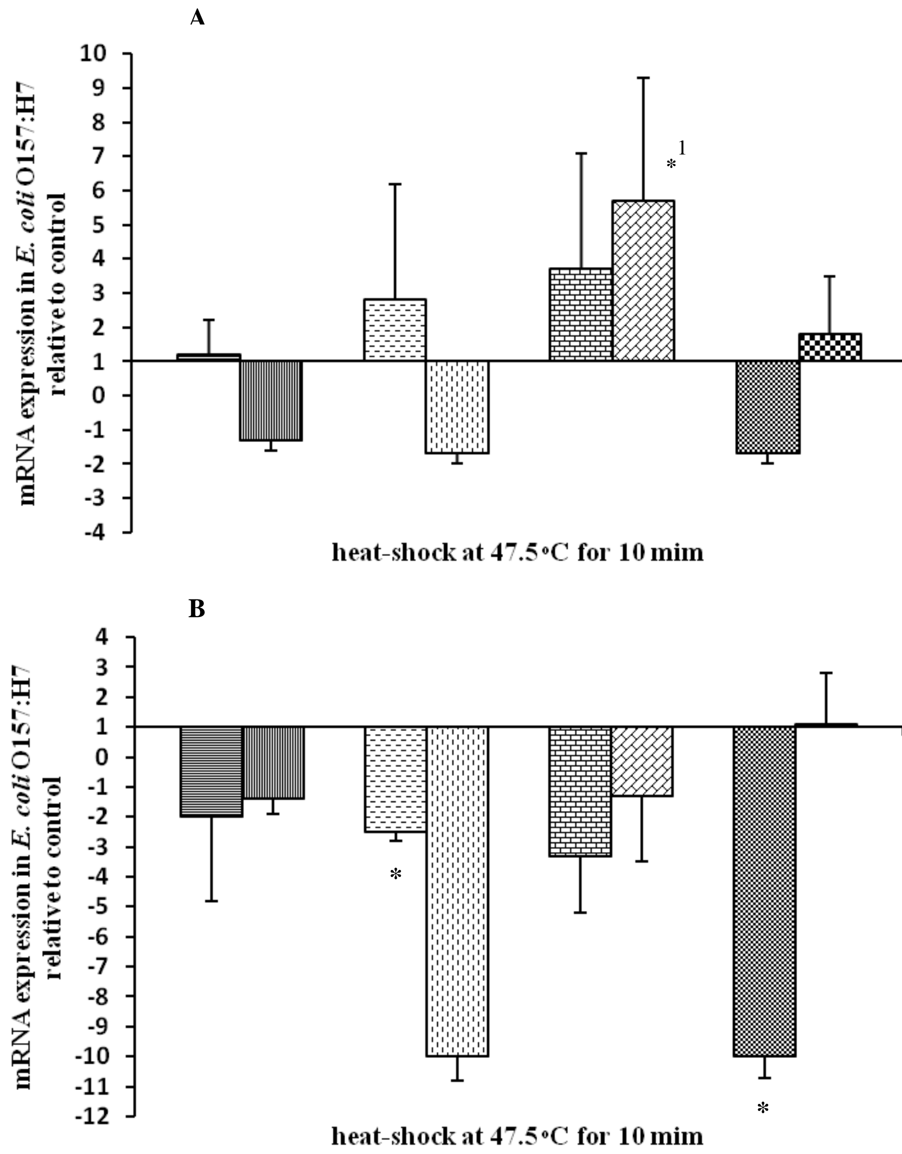


Figure 6.4



[1]* Relative fold change in mRNA level was significantly ($P < 0.05$) different in comparison to control treatment.

Figure 6.5



[1]* Relative fold change in mRNA level was significantly ($P < 0.05$) different in comparison to control treatment.

CONCLUSIONS

Composting is economical means of treating huge amount of animal wastes for use in agricultural practices. High temperature reached during this process also inactivates human pathogens that may be present in manure. However, inefficiency of composting process in inactivating human pathogen has been reported, and has raised concerns on the use of compost as soil amendment or fertilizer for produce productions. In this study we did a thorough study on the factors affecting pathogens during composting and possible mechanisms of pathogen survival. Heat-adaption (heat-shock) of *E. coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* extended their survival to lethal temperature during composting. At the microbial genetic level heat-shock proteins were important in providing protection to pathogens during high composting temperature and prolonging their survival. Temperature rises during initial phase of composting process is also critical as slow rise of temperature extended pathogen survival due to better heat-adaptation. Additionally, low initial moisture and compost nutrient also complemented this effect. In this study, heat-adaptation of *E. coli* O157 was identified as the major microbial stress that affected their survival. Whereas as cross-protection of acid-adaptation to heating was lost during mesophilic phase of the process. In fresh poultry compost inactivation of *Salmonella* was influenced by high ammonia volatilization beside low initial moisture and rate of temperature increase. Overall, physiological state of pathogens, initial moisture level, compost nutrients, and rate of heating (come-up time) affect microbiological safety and compost quality. Monitoring of temperature rise during initial phase of the composting process in addition to use of

mathematical model can help predicting final outcome of the undergoing composting process safety. Overall, results from this research have helped in:

1. Understanding mechanisms of pathogen inactivation during composting and influence of various environmental factors on that.
2. This study has also been important in providing valid data for risk assessment of composting safety.
3. Providing scientific evidence for improving composting operation on farm.