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Thermal Inactivation of Shiga Toxin-Producing *Escherichia coli* in Foods

Malcond David Valladares
University of Tennessee - Knoxville, m.valladares@utk.edu

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I am submitting herewith a dissertation written by Malcond David Valladares entitled "Thermal Inactivation of Shiga Toxin-Producing *Escherichia coli* in Foods." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

Doris H. D'Souza, Major Professor

We have read this dissertation and recommend its acceptance:

P. Michael Davidson, Gina Pighetti, Xiaofei Ye

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

Thermal Inactivation of Shiga Toxin-Producing *Escherichia coli* in Foods

A Dissertation Presented for the
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Degree
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Malcond David Valladares
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DEDICATION

I Dedicated this dissertation research project to the memory of my mother Isabel Cristina Herrera, and family.
And to all of those who contributed on my academic formation since my early years

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ABSTRACT

Emerging non-O157 Shiga toxin-producing *Escherichia coli* (STEC) were recently added to the zero tolerance policy by the USDA-FSIS. Therefore, the precise characterization of their thermal inactivation kinetics in different foods and the effect of stress on thermal inactivation are needed. This research aimed at determining the heat inactivation kinetics of non-O157 and O157 STECs in buffer and model food matrices and the effects of DnaK levels on thermal resistance after acid and heat-shock. Thermal inactivation was carried out in either in 2-ml glass vials or nylon vacuum-sealed bags for buffer and food (spinach, ground-beef, turkey deli-meat, pasta) samples, respectively. Vials or bags were immersed in a re-circulating water bath at various set temperatures for fixed time-intervals. Surviving bacteria were enumerated using Tryptic Soy Agar plates. D-values were calculated using first-order linear and Weibull (for pasta only) models. Total bacterial protein (using Bicinchonic acid assay) and the heat-shock protein (DnaK) concentration were measured (using competitive ELISA) before and after treating overnight-grown cells in Tryptic Soy Broth with either acid- (acetic acid pH 5.5 for 1 h) or heat-shock (46°C for 15 min). All experiments were performed in duplicate and replicated thrice, data were analyzed using SAS ($p<0.05$). For both, *E. coli* O157 and non-O157 in all tested samples, at 56°C D-values ranged from 5.57±0.38 to 15.39±0.14 min; at 58°C D -values ranged from 1.99±0.9 to 7.20±0.55 min and at 60°C D–values ranged from 0.99±0.07 to 2.86±0.22 min. Higher levels of DnaK were detected after sub-lethal injury with heat- or acid-shock that corresponded to enhanced thermal tolerance of all strains, except *E. coli* O111. D-values in buffer for un-shocked cells ranged from 1.49±0.35 to 2.21±0.17 min, heat-

shocked cells from 2.04±0.35 to 2.83±0.35 min and acid-shocked cells from 2.32±0.29 to 4.09±0.29 min. Thus, acid- or heat-shock conditions that might occur during food processing need to be considered during design of thermal inactivation processes and product-formulation to prevent food-borne outbreaks. This study provides insights on the thermal inactivation parameters of O157 and non-O157 STEC in foods that would be beneficial to the food industry.

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INTRODUCTION

More than 70 different serogroups of Shiga-toxin producing *Escherichia coli* STEC are capable of causing illness in humans *(22)*. All strains of *E. coli* O157:H7 are known to produce Shiga-like toxins *(182*,*15*,*151)*. Shiga-like toxins, attribute their name due to their similarities with Shiga toxins, or Verotoxins. *(145*,*248*,*23*,*123)*. *E. coli* O157:H7 can carry one or two distinct Shiga-like toxins, Shiga-like toxin I and Shiga-like toxin II *(165*,*167*,*180*,*225)*. The Food Safety and Inspection Services *(73)* on September 20th, 2011, declared six non-O157 STEC as adulterants in raw meat, non–intact beef product or components, in the same manner as *E. coli* O157:H7 strains *(73)* that were recently included in the zero tolerance policy. According to Wang *et al.(249)* the top six non-O157 serogroups, are O26, O45, O103, O111, O121, and O145, and they account for approximately 70 to 83% of all non-O157 illnesses. Hence, research to determine inactivation of these non-O157 STECs is needed.

According to Doyle *et al.(56)*, *E. coli* O157:H7 is present in 1.5% to 3.7% of the retail meat sold in groceries stores in the United States and Canada, including: beef, pork, poultry and lamb. In addition, other non-food items also have been identified as vehicles of transmission, including: water *(52*,*229)*, person-to-person transmission both in sporadic cases and outbreaks *(193)*. The most common foods associated with *E. coli* are meat, dairy and produce (Table 1.1 and 1.2) *(249)*. *E. coli* outbreaks have been associated with undercooked beef and un-pasteurized milk *(179,39,5,4)*, unpasteurized apple juice *(155, 6)*, cooked meat *(24)*, lettuce *(8)*, unconfirmed food vehicles *(252)*, Fresh spinach *(186)*, fresh curry leaves *(51),* raw clover sprouts *(187)* and more recently

frozen food products *(67).* Therefore, these at-risk food items need to be adequately treated or processed before consumption.

The thermal inactivation of infectious non-spore-forming pathogens is a critical control point in the safe preparation of many foods. Insufficient heating time and inadequate heating temperature as well as unhygienic handling conditions aid in the growth of pathogenic microorganisms in the food. A log-linear first order kinetics model is the most common approach use by the food industry to explain the thermal destruction of microorganisms, where the logarithm of the bacterial number decreases over time at a given temperature *(226*, *240)*. It assumes that microorganisms within a population are identical. However, it is very common to find deviation in the log numbers from the linear model *(184, 240)*. The use of a linear regression approach is still being used in the industry for validation of thermal process despite its poor fit, this may lead to false estimates of heat resistance for non-lineal survival curves. Other models have been proposed to analyze nonlinear survival curves *(116*, *260)*, which include shoulder and tailing effects derived from a logistic based model, making this an ideal approach to estimate the heat resistance when a non-linear behavior is observed *(114)*. The thermal inactivation kinetics of the non-O157 STECs should be compared to the O157 STECs in buffer and at-risk foods and the mechanism of inactivation need to be explored.

It has been proven that pathogenic bacteria are capable of adapting to acid food environments, giving them the chance to stay viable and cause illness if they are present in the sufficient doses *(146)*. *E. coli* O157 and non O157 STEC have similar mechanisms to respond to acid environment, such as the acid tolerance response, the oxidative system, and the glutamate and arginine decarboxylase systems *(37)*. However

some non-O157 STEC strains have an extra acid stress response not present on *E. coli* O157 strains, which is based on chaperon proteins, HdeA and HdeB *(37)*. Even though *E. coli* O157 and non O157 STEC can cause the same disease; the study of their genome reveals that they have different evolutionary histories *(170)*.

The cell defense mechanism against induced stress states like sub-lethal thermal injury causes the acceleration in the production of a group of molecular chaperons, collectively called heat-shock proteins. Chaperon proteins are defined as a group of unrelated proteins that are involved in the correct assembly of other polypeptides, without being part of the final functional structure *(59)*. Bacterial cells trigger the production of chaperon proteins as one of their homeostatic mechanisms against environmental stresses *(76)*. A large group of these chaperone proteins are expressed in response to heat exposure; these are known as heat-shock proteins (HSP) *(58)*. Heat-shock proteins help in prevention of aggregation, degradation of mis-folded proteins and refolding proteins *(162*,*92*,*31)*.

The exposure to heat-shock conditions induces the production of heat-shock protein, which can enhance the cell tolerance to heat *(132)*. These specific proteins will assist the cell to alleviate or reduce damage incurred by the cell *(147)*. Therefore, the role of DnaK levels after sub-lethal injury induced by acid or heat-shock and its corresponding effects on thermal resistance needs to be explored. This research aimed at bridging the existing data gaps that have been identified above including (a) determination of the heat inactivation kinetics of non-O157 and O157 STECs in buffer and model food matrices; (b) determination of the heat inactivation kinetics in pasta

using non-linear models; and (c) exploring the effects of DnaK levels on thermal resistance after sub-lethal injury induced by acid and heat-shock.

CHAPTER I
A REVIEW OF THERMAL INACTIVATION OF *ESCHERICHIA COLI* O157:H7 AND NON-O157 IN FOODS

Thermal inactivation of Shiga toxin-producing *Escherichia coli* (STEC) in foods.

Generalities

Escherichia coli are gram-negative, facultative anaerobic, rod-shaped bacteria, found in the intestinal tract of humans and other warm-blood animals *(53)*. *E. coli* belongs to a diverse group of coliform bacteria of the *Enterobacteriaceae* family *(63)*. They were first discovered in 1885 by the German pediatrician, Dr. Theodor Escherich in human feces of healthy individuals and named *Bacterium coli commune (62)* and later reclassified following the binomial structure as *Escherichia coli* after its original discoverer *(38)*. Despite *E. coli* being a natural member of the colon micro flora and the vast majority being non-pathogenic or inoffensive to humans, a small fraction are known for their pathogenic potential to develop illness in humans *(14)*.

Shiga toxin-producing *Escherichia coli* (STEC) strains are recognized as food-borne infectious agents that cause a number of life-threatening diseases, including post-diarrheal sequelae such as hemorrhagic colitis and hemolytic uremic syndrome *(117 235)*. It is estimated that more than 70 different serotypes of STEC cause disease in humans. STEC are present in a wide variety of animals as part of their natural gut microflora, resulting in a very heterogeneous group of microorganisms.

Escherichia coli through history

The first registered outbreak caused by *E. coli* serotype O157:H7 was reported in Michigan and Oregon in 1982, when 47 persons had bloody diarrhea episodes after eating contaminated hamburgers *(195)*. This microorganism was also isolated in 1975

from sporadic cases of hemorrhagic colitis *(195)*. In 1977, the toxins were discovered for the first time in certain *E. coli* strains known to produce diarrhea *(127)*. An *E. coli* O157:H7 isolate was first reported to produce a toxin in 1983 *(110)*.

These toxins are usually referred as Shiga-like toxins, due to the close relation with Shiga toxin and verotoxins infecting Vero (green monkey kidney) cells in tissue culture *(227)*. Researchers have shown that *E. coli* O157:H7 produces two Shiga-like toxins, *stx1* and *stx2(210)*. Based on their pathologic features *E. coli* can be grouped as enteroinvasive, enterotoxigenic, enteropathogenic and the strains that cause hemorrhagic colitis due to the presence of Shiga-like toxins have been denominated as enterohemorrhagic *E. coli* (EHEC) *(140)*. This distinction is important, because, several strains of *E. coli* can produce Shiga-like toxins, and produce the same disease, besides the O157:H7 serotype *(227)*.

The symptoms of these pathogenic *E. coli* range from mild to severe diarrhea, while others cause urinary infections and other illnesses *(81)*. According to the U. S. Food and Drug Administration *(242)*, the infectious dose for *E. coli* O157:H7 ranges from 10 to 100 cells, however very little is known about the infectious dose for other emerging enterohemorrhagic *E. coli* (EHEC) serotypes. According to Karch *et al. (120)*, the infectious dose of STEC organisms is very low, infectious dose have been reported to be less than 700 organisms for hamburgers, 50 organisms for salami, and even lower in the case of fermented sausage were the infectious dose was less than 1 CFU per 10 g of product.

Hemolytic-Uremic Syndrome (HUS)

The symptoms associated with Hemolytic-Uremic Syndrome include microangiopathic hemolytic anemia, thrombocytopenia and renal failure *(227)*. According to Wong *et al. (262)*, the groups at risk are predominantly infants and young children; that are more prone to develop renal failure. Moreover, hemorrhage and necrosis is usually observed in the cecum and the ascending colon *(227)*, along with non-specific inflammation *(11)*, chronic ischemic colitis *(205)* full-thickness necrosis *(21)*, and colonic perforation *(234)*.

Martin *et al. (151)* suggested that host related factors are involved in the higher incidence of HUS in children younger than 5 years old. The fatality rate of patients with HUS range from 5% *(262)* to 10% *(121)* among children, and a fatality rate of 88% associated with HUS was reported during an outbreak involving elderly patients *(36)*. Moreover, the severity of the infection was reported to be strain dependent, where patients were more likely to develop HUS when the clinical isolates contained Shiga-like toxin type II, suggesting that Shiga-like toxin II is more virulent than Shiga-like toxin I *(177)*.

In addition to microangiopathic hemolytic anemia, thrombocytopenia and renal failure *(227)* complications with the central nervous system have been described to be associated with HUS in 30% of the cases *(79*, *44*, *199)*. The neurological manifestations can range from mild irritability and lethargy *(10)* to more serious complications including seizure and coma *(207)*.

Serology of E. coli

Serological elements are used to categorize different strains of pathogenic *E. coli* are based on the immune response in animals, which includes somatic antigen O, capsular antigen K and flagellar antigen H. The O antigen denotes a thermostable surface antigen found in the cell walls in all *Enterobacteriaceae.* This is a specific polysaccharide that retains agglutinating-binding capacity even after boiling *(175)*. In addition K antigen refers to envelope or capsule antigen based on the loss of the agglutination ability of non-heated bacteria *(175)*. The antigen H denotes the variations found in the single protein polymer that conform the flagellar filament product of the gene fliC gene *(130, 131)*.

Microbiology of Shiga-like toxin I and II

In 1898, Kiyoshi Shiga discovered Shiga toxin from *Shigella dysenteriae* serotype *(214)*. Shiga toxin producing *E. coli* (STEC) has been recognized as the causal agent of several outbreaks in the U.S. and worldwide *(27*, *103)*. However, the toxin is known to be present in other bacteria genera, such as: *Citrobacter*, *Enterobacter* and *Acinetobacter (209*, *183*, *87)*.

All strains of *E. coli* O157:H7 are known to produce Shiga-like toxins *(182*, *15*, *151)* with one exception *(176)*. Shiga-like toxins, attribute their name due to their similarities with Shiga toxins, or verotoxins but their infectivity is not restricted to Vero cells alone. They can have a cytotoxic effect in other cell lines including *HeLa*, intestinal Villus cells, endothelial cells and Burkitt Lymphoma cells. *(145*, *248*, *23*, *123)*. *E. coli* O157:H7 can carry one or two distinct Shiga-like toxins, Shiga-like toxin I and Shiga-like toxin II, with distinctive physicochemical and immunological characteristics *(165*, *167*,

180, *225)*. However, repeated laboratory culturing may result in the loss of the toxin encoding genes, resulting in the absence of Shiga-like toxins, due to the instability of the toxin genes *(77)*.

Shiga-like toxin I share almost identical biological properties with Shiga toxin found in *Shigella dysenteriae* type I *(106)*, they only differ at the nucleotide at the protein level *(166-168, 225)*. Several variants of the Shiga-like toxin have been identified including: Stx1c and Stx1d *(32)*. Shiga-like toxin I and Shiga toxin are identical in terms of heat stability and isoelectric point, also both can be neutralized using antiserum to purified Shiga toxin *(165-168)*. Moreover Shiga toxin and Shiga-like toxin I also share the same units structure, having one A 32 kDa and five 7.7 kDa identical B subunits *(233*, *221*, *70)*. Shiga toxin and Shiga-like toxin I have more than 99% homology in their nucleotide sequence, deferring only by one aminoacids on the subunit A *(225*, *33*, *106)*, and four aminoacids differences in the entire gene alignment *(134)*.

In contrast, Shiga-like toxin II has a different antigen response compared to Shiga-like toxin I, but they still are genetically related *(181*, *225)*. Shiga toxin and Shiga-like toxin II only share 55-60% of nucleotide sequence homology, and in contrast with Shiga toxin I, also it cannot be neutralize using antiserum *(180, 181, 225*, *256)*. Several Shiga toxin II have been identified including: Stx2c, Stx2d, Stx2e, Stx2f, and Sxt2g *(72*, *139*, *208)*.

Regardless of their differences in sequence and immunogenic response, Shiga like toxin I and II still have the affinity and bind the same cell receptor. The B subunits of Shiga-like toxin I and Most of the Shiga-like toxin II bind specifically to glycosphigolipidglobotriaosylceramide (Gb3), this receptors are present in eukaryotic

cell membranes *(50)*. Shiga-like toxin II (Stx2e B subunits) primarily binds to globotetraosylceramide (Gb4), that enables the toxin to bind different cell tissues and therefore infect different cell lines *(50)*. Additionally to the receptor binding similarities they also share the same mechanism of action in vitro as Shiga toxin *(145*, *248)*. The cell receptor for Shiga toxins and Shiga-like toxins I and II is mainly found in human endothelial cells *(248)*, and is highly expressed in the cortex of the human kidney tissue *(23)*. After the toxin is bound to its receptor, it triggers endocytosis producing toxin-containing vesicles, that travel across the Golgi apparatus and the endoplasmic reticulum in the attacked cell *(204)*.

The A subunit is split into A1 (27.5kDa) and A2 (4.5kDa) however, this two components of the A subunit still covalently bound by disulfide bonds between the two cysteine residues *(60)*. Cysteine's residues are reduced, by the catalytic active A1 enzyme, which cleaves a specific adenine form the 28s rRNA of the 60s ribosomal subunit *(60)*. This process will affect the association of the GTP/elongation factor Tu/amino acyl-tRNA complex with the ribosome; that will stop the amino acid chain elongation resulting in cell apoptosis.

The genes responsible for the production of Shiga-like toxin I and II are encoded in the lamboid bacteriophage. It has been suggested that toxin production is a response to lysogenization by one or more toxin-converting phages *(169)*. Even though Shiga-like toxin I and II are similar they cause different levels of damage, Enterohemorrhagic *E. coli* carries Stx2 which is more prone to cause HUS compared to those who just carried the Stx1 toxin *(215)*, It is been proposed that this difference could be attributed to

difference in the accessibility of the active site in the carbonyl end of the A subunit or differences in affinity binding site of the B subunit with the Gb3 receptors *(71)*.

Epidemiology

It is estimated that more than 70 different serogroups of STEC cause disease in humans *(22)*. The Food Safety and Inspection Services *(73)* on September 20th, 2011, declared six non-O157 STEC as adulterants in raw meat, non–intact beef product or components *(73)*. As of March 2012, these top six non-O157 STEC serogroups are included in the zero tolerance policy. According to Wang *et al. (249)*, the top six non-O157 serogroups, namely O26, O45, O103, O111, O121, and O145, cause approximately 70 to 83% of all non-O157 illnesses with an incidence rate of 26%, 5%, 22%, 19%, 6%, and 4%, respectively *(83)*. This incidence rate for non-O157 STEC infections increased from 0.12 per 100,000 population in 2000 to 0.95 per 100,00 in 2010; while the O157 STEC infections decreased from 2.17 to 0.95 per 100,000 *(83)*.

When comparing 2011 with 2006-2008, the overall incidence of infection of the six major foodborne pathogens, *Campylobacter*, *Listeria*, *Salmonella*, STEC *O157*, *Vibrio*, and *Yersinia*, did not change significantly. However, individual pathogens, especially O157 STEC, had a significant decrease of 26% *(206)*. In 2010, 96 cases of HUS were reported in the US, with 1 death and 88% cases being children *(206)*.

Epidemiological studies have shown that Hispanics were the ethnic group more susceptible to non-O157 infections, and infections in general were less severe than those caused by O157 serogroups, but this is serogroup dependent *(83)*. According to Gould *et al. (83)* non-O157 STEC infections associated with outbreaks (not sporadic cases) were 7% compared to 20% of infections caused by O157 STEC. A greater

proportion of the cases of non-O157 were associated with international travel, 14% compared with only 3% from O157 STEC. Therefore, an improved understanding of the epidemiological features of the non-O157 STEC groups is still needed to prevent spread from the food safety stand point and to improve diagnostic tools for rapid detection. There remains an urgent need to develop new sensitive, reliable methods for rapid detection and quantification of these specific “regulated” STEC serogroups, for evaluation/application of beef and other high-risk foods with the zero tolerance policy *(250)*.

More recently another serotype has been added to the list, which is enteroaggregative EHEC O104:H4, identified as the causative agent of the May to June 2011 outbreak in Germany and other European countries *(243)*; where around 4,137 cases were reported and approximately 22% developed Hemolytic Uremic Syndrome (HUS) with 33 deaths. The German authorities associated the outbreak with contaminated sprouts *(264)*. The first documented case of *E. coli* O104:H4 occurred in the US, in the state of Montana in 1995, and prior to that the first case of HUS due to *E. coli* O104:H4 was reported in Korea in 2006 *(42)*. A mixture of specific virulence factors makes the O104:H4 strain extremely pathogenic and resistant to antibiotics and environmental stress. Binding properties of an EAEC and a STEC with aggregative adherence to intestinal epithelial cells was demonstrated, but it does not present the intestinal adherence factor intimin, and also lacks of the *ehx* gene encoding the enterohemolysin toxin *(264)*.

According to Tzchoppe *et al. (243)* STEC and EHEC can carry one or more Shiga toxin (*stx*) genes, the EHEC strains can also be differentiated by the presence or

absence of the LEE (locus of enterocyte effacement), the (*eae*-gene) which is a gene that encodes a mechanism for intestinal colonization, and also by no-LEE (*nle* genes) *(243)*. The presence of EHEC plasmids, such as enterohemolysin (*ehx* gene) can also be used for characterization. Aggregative EHEC such as O104:H4 carry an aggreggative adherence fimbriae (AAF) plasmid related for intestinal colonization, and (aggR) gene responsible for aggregative adherence *(243)*. The German strain (*E. coli* O104:H4) was found resistant to a wide variety of antimicrobials such as penicillin (e.g. ampicillin), third-generation cephalosporin, trimethoprim-sulfa-methoxazole but they present susceptibility to fluoroquinolones (ciprofloxacin) and aminoglycosides (gentamicin or tobramycin) *(264)*.

Transmission

The transmission STEC is via the fecal to oral route, via contaminated food or water, animal contact and person-to-person contact. Dairy and beef cattle are both considered a domestic reservoir of *E. coli* O157:H7, even though this microorganism is more often isolated from dairy cattle *(152)*, as *E. coli* O157:H7 has been isolated from milk and feces of healthy cows *(152, 258)*. It is estimated that in North America the prevalence of *E. coli* ranges from 0 to 60% of the feedlot cattle herd *(108)*. Its presence in cattle is asymptomatic, since cattle do not possess the Shiga toxin receptor, globotriaosylceramide *(188)*.

According to Doyle *et al. (56)*, *E. coli* O157:H7 is present in 1.5% to 3.7% of the retail meat sold in groceries stores in the United States and Canada, including: beef, pork, poultry and lamb. In addition other non-food items also have been identified as vehicles of transmission, including: water *(52*, *229)*, person-to-person transmission both

in sporadic cases and outbreaks *(193)*. In special scenarios such as nursing homes, day care centers, secondary (person to person) transmission becomes a major vehicle of spread *(36, 201)*.

The most common foods associated with *E. coli* are meat, dairy and produce (Table 1.1 and 1.2) *(249)*. *E. coli* outbreaks have been associated with undercooked beef and un-pasteurized milk *(179, 39, 5, 4)*, unpasteurized apple juice *(155, 6)*, cooked meat *(24)*, Lettuce *(8)*, unconfirmed food vehicles *(252)*, Fresh spinach *(186)*, fresh curry leaves *(51),* raw clover sprouts *(187)* and more recently frozen food products *(67).*

PCR detection studies of *E. coli* O157:H7, have shown that filth flies *Diptera, Muscidae* and *Anthomyiidae* including house flies *Musca domestica*, Black dump fly (*Hydrotaea aenescens*), stable fly (*Stomoxys callcitrans* and *Adia cinerella*), are carriers of *E. coli* O157:H7 *(232, 105, 126)*, therefore have the potential to spread the microorganism as well.

Detection

Detection of E. coli O157

Over last decades *E. coli* O157:H7 and other STEC have emerged as important gastrointestinal pathogens *(14)*. The capacity to control outbreaks and illness depends o rapid detection *(14)*. It is estimated that 93% of all *E. coli* from human origin can ferment sorbitol, whereas *E. coli* O157:H7 is sorbitol negative *(257)*. In addition *E. coli* O157:H7 can also be characterized by the inability to hydrolyze 4-methylumbelliferyl-β-D-glucuronide (MUG) *(54)*. Standard fecal coliform methods usually include an incubation

step at 45^{o}C, therefore they are not suitable to isolate *E. coli* O157:H7 due to its inability to grow at 45^{o}C in presence of 0.15% bile salts *(54, 231)*. One of the most widely used method for detection in clinical laboratories is the cultured based method using Sorbitol MacConkey Agar (SMAC) This method does not account for sorbitol fermenter STEC serogroups *(179)* such as the big six non-O157 STEC.

E. coli O157:H7 due to its inability to ferment sorbitol appear colorless on SMAC agar, in contrast to *E. coli* O104:H4 that appear as pink colonies *(264)*. *E. coli* O157:H7 is considered a slow sorbitol fermenter after 42 hours incubation *(66, 149)*. The SMAC medium is consider to be 100% sensitive, 85% specific and 86% accurate for detecting *E. coli* O157:H7 *(149)*. Furthermore the isolated colonies can be picked and analyzed using biochemical test, by serotyping their response to O157 and H7 antisera and the presence or absence of Shiga-like toxins *(257, 54, 88, 194, 238)*.

The process of screening for *E. coli* O157:H7 using SMAC followed by biochemical characterization and serotyping is considered to be laborious and time consuming *(227)*. Moreover, it is known that the rate of isolation decreases over time; if the stool/clinical samples are obtained 6 days after the onset or the use of antibiotics negative results can be obtained *(218, 84, 236, 257)*.

Commercially available latex slide agglutination test offer an efficient reliable and more rapid option compared to the traditional serotyping *(41)*. However the latex slide agglutination test result is recommended to be confirmed by using a direct immunofluorescence antibody test *(41, 150)*. Most of the methods used for enterohemorrhagic *E. coli* (EHEC) identification were developed for animal origin food matrices, food from vegetable origins have totally different composition and also a

different natural microflora. Therefore, methods for detection and enrichment have to be modified to match the respective food matrix being studied. The selection of the enrichment media has a direct impact on the selective growth and isolation of EHEC extracted from food matrices *(243)*.

Selective enrichment has been widely used to isolate *E. coli* O157:H7 from foods, however at the same time these selective agent can inhibit the growth of injured cells of *E. coli* O157:H7, this may explain why some methods developed for clinical samples perform poorly when they are applied to food samples, where processing (low pH, additives, freezing, heating) may cause some damage/injury to the cells *(20, 91)*.

Also, as reported by Weagant *et al. (253)*, high levels of coliforms that are found in the background flora may cause interference with the isolation of *E. coli* O157:H7. Therefore, immunomagnetic separation after the enrichment step has been used to enhance the isolation of the *E. coli* O157:H7 from food matrices. Selective media is required for isolation and detection of specific bacteria among the background natural flora of food, but it also may inhibit the growth of injured cells, this can be overcome by using a non-selective enrichment for 8 h and immunomagnetic separation (IMS). Kanki. *et al. (117)*, described an IMS method for recovery of *E. coli* O157:H7 from foods contaminated with background flora after an enrichment step using paramagnetic particles coated with *E. coli* O157 antibodies .

Other methods can provide similar results in terms of sensitivity compared to the immunological assays, such as IMS–plating method. However this higher sensitivity can be attributed to false positive results. Also false negatives can be due to the low concentration of pathogen and low sensitivity of the assay, suggesting that IMS-plating

using SMAC with cefixime and tellurite (CT-SMAC) can be more sensitive compared with immunological methods *(117)*.

Detection of injured cells

According to Rocelle *et al. (196)*, during processing, foodborne pathogens are exposed to high and low temperatures and also chemical additives. These processing steps are used to enhance the shelf life of a product that may cause cell membrane damage, increasing their sensitivity to selective agents during the enrichment stage. However, Injured cells of pathogenic bacteria may undergo repair and regain the ability to cause illness when conditions are favorable for growth *(196)*.

Most of the commercially available selective media used to detect *E. coli*, often do not support the repair of injured cells as they contain bile salts, crystal violet, which can inhibit the repair of injured cells *(196)*. The introduction of a resuscitation step incubation at room temperature for 3h, using a non-selective broth, prior to enrichment using modified *E. coli* broth + novobiocin (mEC+n), has been shown to increase the recovery of injured cells of *E. coli* O157:H7, with exception of some acidic foods *(91)*. One of the critical aspects of resuscitation is the recovering time, because other microorganisms present in the sample will begin multiplying before the injured cells are fully recovered *(196)*. Kanki *et al. (117)* suggested that, enrichment broths for STEC should be evaluated in terms of their capability for resuscitation, instead of growth, because additionally the UBP enrichment demonstrated to be equally effective for facilitating detection of undamaged strains as other examined media. Modified sorbitol MacConkey Agar (MSMA) is recommended for enumeration of *E. coli* O157:H7, but according to Rocelle *et al. (196)* it does not perform well detecting heat-stressed injured

cells, an also is unable to recover cells from frozen chicken samples. These facts increase the importance of assess a better approach for detection and enumeration of *E. coli* in food matrices.

Detection of E. coli non-O157

According to Jinneman *et al. (109)*, most of the methodologies for screening STEC, are mainly aimed to detect *E. coli* O157:H7. But the increase in the number of outbreaks related to non-O157 *E. coli*, further emphasizes the need to develop more efficient and less time consuming methods for their rapid detection isolation and quantification *(83)*. In the U.S. *E. coli* O157 and non-O157 STEC both have similar trends in terms of the seasonal incidence, with highest incidence rates occurring in the summer months *(26*, *216)*. Human fecal samples usually are not tested for STEC *(99)*. As more clinical laboratories tested routinely for non-O157, more isolates were found *(82)*. Diagnosis of infection with non-O157 STEC is more difficult, as clinical diagnosis is based on detection of the presence of Shiga toxins on the stool samples. For this reason non-O157 STEC infection are often misdiagnosed, that are known to cause the most serious symptoms of STEC illness. The CDC estimates that for every case of O157, 26.1 cases are not diagnosed and for every case of non-O157 STEC there may be 106.8 cases that are not diagnosed *(206)*. Therefore, the CDC is now encouraging and recommending testing for non-O157 STEC in clinical laboratory diagnosis *(82)*.

The lack of phenotypic biochemical characteristic that can distinguish non-O157 STEC from O157 or generic *E. coli* makes detection of non-O157 STEC somewhat challenging *(178)*. The International Organization for Standardization *(220)* and the U.S. Department of Agriculture *(68)* recommend the use of modified tryptic soy broth

(containing 20 mg/L) 1 novobiocin (mTSB + n) for the enrichment of O157 from food samples. However, according to Paddock *et al. (178)*, the inclusion of antibiotics in media has shown to inhibit certain serotypes and strains within serotype. Moreover, novobiocin and bile salts, are known to inhibit the resuscitation of injured non-O157 cells *(173, 222)*. Therefore, other media may be more suitable for the enrichment of non-O157 STEC.

One promising medium for the enrichment of non-O157 STEC is universal pre-enrichment broth (UPB), which lacks inhibitory agents, is highly buffered and contains low amounts of carbohydrates *(9)*. Kanki *et al. (118)* demonstrated that UPB is comparable to or superior than mEC + n for the analyses of food samples containing low numbers (<10 CFU per 25 g of food) of O157 and O26 cells concluding that UPB would be a suitable enrichment medium for non-O157 STEC. Rocelle *et al. (196)* evaluated other media (MSMA and TSA) for recovery after exposing cells to heat-stress. Despite the heating time as expected MSMA exhibited the poorest performance indicating that a fraction of the injured cells were not able to resuscitate. In contrast the TSA media exhibited the best results, giving the highest recovery of non-O157 cells regardless of the heating time, when the exposure temperature was 52^{o}C. The difference in the number of cells recovered became more apparent as the heating time was increased to 30 min, when the population of viable cells detected on TSA exceeded that of those on MSMA by approximately 10^3 CFU ml *(196)*. Therefore, extreme caution must therefore be exercised in interpreting results of studies with stress-injured organisms, especially when only one strain is being evaluated and selective media are used for enumeration *(196)*.

The screening for Shiga-like toxins offers another sensitive alternative for diagnosis of infection, that allows the detection of O157:H7 as well as other Shiga-tlike toxin producing serotypes such as the emerging big sis non-O157 STEC *(122, 129)*. These toxins can be detected in broth suspensions and stool extracts *(110*, *168*, *122)*. In addition, other advantages of detecting the Shiga-like toxins is that there are more persistent, since they can be detected even, when *E. coli* cannot be cultured from the stools samples *(122, 129)*. After 4 to 9 days the shedding of *E. coli* O157:H7 in the fecal matter decrease to undetectable levels, but free fecal Shiga-like toxins could still be detected even after 4 to 6 weeks *(36, 121)*. There is plenty of literature available regarding the molecular approaches for the detection of *stx1* and *stx2* using PCR, real time PCR (RT-PCR) and another isothermal amplification (loop-mediated isothermal amplification LAMP) methods, that offer a quick option for the detection of STEC however they require highly trained lab personnel to perform the tests.

Thermal inactivation

The thermal inactivation of infectious non-spore-forming pathogens is a critical control point in the safe preparation of many foods. Insuficient heating time and inadequate heating temperature as well as unhygienic handling conditions aid in the growth of pathogenic microorganisms in the food.

At a given temperature, the processing time will depend on the initial microbial population present in the food system, and therefore the thermal process has to be planned based on the anticipated microbial load. Bacterial thermal resistance can be

explained using two numerical terms D- and z values. D-value is defined as the time required to destroy 90% or one $\log_{10}$ of the initial microbial load of a specific microorganism at a given temperature, while the z-value is a reference to the thermal resistance of the microorganism at different lethal temperatures and is the change in the temperature required to change the D-value by 1 log *(113*; 61*)*. The decimal reduction time or D-value varies with microbial species, spores, temperature, age of the culture and suspending media *(228)*. The D-value can be calculated using the following equation

$$D = \frac{t_2 - t_1}{logN_1 - logN_2} \quad \text{(Eq. 1.1)}$$

Where (N) is the number of viable cell present at (t) time.

The calculation of the D-values at different temperatures facilitates the calculation of the z-value, this allows integrating the lethal effect of temperature within a given thermal process *(18)*. The z-value is calculated by constructing a thermal death time (TDT) curve by plotting the logarithm of D-values versus exposure to temperatures *(61)*.. The z-value can be calculated from the following equation

$$z = \frac{T_1 - T_2}{logD_2 - logD_1} \quad \text{(Eq 1.2)}$$

Where D2 and D1 are values corresponding to the temperature T2 and T1, respectively *(228)*.

The F-value for a process is the length of time required in order to inactivate a known population of microorganism in a given menstruum under specific conditions. The F-value is commonly set to be 12D for sterilization, meaning a theoretical 12 $\log_{10}$

reduction of the most heat resistant species of spores in canned food *(246)*. In other words there is a chance of getting one contaminated can out of 1,000,000,000,000 cans. The F-value is calculated by the D-value by the number of log reductions desired, given a $D_{121°C}$-value of 1 min a 12D will be of 12 min *(246)*.

There are different factors affecting the heat resistance of the microorganisms, some can be attributed to intrinsic factors such as inherent heat resistance of the microorganism, for example difference in thermal resistance among different species and strains, and their physiological states. Extrinsic factors such as environmental conditions during the cell conformation stage: growth temperature, nutrient availability, previous stress exposure prior thermal treatment, and during the thermal treatment of the cell suspension such as: composition of the heating menstruum, water activity (a_w), pH, added preservatives, heating method, and recovery of survivor methodology all play a role in heat resistance *(113)*.

The recovery of heat injured cells has an impact on the calculation of the heat resistance of the microorganism, therefore the choosing an inappropriate method of enumeration of survivors of heat inactivation experiments can lead to inaccurate values. In a study by Murano and Pierson *(163)*, using anaerobic incubation of agar plates for *E. coli* O157:H7 resulted in D-values up to twice as great as those from aerobically incubated plates, similar behavior has been reported for *Salmonella (265)*.

It is acknowledged that in general when the water activity decreases, the bacterial heat resistance will increase, however the heat resistance of *E. coli* O157:H7 was shown to increase with increasing NaCl up to 8.5% w/w *(19)*. This is important for food products that have a low water activity such as peanut butter. Weagant *et al. (254)*

discovered that some strains of *E. coli* O157:H7 were able to survive for relatively long time in food with pH ranging from 3.7 to 4.4. Moreover Hill *et al. (98)* proved that the adaptation of these bacteria to acidic environments occurs with relative ease. In addition to acquisition of resistance to a single factor like pH, may also trigger enhanced resistance to other environmental factors including high temperature or high salt concentration *(198)*.

A significant relationship between the final pH of the culture medium and thermal resistance when *E. coli* is heated at 58 °C in milk or chicken broth was reported *(30)*; the D-value at 58 °C for *E. coli* O157:H7 at the final pH of media between 4.6-4.7 was 3 folds higher, with a 5D of 40.6 min, compared to *E. coli* in a final pH of the cultured media between 7.0-7.2. This acid adaptation follow by the increase in the thermal resistance of *E. coli* is referred as cross protection *(98)*.

Traditionally the heat resistance of microorganisms is based on data obtained from experiments conducted on menstruum at neutral pH and high $a_{w,}$ however pH and a_w of food products is very heterogeneous and therefore it will influence the heat resistance of microorganisms present in the food matrix *(19)*. In this regard, validation of the thermal process of each thermally processed product is necessary.

Generally gram-negative bacteria have been considered to be sensitive to high temperature, but it has been demonstrated that they do not always undergo complete inactivation during heating, this may represent a threat specially in the case of pathogenic strains whose infection dose is very low, since these cells may not be detected during routine food screening analysis, but sub-lethally damaged cells capable of regeneration can be still present *(244)*

When the inactivation test is carried in food systems, the food characteristics can provide a thermo- protectant effect. This effect can be attributed to the presence of the different compounds including: carbohydrates, lipids salts, etc. It is known that thermal resistance can be affected by alteration in the fatty acid profile of the cell membrane; therefore the target of thermal inactivation is to damage the nucleic acid, proteins, enzymes and the cellular membranes *(113)*.

In past years extensive research has been done regarding *E. coli* thermal inactivation kinectics in several food systems. Clavero *et al. (45)* reported different D-values among different strains of *E. coli* O157:H7. in meat at 50-64^{o}C ranging from 96.67 to 0.16 min *(2*, *144*, *55)* suggesting that the microrganism behavior is under the normally expected heat resistance. The majority of thermal resistance studies of enterohemorrhagic *E. coli* have been focused on the effect of temperature alone on different foods, principally in meats due to epidemiological importance *(244)*.

These studies include the counts reported by Abdoul-Raouf *et al. (1)*, *E. coli* O157:H7 decreased by 6 log cycles within 14 min in ground meat heated at 56^{o}C. Duffy *et al. (57)* determined D-value at 50, 55 and 50°C in minced salami (pH 4.7-5.1) for three *E. coli* O157:H7 strains. At 50°C the D-values ranged from 116.93 to 91.14 min,, calculated by subtracting the numbers obtained for TSA plates minus SMAC agar plates (TSA-SMAC) agar plates while in SMAC agar plates the vales range from 130.46 to 41.55 min.at 55°C the D-values range from 17.00 to 21.91 min for the TSA-SMAC agar plates; while in SMAC agar plates the values ranged from 12.25 to 21.07 min. AT 60°C D-values ranged from 1.18 to 2.21 min in TSA-SMAC, however they were not able to recovery any survivor using SMAC agar plates *(57)*. The surviving populations

recovered from all the heating temperatures, specially at 60°C were mainly composed of damaged cells which were unable to grow on selective agar, however they still can undergo a repair process under favorable conditions *(57)*.

Ahmed *et al. (2)*, studied the thermal inactivation of *E. coli* O157:H7 in pork sausages with different fat content, the D-values were 6.37, 7.83 and 11.28 min at 55^{o}C and 0.37, 0.46, and 0.55 min at 60^{o}C in 7%, 10%, and 30% fat content respectively. Line *et al. (144)*, reported D-values for *E. coli* O157:H7 in ground beef with 30% and 2% fat content at 57.2^{o}C of 5.3 and 0.47 min respectively; at 62.8^{o}C, the D-values were 4.1 and 0.3 min for 30% and 2% fat content respectively. Juneja *et al. (115)*, reported D-values for *E. coli* O157:H7 of 21.13, 4.95, 3.17, 0.93 and 0.39 min in ground meat with 10% fat and pH 6.0 at 55, 57.5, 60, 62.5 and 65^{o}C, respectively; and D-values for *E. coli* O157:H7 of 11.83, 3.79, 1.63, 0.82, and 0.36 min in ground chicken pH 6.0, using the same temperatures, respectively. It was shown that the higher the fat content the higher the D-value at 50 and 55^{o}C compared to the lower fat content meats *(2)*. However, Rajkowski *(190)* obtained opposite results for thermal resistance of *E. coli* O157:H7, when comparing the D-values at 55^{o}C of tilapia with a fat content of 1.7% and cat fish with a higher fat content of 7.6% both at pH of 6.3, moreover there was no significant difference in the D-values at 60 and 65^{o}C for both fish samples.

Modeling

A predictive microbial model is a mathematical approach to explain the growth, survival, inactivation or biochemical process of a foodborne microorganism. An alternative is to fully understand the responses of the microbes of concern to the key controlling factors in the food environment, to build a cumulative store of knowledge,

and to develop the means to interpolate calculated microbial responses *(80)*. The rapid development of microbial models and their ability to predict microbial inactivation makes modeling an invaluable research tool. Use of models can provide information quickly and so the real value and usefulness of predictive models should be appreciated *(80)*.

A log-linear first order kinetics model usually explains thermal destruction of microorganisms, where the logarithm of the bacterial number decreases over time at a given temperature *(226*, *240)*. It assumes that microorganisms within a population are identical. However, is very common to find deviation in the log numbers from the linear model *(184, 240)*. The use of a linear regression approach is still being used in the industry for validation of thermal process despite its poor fit, this may lead to false estimates of heat resistance for non-lineal survival curves. Other models have been proposed to analyze nonlinear survival curves *(116*, *260)*, which include shoulder and tailing effects derived from a logistic based model, making this an ideal approach to estimate the heat resistance when a non-linear behavior is observed *(114)*.

According to Pflug and Gould *(185)* deviations from the linear declines in the log numbers can frequently occur. Shouldering in the survivor curve can be attributed to an initial lag period *(115)*. Additionally Kontrola and Conner *(128)* explain the shouldering phenomena to be cause by poor hear transfer through the heating menstruum. Chung *et al. (43)* through computer simulation confirmed that the shouldering observed in the survival curves was a result of the thermal lag caused by slow heat transfer in the samples. These studies clearly suggest that there is an initial lag period or shoulder where the bacterial numbers stay at the inoculum level, followed by an exponential reduction and sometimes an even a tailing effects is also observed due to more

persistent bacterial sub-population that decrease in numbers at a slower rate compared to the rest of the microorganisms of the initial inoculum *(114*, *107)*. Hansen *et al. (90)* reported that tailing could be attributed to the clumping of cells in the heating menstruum, therefore providing a protective effect acting like a shield allowing the cells in the center to survive longer during the heat treatment *(40*, *125)*. Although, this was confirmed by Cerf *(40)* and Klijin *et al.. (125)*; a bacterial clump in average has a 2 µm in diameter, this was demonstrated to be not big enough to provide a significant delay in the heat transfer to the center of the bacterial clump *(49)*. There are many potential causes that could help to explain the deviations from the first order inactivation model, including: the existence of multiple critical sites of bacterial cell *(157)*, heterogeneity of heat resistance *(89)* and experimental artifacts *(40*, *226*, *247)*.

In a study conducted by Black *et al. (17)*, potential stress adaptation is indicated by longer inactivation times, this behavior was more evident at 58^{o}C. When comparing statically versus chemostat grown *E. coli,* the shape of the response curve was significantly different, where cells that were statically grown had a decreasing thermal sensitivity over time, in contrast chemostat cells exhibit the opposite effect, showing an increasing thermal resistance *(17)*. Less variability of the data points was observed when the heating menstruum was buffered to a final pH of 7.0, and depending on the stage of reduction time to a specific log reduction value for the Weibull model were conservative relative to the log-linear model. In general, the Weibull model expresses a most accurate fit of the model for the data, since Weibull model is a variation of the log-linear model with a fix shape factor of 1 *(17)*.

Black *et al. (17)* reported a higher data scatter when the heating menstrumm was peptone buffer in comparison to phosphate buffer, which could be attributed to the result of stress protection, greater genetic diversity or more significant pH changes in the growth environment, but the exact reason still unknown *(17)*. Black *et al. (17)* concluded that the differences in inactivation of *E. coli* depend on the physiological state of the cells at the time of exposure to the heat treatment; as well to the conformation of the heating menstruum, indicating the importance of the environment conditions which the cell were exposed before and during the thermal inactivation experiment is conducted.

Therefore, is necessary to accompany the thermal resistance results with a complete description of how the cells were grown and kept before and during the thermal inactivation trial, in order to avoid errors for the fit of the model calculations.

Weibull model

While the conventional first-order model implicitly assumes that microbial populations are homogeneous from the point of view of their heat resistance, some researchers *(148)* assumed that at a given temperature, the time of heat exposure, which caused the death of a microbial cell or a bacterial spore, is variable from one individual to the other, and that the dispersion of individual heat resistance was governed by a Weibull distribution. The model can be expressed as:

$$\log\big(S(t)\big) = -\frac{1}{\log(10)}\left(\frac{t}{\alpha}\right)^{\beta} \qquad \text{(Eq. 1.3)}$$

For β>1 (the shape parameter) results in convex curves; β<1, results in concave curves and when β=1 the model becomes a first order kinetic model. Then α, the Scale parameter can be denoted as the time for the first decimal reduction (pseudo D-value).

β<1 suggest that the cell at any point in the inactivation curve are capable to adapt to the applied stress this is known as tailing effect. β>1 can be interpreted, as the remaining cells are becoming increasingly damaged this is known as shouldering effect. The advantage of the Weibull model is the ability to explain linear models as well as deviation when shouldering and tailing is observed

Thermal resistance enhancement -Cross protection

It is generally *(154)* acknowledged that fruit juice acidity was an effective control against pathogenic microorganism *(124, 154)*. There is a growing concern about the potential survival of acid adapted *E. coli* O157:H7, especially in minimally processed foods, due to the cross protection effect *(202)*. *E. coli* outbreaks have been linked to acid foods including fruit juices, fermented sausage *(46)*, mayonnaise *(254)* and yogurt *(161)*. It has been proven that pathogenic bacteria are capable to adapt to acid food environments, giving the chance to stay viable and capable to cause illness if they are present in the sufficient doses *(146)*.

The acid adaptation is a metabolic response to pH that results in physiological changes in the cells structure, enhancing the acid resistance of the sub lethal exposed population *(259)*. According to Rowbury *et al. (197)*, the production of lower amounts extra cellular components mainly stress proteins and or different proteins in response of chemical and physical stresses can influence the level of acid tolerance of *E. coli.* Studies also showed that sub-lethal exposure to acidic conditions induce cross

protection against thermal inactivation in food products including: fruit juices, milk and chicken broth *(30, 154, 202, 213)*. Gabriel and Nakano *(74)* reported that the D-value of *E. coli* O157:H7 in apple juice was higher than PBS, that can be explained by the ability of the cells to adapt to acidic conditions *(154, 213)*. Even if the initial inoculum has not been adapted to acidic conditions prior to the thermal inactivation, the cells are able to acclimatize in the suspending menstruum. The D-values varied depending on the suspending medium and mode of inactivation, for *E. coli* O157:H7 were 4.41 and 4.43 min in PBS and apple juice, respectively, and for *E. coli* K-12 were 0.22 and 0.55 min in PBS and apple juice, respectively. Gabriel and Nakano *(75)*, demonstrated that duration of the lag time was influenced by the exposure of previews stresses in the resuscitation stage of the *E. coli* O157:H7 cells, the exposure to low pH that caused injury of even 1 log reduction in the cell viability, could enhance the thermal resistance of *E. coli* O157:H7 *(75)*. In addition, Gabriel and Nakano *(74)*, compared the D-value in PBS and apple juice of other pathogenic bacteria, including: Salmonella, *E. coli* K-12 and *L. monocytogenes* that exhibited a lower D-value in juice compared to the PBS suspension; this could be attributed to the relative rates by which the tested microorganisms adapted to the acidic juice.

Studies have shown that the D-values at 52^{o}C of acid adapted *E. coli* O157:H7 were considerably higher than those of acid-shocked or control cells both in apple cider and orange juice *(202)*. The D-values for *E. coli* O157:H7 in apple cider reported by Splittstoesser *et al. (219)* at 52, 55, and 58^{o}C were 12, 5 and 1 min, respectively. The D-values at 58^{o}C were significantly lower ($P \leq 0.05$), compared to those reported by Ryu and Beuchat *(202)*, which were 53.5 and 22.7 min in control cells heated in apple cider,

which suggest that the composition of the juice as well the condition of the apples used to make the cider can influence the survival characteristics of *E. coli* O157:H7 *(202)*. In addition, Ryu and Beuchat *(203)*, reported D-values of 100.2, 28.3 and 6.1 min at 52, 54 and 56^{o}C respectively with a z-value of 3.38^{o}C, in Tryptic soy broth for *E. coli* O157:H7 (E0139), an unusual heat tolerant strain.

It has been reported that acid-shock with hydrochloric acids can significantly increase the thermal resistance of *L. monocytogenes*, However, this was not true when the acid-shock treatment was performed using lactic or acetic acid *(65)*. Similar results were reported for *E. coli* O157:H7, where, the acidulant agent and the exposing procedure of the cells affected the tolerance to subsequent stresses. *(29)*.

Ryu and Beuchat *(203)* reported that both acid adapted and acid-shocked cells of *E. coli* O157:H7 and non O157:H7 showed enhance thermal resistances suggesting that different mechanisms can protect acid adapted and acid-shocked cells against later heat or acid stresses. Heat resistance enhancement, was reported by Wang and Doyle *(251)*, who suggested that a mild sub-lethal heat exposure at 48^{o}C for 10 min, enhances the acid tolerance. It was hypothesized that the heat-shocked cells synthesis outer membrane proteins, as a heat stress response, probably involved in the H+, transport inducing a higher acid tolerance *(251)*.

In contrast with the acid adapted and acid-shocked cross-protection effect on the heat tolerance of *E. coli* O157:H7, an opposite behavior has been reported by Williams and Ingham *(261)*, were short temperature abuse (heat –shocked cells), did not significantly enhance the thermal resistance of *E. coli* O157:H7 cells in ground beef. Layer *et al. (141)* reported that acid adapted *E. coli* O157:H7 cells at pH 5.0 showed a

higher tolerance to lactic acid during processing of fermented sausage pH 5.0 and apple cider pH 3.0. Therefore Gabriel and Nakano *(74)*, recommend considering the acid adaptation as a prerequisite for the inoculum used in thermal inactivation studies.

According to Juneja *et al. (112)* the future research on dynamic pasteurization a thermal processing should be focus on low-temperature-long cooking time to evaluate the integrated lethality of cooking and develop predictive models that include injury and repair behaviors in ready to eat meats. The food industry and governments are forced to increase vigilance and the microbiological safety offered to the consumer because of the serious consequences in case of non compliance with the safety standards for specific types of product, this consequences include potential deaths, commercial losses and image detrimental effects for the food manufacturing company *(47)*.

Food processor must fulfill the market expectations as well the current legal food safety regulations, and take full responsibility of the safety of their products; the microbiological safety of food products requires an integrated approach, combining knowledge about natural sources of contamination of raw materials and process foods, knowledge about the survivability of the microorganism during thermal processing *(47)*. Given this scenario, predictive microbial models as a tool for predicting the microbial behavior of the microbial population during food processing is useful *(47)*. The predictive microbiological models are useful tools for industry, academia and regulatory agencies, for developing new food products, designing new processing technologies, as well for assessing the microbial risk for foods and the set up of new guidelines for the industry and to overall maintain the food safety of the food products.

Sterilization and pasteurization are the two most commonly used heating processes for the preservation of food, extending the shelf life and ensuring the microbial safety of the product by eliminating or inactivating microorganisms and enzymes. The thermal inactivation data obtained from predictive models needs to be validated in each process product, with the use of thermocouples to monitor the temperature, ensuring the cold spot of the product reach the desired temperature and that is held for the adequate time, followed by confirmation with microbiological analysis with traditional culturing and plating/enumeration.

Stress response and heat-shock proteins

After the declaration of the big six non-O157 STEC as adulterants, there is a need to characterize their stress response. Very few studies have been conducted to determine if the heat and acid treatments designed for *E. coli* will also be effective against non-O157 STEC *(217)*. However, the non-O157 STEC are a very heterogeneous group, and there is variability in their stress tolerance, for example it has been shown that non-O157 STEC can survive in fermented products such as sausage and cheese, requiring other treatments for their inactivation *(159, 160, 174)*.

E. coli O157 and non O157 STEC have similar mechanisms to respond to acid environment, such as the acid tolerance response, the oxidative system, and the glutamate and arginine decarboxylase systems *(37)*. However some non-O157 STEC strains have an extra acid stress response not present on *E. coli* O157 strains, which is based on chaperone proteins, HdeA and HdeB *(37)*. Even though *E. coli* O157 and non O157 STEC can cause the same disease; the study of their genome reveals that they have different evolutionary histories *(170)*.

Because of the heterogeneity of the non-STEC group and their variability in terms of virulence and stress response, it becomes difficult to predict their behavior under environmental stress, in food systems and during processing, making it necessary to study their behavioral kinetics *(217)*. The thermal inactivation process is normally studied by using plating of thermally challenged cultures and further construction of thermal death time curves. The exact mechanism by which temperature inactivates bacterial cells, is still not fully understood, therefore research in this field still relevant.

Food products are often preserved by a combination of processing technologies in order to avoid the negative aspects of thermal processing alone. This is known as the hurdle approach, which is the combination of mild heating with other treatments such as pH, low water activity, and packaging. This is done with the purpose of preserving the nutritional value and the desirable flavor and texture characteristics of the food product. The hurdles technology approach serves different purposes, first is extending the shelf life and ensuring the safety of the product *(135)*. The hurdle technology approach helps to lower the thermal resistance of microorganisms by treating them with either chemical or physical means before the heat treatment *(135*, *119)*.

Heat stress

Cells respond to the exposure to supra-optimal temperature or heat-shock in several ways, severe exposures can lead to cytotoxicity and cell death *(138)*. The mechanism of *E. coli* against heat stress is regulated though several genes (Table 1.3). Stress exposure of cells will lead to mis-folding and aggregation of proteins, losing their

biological functionality, this stress includes heat-shock and pathophysiological states *(162*, *102*, *143)*.

The cell defense mechanism against induced stress states like sub lethal thermal injury causes the acceleration in the production of a group of molecular chaperons, collectively called heat-shock proteins. Chaperon proteins are defined as a group of unrelated proteins that are involved in the correct assembly of other polypeptides, without being part of the final functional structure *(59)*. Bacterial cells trigger the production of chaperon proteins as one of their homeostatic mechanisms against environmental stresses *(76)*. Environmental stresses, including heat and organic solvents, can perturb the internal folding conditions of the cell *(92)*. A large group of these chaperone proteins are expressed in response to heat exposure; these are known as heat-shock proteins (HSP) *(58)*. Heat-shock proteins help in prevention of aggregation, degradation of mis-folded proteins and refolding proteins *(162*, *92*, *31)*. The exposure to heat-shock conditions induces the production of heat-shock protein, which can enhance the cell tolerance to heat *(132)*. These specific proteins will assist the cell to alleviate or reduce damage incurred by the cell *(147)*.

There are two groups of HSP in *E. coli*, transcribed by the holoenzymes Es32 (*E. coli* sigma 32 (σ32, RpoH) and EsE (*E. coli* sigma σ 24 RpoE) *(237)*. The enzyme Es32 is associated with the regulation of stress in the cytoplasm, while the EsE enzyme regulates the stress response proteins in the periplasm *(85)*. Most of the holoenzymes members of the Es32 family belong to two groups, which are molecular chaperons and ATP-dependent proteases *(85)*. This group of molecular chaperons include: DnaK-DnaJ-GrpE and GroEL-GroES systems, that function to promote the proper folding and

conformation of newly synthesized proteins and help heat damage proteins and to recover their biological activity by helping them to recover their natural conformation *(92)*. The main heat-shock protein is DnaK, which is highly conserved during evolution sharing 50% of it amino acid sequence with its eukaryotic homologous HSP70 protein *(142)*. DnaK is known to work synergistically with DnaJ and GrpE, which together form the “chaperone machine” *(78)*.

DnaK is homologous to the HSP70 heat-shock protein being approximately 70 kDa in size, and interacts with DnaJ (HSP40) proteins, increasing the ATP consumption and DnaK activity *(153)*. Dnak consist of five consecutive hydrophobic residues. DnaK interacts with unfolded extended proteins by attaching them to the positively charged residues outside the substrate-binding cavity *(200)*. DnaJ is homologous to the heat-shock protein 40 (HSP40), expressed in a wide variety of organisms including humans *(34*, *172)*. DnaJ belongs to the J domain protein family, which has a 70 amino acids consensus sequence in the N-terminal. The J domain allows DnaJ to interact with the HSP70 type of proteins *(94)*. DnaJ also assists in the regulation of the ATPase activity of HSP70 proteins *(64*, *171)*.

GroEL also known as chaperonin is also found in a large variety of microorganisms *(267)*. Its function is to refold damaged proteins; it requires the presence of GroES in order to perform its functions. The combination of these proteins forms the GroEL/GroES chaperonin complex, which is homologous to the complex found in eukaryotes with the interaction of HSP60 and HSP10 *(268)*. The GroEL/GroES complex is approximately 1 MDa in size. The structure of GroEL consists of a dual-ring tetradecamer, each composed of seven subunits. While GroES is a single-ring

heptamer, that bind to GroEL in the presence of ATP or ADP forming the chaperonin complex *(69)*. GroEL undergoes conformational changes within its central cavity due to interaction of its subunits making the inside of GroEL become hydrophilic allowing the interaction with mis-folded proteins facilitating their re-folding. This is believed to facilitate the protein folding. The unfolded proteins attaches to the hydrophobic portion of the chaperonin complex in the interior of the cavity of GroEL *(101)*.

The expression of the heat-shock genes is regulated by the alternative sigma factor RpoH (σ^{32}) *(85, 266)*. At normal growth conditions of 30^{o}C, its level is very low, however, the levels rise after a quick increase in temperature *(86)*. The translation of the rpoH mRNA is prevented by its secondary structure, however once it is exposed to heat stress, it loses its secondary structure, allowing the interaction of the translation site with the translation machinery, this process is aided by a secondary transcription factor, σ^{24} involved in the heat-shock response, once the cell reaches temperatures over 50^{o}C *(266)*.

The *rpoE* gene is responsible for the transcription of σ24; that interacts with one of the *rpoH* gene promoters, which is still active while other promoters are inactive during heat exposure. Therefore at high temperature σ^{24} causes *rpoH* transcription and consequently the production of heat-shock proteins *(266)*. At optimal growth temperatures, association of DnaK with σ^{32} prevents the formation of the RNA polymerase- σ32-complex, resulting in the down-regulation of the expression of heat-shock proteins *(48*, *93)*. Therefore the production of heat-shock proteins is regulated by the balanced interaction of DnaK with denatured proteins and σ^{32} *(7*, *48*, *76)*.

Since DnaK has a preference for denatured proteins after heat stress exposure, DnaK will disassociate from $\sigma^{32,}$ this allows DnaK to interact with the RNA polymerase core protein *(211)*. This is followed by the transcription of heat-shock genes, which will translate in an increment in the heat-shock proteins production including DnaK. Once the stress is remove and the cells go back to their normal growing conditions the amount of denatured proteins decreases and the free DnaK binds with σ^{32}, this stop the translation of heat-shock genes, thereby production of heat-shock proteins *(96, 136, 263)*.

The rpoH is a polypeptide, product of the *htpR* gene, which is a σ^{32} a sigma subunit of the RNA polymerase (RNAP) *(189)*. rpoH regulates the expression at the transcriptional level of approximately 20 known heat-shock genes, thus accelerating the production of over 20 heat-shock proteins. *(142, 164)*. The σ32 is very susceptible and unstable at normal temperatures; it has a half-life of approximately of 1 min; the heat-shock response is correlated with the level of the σ32 polypeptide *(224, 223, 239)*. At temperatures above 45^{o}C, normal cell growth is not possible and the cell focuses on synthesis of heat-shock proteins. Thus, the synthesis of the σ^{32} is needed in order to promote the transcription of heat-shock genes at higher temperatures *(189)*.

Exposure to different stress factors such as starvation, heat, cold or acid-shock, and entry into the stationary phase, can induce the *RpoS* sigma factor, which can lead to resistance to other types of stress *(12)*. Sensitizers or protectors to heat-shock, include: methanol, H^{+}, Solvents, (DMSO), Sodium arsenite among others, that can trigger the synthesis of heat-shock proteins at normal growth temperatures, while protectors such as polyhydroxyl alcohols (e.g. glycerol) are known to inhibit the

production of HSP and therefore reduce the thermal tolerance of the bacterial cell when present during heat treatment *(137)*. The common feature about the sensitizer agents is that all of them alter the protein structure, and the protector agents are protein structure stabilizers *(137)*. Numerous chemical agents that sensitize cells to heat-shock and induce HSP synthesis and thermo tolerance suggests that common initiating signals are responsible for triggering these responses as observed by Lepock *(137)*. Hightower *(97)* suggested that the HSP production is induced by damaged or denatured protein, where introduction of denatured proteins in to the cell induced the production of HSP *(3*, *156)*. Protein denaturation is the trigger event for both HSP production and thermal tolerance and thermal damage and further cell death *(138)*.

In food systems a study using ground beef inoculated with *E. coli* O157:H7 pre-treated with a heat-shock at 46 °C for 15 min to 30 min, showed an increase in the thermal tolerance when exposed to 60°C, compared with non pre-treated samples *(111)*. The enhanced thermal tolerance was correlated with the increment in the concentration of two 60 and 69 kDa proteins immunologically related to GroEL and DnaK, respectively in the heat-shocked samples *(111)*. Herendeen *et al. (95)* estimated that at the log phase of growth, DnaK represents approximately 1% of the total proteins present in the cells grown at 30^{o}C. While if cells are expose to sub lethal temperature at approximately 42^{o}C, the amount of Dnak present will rise up to 13%.

Acid stress

When exposed to acidic conditions bacterial cells will maintain the internal pH close to neutrality, known as homeostasis *(98)*. It is known that at pH 3 the penetration rate of hydrogen ions across the cell membrane is higher than the rate they are pumped

out of the cell *(13)*. When an organic acid is used as a hurdle, if the pH is lower than the pKa of the organic acid the protonated part of the organic acid molecule will be transported across the cellular membrane and the bacterial cell will pump out anions and protons from the organic molecule causing bacterial cell inactivation *(28)*. Smaller molecular weight organic acids are more effective, because the diffuse easier through the cell membrane *(104)*.

The effect of acetic acid and/or citric acid on different STEC serotypes including O26:H11, O111:H2, O91:H2, O145:H2, and O157:H7, was determined in Luria Broth at different pH *(158)*. Citric acid at pH 4.5 showed little inhibitory effect, where all the strains grew with the exception of O111:H2; and at pH 5.0 and pH 5.5, all the strains grew *(158)*. However, there was no growth at pH 4.5 and 5.0 in the presence of acetic acid; at pH 5.5 all grew with exception of O111:H2 and O145 *(158)*. From this it was concluded that there is a substantial variability in the tolerance of both *E. coli* O157:H7 and non-O157 *E. coli,* when exposed to acetic and citric acid *(158)*.

A study comparing the survival rates of *E. coli* O157:H7 against non-O157 STEC (O26, O111 and O121:H19), using minimal medium at pH 2.0 and 2.5, indicating that the survival rates of O157:H7 were less than those for non-O157 STEC *(133)*. A study by Carter *et al. (37)* demonstrated that the genes hdeA and hdeB were necessary for acid tolerance of STEC serotypes O145:NM and K-12 but not for serotypes O157:H7 and O55:H7; despite the genes being present in all serotypes, the HdeB protein was not produced in the O157:H7 serotype.

An additional single nucleotide polymorphism G→A transition on the putative start codon of the hdeB gene explained the absence of the HdeB protein on the O157

serotype *(37)*. Moreover this G→A transition in the +3 position, was found in 20 strains of the O157:H7 serotype but not on 12 of the non-O157 STEC including: O26:H11, STEC O111:H8, O111:H11, STEC O111:HN, and STEC O45:H2). The study concluded that the acid resistant mechanisms of *E. coli* O157:H7 and non-O157 STEC follow different evolutionary paths; *E. coli* O157:H7 acid survival is not HdeB/A dependent since the *hdeB* gene is silenced *(37)*. In order to understand the mechanism of heat inactivation of STECs, various approaches are available and some are described below.

Analysis approaches

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE is universally accepted for biomedical research for characterization and/or identification of proteins and nucleic acids based on their molecular weight. This technique requires that the proteins use molecular weight calibrations and the loaded samples on the gel adopt equivalent shapes. This is achieved by using a Sodium Dodecyl Sulfate (SDS), which is an anionic detergent that induces a partial unfolding of the proteins and the aggregation of the SDS molecules at the hydrophobic binding sites of the proteins. SDS linearizes the proteins by polarizing them with a negative charge and denaturation of the secondary and non-disulfide-linked tertiary structures *(255*, *212)*.

In the SDS- PAGE method the proteins are bound to the SDS, that facilitates the even distribution of the electric charge per unit of mass, allowing the fractionation of the polypeptides by their approximate molecular weight, during the electrophoresis *(191)* and inducing what is called a reconstructive denaturation, where the proteins are in a

mix state of alpha-helix and random coil *(192)*. The separation of the macromolecules is based in function of the length, conformation and charge of the molecule *(191)*. The sample preparation consists of extraction of the protein or nucleic acid. This can be achieved by mechanical action using a blender, particularly for solid tissues or cells in large volumes, or using a homogenizer for smaller volumes, and also by sonication. Other methods include combinations of biochemical and mechanical procedures including filtration centrifugation and enzymatic treatments in order to concentrate the amount of the analyte and remove the cell debris *(255*, *212)*.

There is a variation of the SDS-PAGE, known as reducing SDS-PAGE. This method consists of the use of diothiothreitol (DDT) or 2-mercaptoethanol (beta-mercaptoethanol) while the samples are being heated, as these compounds act as reducing agents, enabling the protein denaturation by reducing the disulfide bonds and disrupting some tertiary structures and splitting the oligomeric subunits of the quaternary protein structure *(35)*. In addition the treated samples are mixed with a tracking solution to allow visualizing the progress of the sample through the gel, during the application of the electric field. The electric field allows the migration of the charged proteins across the gel matrix from the negative towards the positive electrode. Proteins are separated by their molecular weight, smaller molecules van move faster though the gel, while bigger molecules move slower. Consequently larger molecular weight proteins will appear in the top part of the gel, while smaller molecule will appear at the bottom.

After the proteins have been separated through the gel they are commonly stained using either Coomassie brilliant blue or silver staining *(230)*. This allows the

visual interpretation of the separated proteins from the sample and comparing them with a standard ladder of known molecular weights. Instead of staining the electrophoretically separated proteins can be transferred to a either a nitrocellulose, nylon or Polyvinylidene fluoride (PVDF) membrane for immune-detection, known as western blotting, which allows the visualization of the transferred proteins and the correct identification as well *(241)*.

SDS-PAGE has been successfully used to analyze heat shock proteins. Juneja *et al. (115)* used SDS-PAGE to analyze HSP and a western blot assay to detect both Dnak and GroEL proteins on *E. coli* O157:H7. After the heat-shock treatment, both proteins showed an increment in levels, however a densitometric scanning of the blot revealed differences between strains in terms of the percentage increase after heat treatment; on average there was approximately an increase of 30 and 17% in the concentration of GroEL and DnaK respectively compared to the untreated cells*(115)*.

SDS-PAGE can be used to separate similar proteins based on their molecular weight through a western blotting technique or more precisely by doing a 2D-electrophoresis. However, the measurements obtain though this technique are considered semi-quantitative, while mass spectrometry can deliver more precise measurements of the protein mass. Moreover, the quantification of protein is made by comparison of the density (darkness) of the bands in the gel, therefore some error can be expected in the accuracy of this method *(16)*. Although, SDS-PAGE can be very effective separating large proteins however smaller sized proteins could rush to the bottom of the gel or react improperly with the SDS-PAGE chemistry. For these cases usually is recommended to use an enzyme –linked immunosorbent assay (ELISA).

Enzyme Link Immunosorbent Assay (ELISA) detection for heat-shock proteins

Enzyme linked immunosorbent assay (ELISA) is immunological detection method, which uses antibodies to attach to a specific target antigen and color development for detection and quantification. ELISA is commonly referred as wet lab biochemistry that detects the presence of a specific antigen in a sample usually liquid. In a sandwich ELISA test, first the primary antibody, also known as the solid phase reactant, is adsorbed usually onto a polystyrene microtiter plate. This is followed by the addition of the antigen. The next step is to incubate with the secondary antibody, which is covalently bound to an enzyme *(100)*. Between each step the plate is washed using a mild detergent to remove any unbound antibody or antigen. The unbound conjugates are removed by washing and color development is achieved with the addition of Tetramethylbenzidine (TMB), which is a chromogenic substrate. After this the plate is read using a spectrophotometer at 370 nm wavelength or at 450 nm wavelength after the addition of sulfuric or phosphoric acid stop solution. The signal intensity is correlated with the concentration of the antigen present in the sample.

Classic thermal inactivation is based on a first order kinetic model, which has some limitations specially when bacteria exhibit either a more sensitive or resistant adaptation to a particular stress *(211)*, in some cases sub lethal exposure to stress can enhance tolerance to later severe heat treatment *(25)*. Therefore, prediction of thermal inactivation becomes a challenge especially at moderate processing temperatures (50 to 65^{o}C) like those use in *sous vide* cooking style, which allow stress adaptation *(211)*.

Considering the crucial role of DnaK in the heat-shock response and the changes in concentration upon heat stress, Enzyme Linked Immunosorbent Assays (ELISA)

represent a rapid way ELISA represents a rapid way to detect and measure the intra cellular levels of DnaK in *E. coli*. This will help establish the correlation between thermal inactivation kinetic parameters after sub-lethal exposure to heat and acidic conditions to DnaK levels and total protein current. Therefore, the objectives of this study were (1) Compare the thermal inactivation parameters (D- and z-values) between *E. coli* O157:H7 and non-O157 STECs in buffer and model foods (spinach, turkey deli meat, pasta, ground beef) and (2) Use linear and Weibull models to determine thermal inactivation parameters of O157 and non-O157 STEC in pasta and (3) Determine the effects of sub-lethal heat and acid-shock on thermal inactivation parameters with correlation to the DnaK levels and total protein content of O157 and non-O157 in buffer.

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Appendix

Table 1. 1 Outbreaks of *E. coli* O157:H7.

Year	Month	State	Serotype	Location of Consumption	Total Ill	Total Hospitalization	Total Death	Food Vehicle	Methods	Reference
2014	April	Michigan, Ohio, Massachusetts, Missouri	O157:H7		12	58%	0	Ground beef		*(59)*
2013	October	Pennsylvania	O157:H7	Restaurant	21	8	0	Ground beef		*(76)*
2013	October	Colorado (Denver)	O157:H7	Restaurant	8	0	0	Produce		*(75)*
2013	September	California, Arizona, Washington	O157:H7	Grocery stores (trader Joes)	33	7	0	Ready to eat salads		*(58)*
2013	September	United Kingdom	O157:H7	Retailer	19	0	0	Bagged salads watercress	MLVA	*(183)*
2013	August	California	O157:H7	Restaurant	22	7		Garlic Noodles		*(15)*
2013	July	Arizona	O157:H7	Restaurant	59	22	0	Lettuce		*(14)*
2013	June	Sweden	O157:H7	Hotel	19	0	0	Mixed salad	PFGE, MLVA	*(321)*
2013	May	Georgia, South Carolina	O157:H7	Restaurant	18	7	0	Unknown		*(13)*
2013	April	Texas	O157:H7	Restaurant	10	5	0	Ground beef		*(16)*
2013	January	Wisconsin	O157:H7	Meat market	17	0	0	Ground beef	PFGE	*(71)*
2012	October	New York, Pennsylvania, Virginia	O157:H7		33	13	0	Organic spinach and spring mix blend		*(257)*
2012	November	Michigan	O157:H7	Farm	4	4	0	Apple cider		*(17)*
2012	April	Multistate		School cafeteria	17	6	2	Bagged salad		*(207)*
2012	April	Oregon	O157:H7	Farm	21	4	0	Fenugreek seed/Sprouts	PFGE	*(11)*
2011	January	Multistate outbreak reported by CDC	O157:H7	Private Home	14	3	0	Deli meat, bologna		*(8)*
2011	October	St. Louis	O157:H7	Grocery store	22				PFGE, MLVA	*(329)*

Table 1.1 Continued.

Year	Month	State	Serotype	Location of Consumption	Total Ill	Total Hospitalization	Total Death	Food Vehicle	Methods	Reference
2011	October	Arizona, Minnesota, Kansas, Georgia, Indiana, Nebraska	O157:H7	Grocery Store; School	60	35	0	Romaine lettuce, unspecified		*(256)*
2011	August	California	O157:H7	Private Home	5	3	0	Raw Milk		*(10)*
2011	July	Oregon	O157:H7	Other; Private Home	15	7	2	Strawberries		*(12)*

Table 1. 2 Outbreaks of *E. coli* non-O157.

Year	Month	State	Serotype	Location of Consumption	Total Ill	Total Hospitalization	Total Death	Food Vehicle	Methods	Reference
2013	September	United Kingdom	O104, O131, O20, among others	fair	413			Fresh curry leaves	PCR, Sequencing (illumina, nextera kit)	*(79)*
2013	March	China	0112ac:H19	Cafeteria	170	14			RT-PCR, Chrome agar, Slide agglutination	*(142)*
2013	January	Multistate outbreak reported by CDC	O121:H19	Grocery stores	35	9	0	Frozen products		*(96)*
2012	September	Osaka, Japan	O169:H41	Restaurant	102				Multiplex PCR, spread-plated on (DHL) agar plates	*(127)*
2012	January	Multistate outbreak reported by CDC	O26	Restaurant - "Fast-food"(drive up service or pay at counter)	29	7	0	Sandwich, ham (raw clover sprouts)		*(258)*
2012	May	Multistate outbreak reported by CDC	O145		18	4		not identified		*(259)*
2011	July	Michigan	O157:NM	McNees Meats and Wholesale LLC	4 confirmed 5 possible			Ground beef		*(9)*
2011	June	Bordeux, France	O104:H4		24			Fenugreek sprouts	PCR, STEC Serology	*(79)*

Table 1. 2 Continued.

Year	Month	State	Serotype	Location of Consumption	Total Ill	Total Hospitalization	Total Death	Food Vehicle	Methods	Reference
2011	April	Japan	O111:H8	Restaurant	181			Raw beef	PFGE, MVLA	*(341)*
2011	May	Europe/U.S.	O104:H4		4000			Fenugreek seeds/Sprouts	PFGE	*(72)*
2010	April	New York, Ohio, Pennsylvania	O145	Restaurant, school	33	13	0	Romaine lettuce, unspecified		*(321)*
2010	November	Minnesota	O103:H2; O145:NM	School	29	2	0	Venison		*(271)*

Table 1.3 Genes involved in heat resistance of *E. coli*.

Gene	Strain	Function	Status	Methods used to detect and quantify	Conditions	Reference
clpB	*E. coli* K-12	ClpB belongs to the superfamily of AAA+ (ATPase associated with a variety of cellular activities) proteins. ClpB together with the DnaK chaperone is able to re-solubilize aggregated proteins.	upregulated	SDS-PAGE, alkaline phosphatase conjugated anti-rabbit IgG, Developed immunoblots were scanned using a fluoro-imager	30°C Luria broth (LB)	*(218)*
hsLJ	*E. coli* K-12 (159 uvrA)	Heat-shock proteins HsLJ		DEAE-cellulose (Ion exchanger) 50C for 7 min, SDS-gel electrophoresis		*(65)*

Table 1.3 Continued.

Gene	Strain	Function	Status	Methods used to detect and quantify	Conditions	Reference
hsLR	*E. coli* K-12 (BL21-DE3)	Hsp15, heat-shock protein with DNA RNA binding activity, interacts with 50s ribosomal subunit	Strongly upregulated	Heat-shock at 42°C for 10 min, Membrane-bound PCR, The cellular abundance of Hsp15 was determined by quantitative Western immunoblotting.	42°C	*(173)*
hspQ		Heat-shock protein, hemimethylated DNA-binding protein = HspQ. A hspQ mutation causes overproduction of DnaA and suppresses the temperature sensitivity of dnaA mutants	upregulated	Restriction enzyme analysis, Gene mapping membrane (EcoRI, BamH1 and Xhol) SDS-polyacrylamide (12%) gel electrophoresis, blotted on to a PVCF membrane, polyclonal anti DnaA antiserum	LB plates 40°C	*(296)*

Table 1. 3 Continued.

Gene	Strain	Function	Status	Methods used to detect and quantify	Conditions	Reference
htpX	*E. coli* K-12	Heat-shock protein, integral membrane protein, (degrading mis-folded proteins location: inner membrane	upregulated	DNA sequencing (termination method), Dot Blot, Northern blot	Minimal medium at 42°C	*(174)*
dnaK	*E. coli* K-12 (B178)	Component of the DnaK-DnaJ-GrpE chaperon system (Hsp70) assist folding of nascent polypeptide chains, and rescue of mis-folded proteins	upregulated	SDS-PAGE phosphatase-conjugated anti-rabbit IgG	Luria Broth 42°C for 10 min	*(288)*

Table 1. 3 Continued.

Gene	Strain	Function	Status	Methods used to detect and quantify	Conditions	Reference
clpP	*E. coli* K-12	Enzyme serine protease with a chymotrypsin-like activity that is a part of the ClpAP, ClpAPX and ClpXP protease complexes (location: membrane)	upregulated	SDS-PAGE (2-D gel) DEAE Affi-Gel Blue	43°C	*(177)*
groL	*E. coli* K-12	GroEL, chaperone Hsp60, peptide-dependent ATPase, heat-shock protein	1.6 folds upregulated	SDS-PAGE and autoradiography	MOPS minimal medium 42°C for 1h	*(178)*
grpE	*E. coli* K-12	Part of the DnaA-DnaJ-GrpE chaperon system, essential for regulation of release of ADP	upregulated	SDS-PAGE, Circular Dichroism, Differential Scanning Calorimetry	>40°C	*(115)*

Table 1.3 Continued.

Gene	Strain	Function	Status	Methods used to detect and quantify	Conditions	Reference
yibA	*E. coli* K-12 9AB1157)	Predicted lyase containing Heat-repeat, reduce lethal effect of stress		Asymmetric PCR	Luria Broth	*(122)*
ibpA	*E. coli* K-12 (B178)	Small heat-shock protein IbpA, suppress thermal aggregation of model proteins	upregulated	SDS-PAGE, PVDF membrane protein microsequencing, EASY Desitometry	45C for 15 min grown in LB	*(65)*
ibpB	*E. coli* K-12	Small heat-shock protein IbpB, binds to aggregated and denatured proteins		6B gel, enzymatic assays to measure refolding of proteins	LB	*(66)*
rpsB	*E. coli* K-12	30s ribosomal subunit protein, Ribosomal proteins - synthesis,		2D polyacrylamide gels, chromatography	42C	*(37)*

Table 1. 3 Continued.

Gene	Strain	Function	Status	Methods used to detect and quantify	Conditions	Reference
mopB	*E. coli* K-12	GroES, 10 Kd chaperone binds to Hsp60 in pres. Mg-ATP, suppressing its ATPase activity	upregulated	Plasmid restriction endonuclease digestion, SDS-PAGE	Luria Broth at 30C	*(95)*
dapE	*E. coli* (CG698)	N-succinyl-diaminopimelate deacylase, enzyme; Amino acid biosynthesis: Lysine	upregulated	Sequencing by dideoxy chain termination method, SDS-PAGE	at 42C Luria broth, and Luria agar, were supplemented with appropriate antibiotics	*(356)*

Table 1. 3 Continued.

Gene	Strain	Function	Status	Methods used to detect and quantify	Conditions	Reference
rpoH	*E. coli* K-12 (MG1655K1)	Sigma 32 is the primary sigma factor controlling the heat-shock response during log-phase growth.	upregulated	RNA microarray, Affymetrix GeneChip, biotin-bound anti-streptavidin antibody, Arrays were scanned at 570 nm with a 3- m resolution using a confocal laser scanner	Supplemented media with ampicillin (100 g/ml), chloramphenicol (30 g/ml),or kanamycin (50 g/ml) as appropriate	*(363)*

CHAPTER II
DETERMINATION OF THE THERMAL INACTIVATION KINETICS OF *ESCHERICHIA COLI* O157:H7 AND NON-O157 IN BUFFER AND A SPINACH HOMOGENATE

This chapter has been submitted as part of a manuscript to the *Journal of Food Protection* and has been accepted for publication.

Abstract

There has been an increase in foodborne outbreaks associated with pathogens in leafy greens, associated with the increase in consumption of minimally processed foods. The objective of this research was to determine the thermal inactivation kinetics of *Escherichia coli* O157 and non-O157 in phosphate buffered saline (PBS; pH 7.2) and a spinach homogenate. Five individual strains of *E. coli* O157 and non-O157 were tested in PBS in 2 mL glass vials. The blended spinach inoculated with bacterial cocktails was aliquoted in vacuum-sealed bags. Thermal inactivation was assessed at 54, 56, 58 and 60°C. D-values for *E. coli* O157 ranged from 5.18±0.21 to 0.53±0.04 min at 54 to 58^{o}C and for non-O157 values from 5.01±0.60 to 0.60±0.13; while D-values at 56 to 60^{o}C ranged from 7.21±0.17 to 1.07±0.11 min and 5.57±0.38 to 0.99±0.07 min for *E. coli* O157 and non-O157, respectively. In spinach, z-values were 4.80±0.92 and 5.22±0.20^{o}C for *E. coli* O157 andnon-O157 STEC, respectively; while in buffer ranged from 4.41±0.13 to 5.04±0.16 and 5.26±0.76 to 7.09±0.55^{o}C for *E. coli* O157 and non-O157 STEC, respectively. Results indicated that mild thermal treatment of spinach at 70^{o}C for less than 1 min would result in a 6D reduction of all pathogens tested. These findings will help the food industry in the design of suitable mild thermal processes to ensure food safety without compromising the quality of the products.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) are pathogens that frequently have been associated with foodborne illness outbreaks. Illnesses caused by these microorganisms not only have a large impact on public health, but also have a major associated economic cost. Scallan *et al. (23)* estimated that there are approximately 175,000 cases of O157 and non-O157 Shiga toxin-producing *E. coli* (STEC) annually in the US. According to the model created by Scharff *(24)*, in which the financial losses from lost productivity and lost utility due to death, medical expenses, and pain and suffering have been included, costs associated with O157:H7 STEC, non-O157:H7 STEC are $607 million, $101 million, respectively.

Produce, such as leafy greens, is one of the types of foods that is generally more at risk of pathogen contamination due to its growth on soil, possible contamination during cutting and minimal processing, attachment of bacteria to damaged sections of the leaves and often the lack of cooking by consumers prior to consumption *(10)*. Several foodborne outbreaks due to the consumption of leafy greens have been associated with *E. coli* O157:H7 in North America; between 2001 and 2009, there were 27 foodborne disease outbreaks associated with produce in Canada, 3 of which were caused by *E. coli* O157:H7 in lettuce *(14)*. In the U.S., there were 49 foodborne outbreaks between 1998 and 2008 caused by bacteria in leafy vegetables alone, 22 of which were caused by STECs and 11 of which were caused by *Salmonella(9)*.

Non-O157 STECs are also emerging as more frequent causes of foodborne disease from produce. U.S. multistate outbreaks were attributed to *E. coli* O145 in shredded Romaine lettuce and in February and April 2012 from *E. coli* O26 in raw clover sprouts *(5, 6)*. The source of these microorganisms can be pre-harvest, such as irrigation water, field workers, insects, fecal contamination by wild animals, or run-off from nearby animal farms *(18)*. There are also post-harvest sources such as insects, handling by workers, transport vehicles and processing equipment, wash water and cross-contamination from other foods *(3)*, leading to high initial levels of microorganisms in produce.

Although a large portion of both intact and fresh-cut produce is consumed fresh with no processing and thus no lethality step to eliminate foodborne pathogens, some produce is treated with mild heat (e.g., blanching, which generally occurs at 75 - 100°C *(17)* to prepare it for freezing, or sous-vide). Mild heat processes may also include washing minimally processed vegetables such as lettuce and cabbage at 45-70°C for less than 5 min to reduce enzymatic browning and extend shelf life *(16, 21, 22)*.

In addition, frozen produce may be thawed and consumed directly without cooking, such as in the case of homemade dips. Therefore it is these processes for which a defined thermal process would be of great value to improve food safety. In order to calculate a thermal process for a food product, it is necessary to assess the thermal inactivation kinetics of the target microorganism in buffer and food *(11)* to determine the effect of the food system on thermal resistance. The objective of this research was to determine the thermal inactivation kinetics of cocktails of *E. coli* O157 and non-O157 in a buffer compared to a spinach model created using frozen chopped spinach homogenate.

Material and methods

Bacterial strains

Bacterial strains were obtained from ATCC (Manassas, VA), Dr. Pina Fratamico, USDA-ARS (Wyndmoor, PA), and from the culture collections of the Departments of Animal Science and Food Science and Technology at the University of Tennessee, Knoxville. A total of five strains were evaluated for each bacterium used. ATCC 8326. *E. coli* O157:H7 strains were ATCC EDL 933, ATCC 43888, ATCC 43894, ATCC 7927 (cider isolate) and ATCC 2175 while non-O157 strains included *E. coli* O145:H18 (ATCC 07865), *E. coli* O26:H11 (ATCC 05-6544), *E. coli* O121:H19 (ATCC 03:2832), *E. coli* O111:H8 (ATCC 01387), *E. coli* O103:H11 (ATCC 043973) and *E. coli* O45:H2 (ATCC 5J8).

Growth conditions

Tryptic soy broth (TSB; Difco, Becton Dickinson) was used for *E. coli*. Stock cultures were kept in their respective broth medium with 15% (vol/vol) glycerol and stored at -80°C. Active cultures for thermal inactivation experiments were prepared by streaking material from the frozen cultures onto tryptic soy agar (TSA; Difco, Becton Dickinson) plates. Single colonies were transferred to appropriate broth media and incubated at 37°C for 18-24 h for both *E. coli*O157 and non-O157 STEC. These strains were sub-cultured overnight twice prior to thermal inactivation experiments.

A 1 ml aliquot was removed from the active broth culture and the cells were harvested by centrifugation (3 min at 5,000 x *g*) and washed twice in an equal volume of sterile phosphate buffered saline (PBS) (Difco, Becton Dickinson). After a second washing, the cell pellets of the cultures were re-suspended to ca. 1 x 10^8 CFU/ml in

sterile PBS (based on prior determinations of cell density using plate counts of 18-24 h cultures of individual strains), and in the case of cultures to be used in spinach, combined to produce a cocktail of ca. 5 x 10^8 CFU/ml. Cultures were plated on TSA to determine the initial number.

Thermal treatment in buffer

A 2 ml volume of the washed and re-suspended cocktail was placed into a 2 ml glass screw-capped vial for each of the time points. To monitor temperatures in the heating medium, one vial was filled with PBS and a type-T thermocouple (Omega Engineering, Inc., Stamford, CT) was placed in the geometric center of the vial and connected to an MMS3000-T6V4 type portable data recorder (Commtest Ins., New Zealand). This was then placed in the geometric center of the water bath. The water bath temperature was confirmed with a mercury-in-glass (MIG) thermometer (Fisher Scientific, Pittsburgh, PA). All tubes were placed in an isothermal water bath (Lauda Eco Silver, Type E 40S, Karlsruhe, Germany).

To ensure a constant temperature of the water bath during each experiment, tubes were completely immersed in the water. The time required for the internal temperature of the tubes to reach the target was measured. Once this temperature was reached, the first tube was removed from the water bath and the heating time started (i.e., time = 0). The come-up time ranged from 45 s to 1.5 min, depending upon the heating medium temperature. Samples were heated at 54°C, 56°C, 58°C and 60°C and the tubes were removed at different time intervals based on the microorganism and inactivation temperature used.

After removal from the water bath, the vials were placed in an ice water bath for 5 min to cool. They were then sprayed with 70% ethyl alcohol prior to opening aseptically. For determination of the number of surviving bacteria, tenfold serial dilutions of the samples in PBS were plated on TSA and incubated at 37°C for 48 h before colonies were enumerated. Initial cell counts were compared to cell counts at time = 0 to determine the lethality of come-up times (data not shown)

Thermal treatment in spinach

Frozen chopped spinach was purchased at a local grocery store. Spinach samples of 250 g were mixed with an equal volume of distilled water and blended using a Waring blender (Model 1063, Waring Commercial, USA). The mixture was sterilized at 121°C for 15 min, aliquoted in 50 g batches in sterile tubes, and stored frozen (-20°C) until needed. Samples were thawed overnight at 4°C before use. Two 10 g samples were aseptically weighed into 13 cm x 19 cm polyethylene/nylon bags (Seco Industries, Los Angeles, CA) for vacuum packaging. One was used for an un-inoculated negative control and in the other a Type T thermocouple (Omega Engineering, Inc., Stamford, CT) was placed to monitor the internal temperature at the geometric center of the bag.

Next, 70 g of blended spinach were weighed into sterilized beakers and 5 mL of 8 log CFU/ml of the test bacteria was added. The mixture was stirred by hand for 30 s. The same plastic bags were aseptically filled with 10 g of the inoculated sample and sealed to -100 kPa with a Multivac A300/16 vacuum-packaging unit (Sepp Haggemuller KG, Wolfertschwenden, Germany). The bags were flattened to approximately 1 – 2 mm in order to ensure a consistent thickness for even heat distribution. The initial inoculum level prior to the heat treatment was determined by plating as described above.

The sealed bags were rinsed with 70% ethanol, placed into a holding unit and then the holding unit with the bags was immersed in the thermostatically controlled water bath described above. Temperature was monitored at immersion until the target temperature was reached to register the come-up time and bags were removed at appropriate time intervals and immersed in an ice bath. Bags were aseptically opened, by cleaning the outer surface with 70% ethyl alcohol and samples removed for plating as described above.

Determination of thermal inactivation kinetics

Survivor curves were constructed by plotting CFU/ml or CFU/g versus heating time for each of the bacteria at a given temperature. The thermal death kinetics follows a first order model that according to Bigelow and Esty *(4)* can be expressed as:

$$log_{10}\frac{N_{(t)}}{N_o} = -\frac{t}{D} \quad \text{(Eq.1)}$$

Where $N_{(t)}$ is the number of survivors after exposure time t in CFU/ml or g, N_o is the initial population (CFU/ml or g) and the D-value is the time for a 90% population reduction at the isothermal process temperature. D-value was determined as the negative reciprocal of the slope of the plotted survivor curves. The z-value indicates the sensitivity of the organisms to changes in temperature and is defined as the change in temperature required in order to change the log D-value by plus or minus one-log. The z-values of the bacteria were calculated by plotting log D-values versus temperature for each cocktail. The z-value was then calculated as the negative reciprocal of the slope, as the log D-value is a linear expression of temperature:

$$z = \frac{T_1 - T_2}{logD_2 - logD_1} \quad \text{(Eq.2)}$$

Where T is temperature (°C?).

The 6D projections at 60, 70, 80 and 90°C were calculated using the linear regression equation and multiplying each D-value by 6. These projections are often used in industry to determine the ability of thermal processing steps to ensure the microbiological safety of the food product *(7)*

Statistical analysis.

The statistical analysis of the heat resistance data was conducted using an analysis of variance (ANOVA) *(20)* with SAS software (version 9.3, SAS Institute, Cary, NC, USA.) to determine if there were statistically significant differences in thermal resistance among the organisms. Means comparisons were analysed using a Tukey's test *(19)* based on non-overlapping 95% confidence intervals for the medians.

Results and discussion

D-values for individual strains of *E. coli* O157:H7 and non-O157 STEC strains from 54 to 60°C are summarized in Table 1. The D-values for the single strains in buffer O157:H7 STEC and non-O157 STEC ranged at 54°C from 5.18± 0.21to 3.94± 0.75 min; at 56°C from 2.10 ± 0.34 to 1.41 ± 0.23 min; and at 58°C from 1.19 ± 0.16 to 0.60 ± 0.03 min. The z-values showed statistical differences among the different strains of STEC. For O157 STECs, the z-value for strain Cider was significantly greater than that for E2175 but there was no difference in z-values among non-O157 strains. Figures 1 and 2 showed the variability among the strains of *E. coli* O157 and non-O157 STEC, respectively. However, the R^2 (Table 2.1) showed the adequacy off the linear model

explaining the thermal behavior of the individual strains of *E. coli* O157 and non-O157 STEC. The R^2 values ranged from 0.93 to 0.99 for *E. coli* O157 and from 0.95 to 0.99 for the non-O157 strains; meaning that the log-linear model was able to explain at least 93 and 95% of the data, for *E. coli* O157 and non-O157 strains, respectively.

In blended spinach, the respective D-values at 56, 58, and 60°C (Table 2.2) were 7.12±0.32, 1.95±0.20 and 0.97±0.33 min, respectively for O157:H7 STEC and 5.79±0.77, 2.01±0.17 and 0.93±0.07 min, respectively for non-O157 STEC. There were no significant differences between D-values of O157:H7 and non-O157 STEC cocktails within each temperature tested, expcept for 56°C, were the values for the O157 STECs cocktail was significantly higher ($p \leq 0.05$). There was no statistical difference observed between the O157 and non-O157 STEC z-values. The values were 4.80±0.92and 5.22±0.20. for the *E. coli* O157 and the non-O157 STECs cocktails, respectively. The R^2 values for the z-values in spinach were 0.96 and 0.98; meaning that the log-linear model was able to explain 96 and 98% of the behaviour *E. coli* O157 and non-O157 STEC, respectively.when they are heated in a spinach homogenate.

A 6-D value (6 x the D-value at a specified temperature) was calculated for processes at 56 58 60, 70, 80 and 90°C using the z-values determined in spinach (Table 2.3). 6-D projections have been used to evaluate the thermal inactivation potential of heating time-temperature combinations by several researcheres *(2, 25, 26)* and are important as they are often used as performance criteria to assess the ability of thermal processing steps to ensure the safety of the food product *(27)*. For example, based on the thermal inactivation data from this study; at 60°C the 6D projection for *E. coli* O157 and non-O157 STECs cocktails were 5.82 and 5.58 min, meaning that it is the

time required to achieve a 6 log reduction which is commonly used as the standar for pasteurization.

Generally, a 6 log reduction is recommended for pasteurization *(7, 12)*, although this dependes on the target microorganism and the food product being process, both the USDA and FDA suggest a target of 7D for *Salmonella* in meat and poultry *(25)*. It is important to note that the values for 70, 80 and 90°C are projections beyond the data gathered and would need to be verified in actual products.

The results of this study are also similar to those conducted by Gabriel and Nakano *(8)*, who determined the thermal resistance in a PBS suspension at 55°C of *E. coli* K-12 and *E. coli* O157:H7. The D-values in minutes were 1.18 and 2.55, respectively. Amado *et al. (1)* documented the D-values for *E. coli* isolated from vegetable base animal feed ingredients; the thermal inactivation was performed in PBS at 55°C and the values ranged from 34.08 to 6.04 min

The general increase in D-value exhibited in this study when the organisms were heated in spinach as compared to PBS was to be expected. When the inactivation test is carried out in food systems, the food characteristics can provide a thermo-protectant effect. This effect can be attributed to the presence of different compounds including carbohydrates, lipids, proteins, salts, etc. Data on the *E. coli*O157 and non-O157 STEC in produce is limited.

The 6D projections show that even under what is considered a “mild” thermal treatment such as blanching or sous-vide, the pathogens studied should be eliminated. Sous-vide achieves at least 70°C for 2 min at the center of the food product *(13)*. Of the organisms tested in this study, the highest 6D value in blended spinach at 70°C was

exhibited by *E. coli* O157:H7 at 2.66 s, which is well under 2 min.

The results indicate that although the heat resistance of the bacteria in spinach was higher than that in buffer, even a mild thermal process of spinach such as 7.5 min at 60ºC would be sufficient to inactivate potential pathogens such as *E. coli* O157 and non-O157 STECs that may contaminate leafy green vegetables. In order to decrease the risk of foodborne illness, it is important that thermal inactivation of these pathogens in other foods that are of risk are studied as well, so that we can validate thermal processing parameters for those specific food products.

Acknowledgements

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Appendix

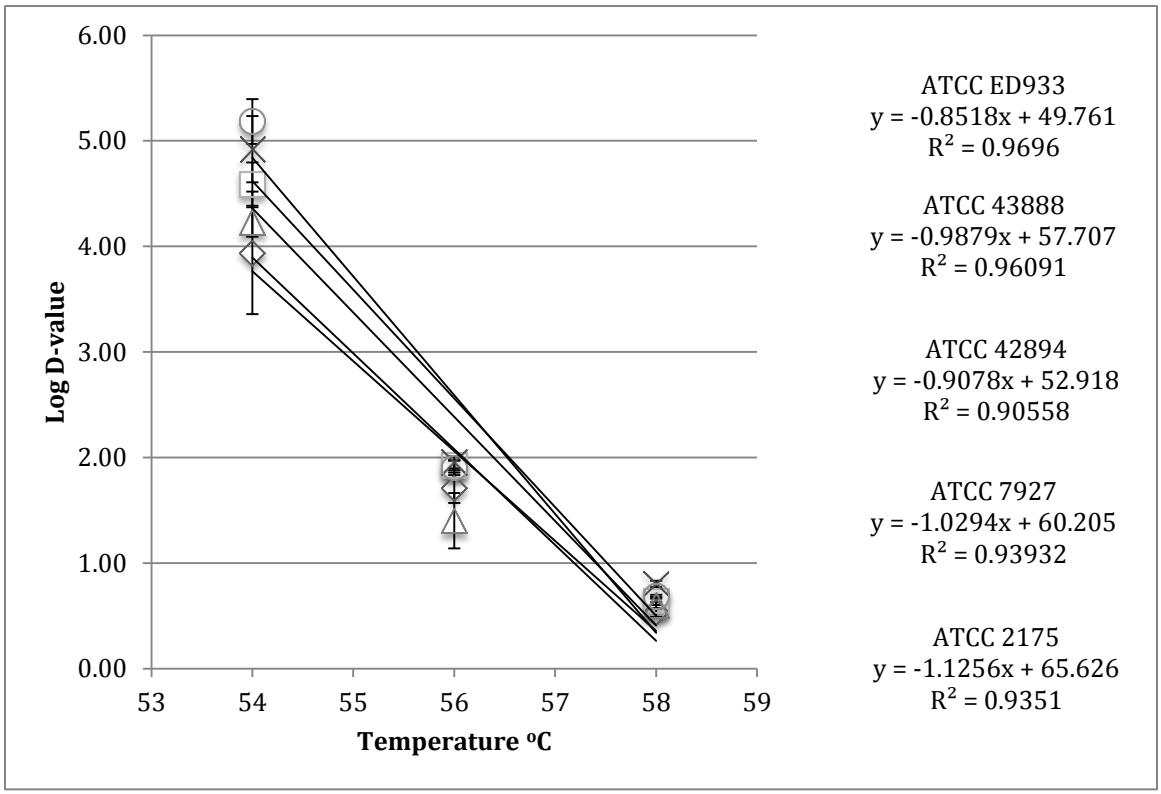

Figure 2.1 Thermal Inactivation of *E. coli* O157:H7 single strains: EDL933 (◊), 43888 (□), 43894 (Δ), 7927 (X) and 2175 (O) in phosphate buffered saline (PBS)

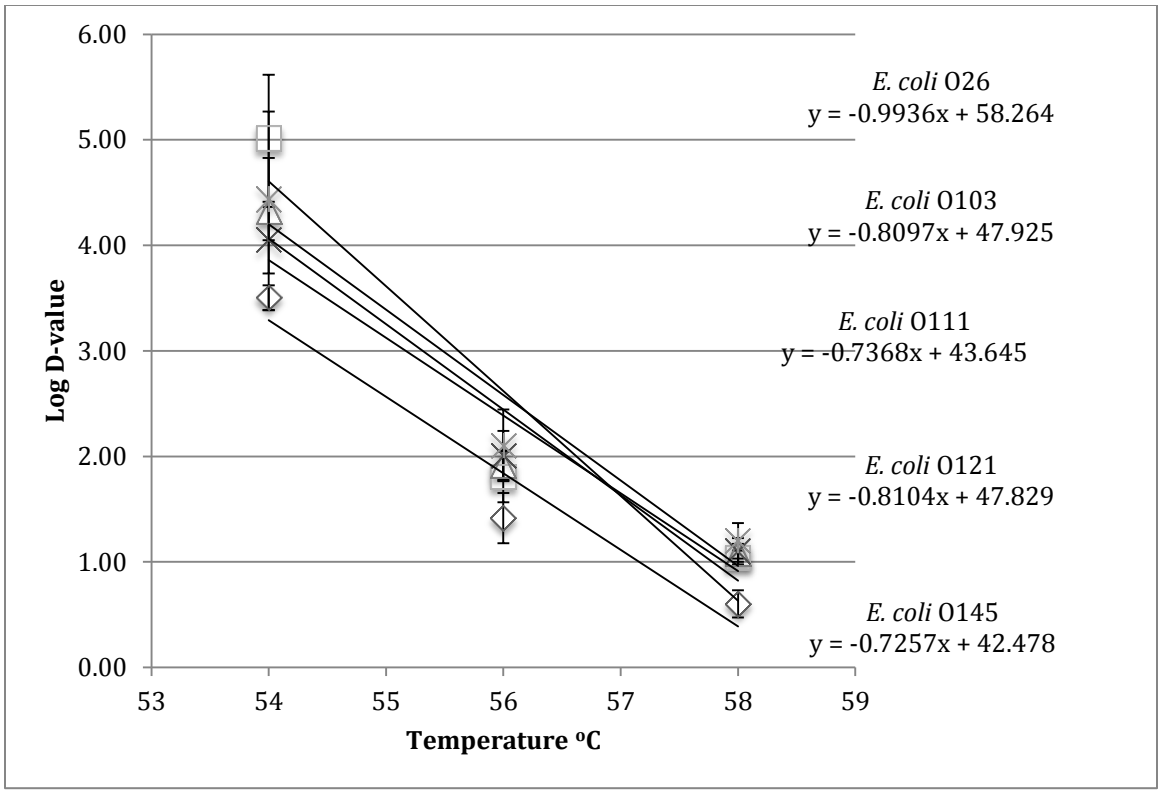

Figure 2.2 Thermal Inactivation of non-O157 *E. coli* single strains::O145 (◊), O26 (□), O121 (Δ), O111 (X) and O103 (O) in phosphate buffered saline (PBS)

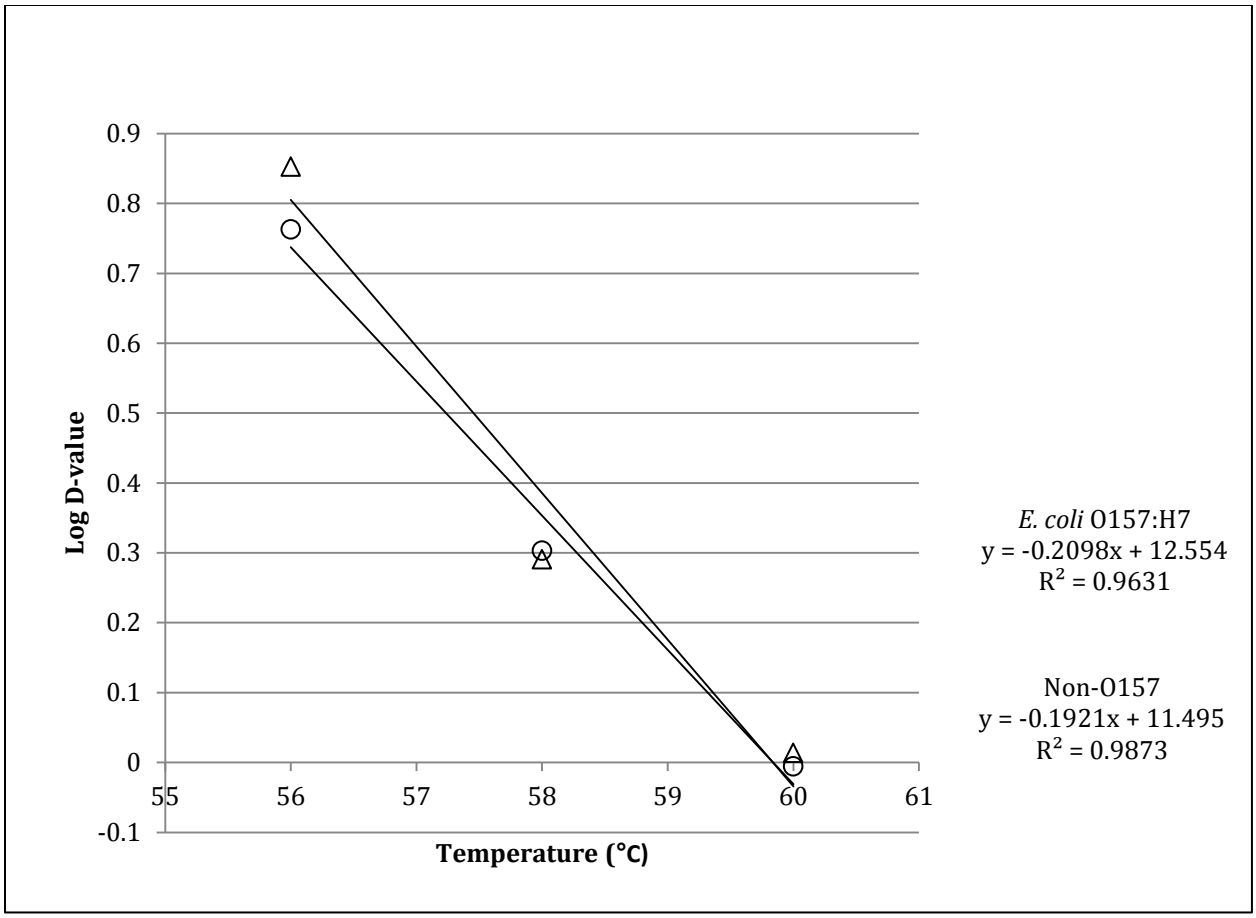

Figure 2.1 Thermal Inactivation of *E. coli* O157:H7 (Δ) and non-O157 (O) in blended spinach homogenate

Table 2. 1 D-values (min) and z-values (°C) of individual strains of *E. coli* O157 and non-O157 *E. coli* strains in phosphate buffered saline (PBS) at 54, 56 and 58°C.

Strain	D_{54}	D_{56}	D_{58}	z-value	R^2 of z
E. coli					
O157					
EDL933	3.94± 0.75 ^{AB,1}	1.71 ± 0.14 ^{A}	0.53 ± 0.04 ^{C}	4.62 ± 0.28 ^{AB}	0.99
ATCC43888	4.59 ± 0.20 ^{AB,2}	1.92 ± 0.04 ^{A}	0.64 ± 0.04 ^{BC}	4.67 ± 0.21 ^{AB}	0.99
ATCC43894	4.23. ± 0.13 ^{B}	1.40 ± 0.26 ^{A}	0.60 ± 0.03 ^{BC}	4.72 ± 0.15 ^{AB}	0.99
Cider Isolate	4.92 ± 0.31 ^{AB}	1.96 ± 0.05 ^{A}	0.80 ± 0.03 ^{ABC}	5.04 ± 0.16 ^{A}	0.99
E2175	5.18 ± 0.21 ^{A}	1.90 ± 0.07 ^{A}	0.68 ± 0.01 ^{ABC}	4.41 ± 0.13 ^{B}	0.93
Non-O157					
O145:H18 (07865)	3.50 ± 0.11 ^{A}	1.41 ± 0.23 ^{B}	0.60 ± 0.1 ^{BC}	5.26 ± 0.76 ^{A}	0.99
O26:H11 (05-6544)	5.01 ± 0.60 ^{A}	1.81 ± 0.23 ^{AB}	1.03 ± 0.0 ^{ABC}	5.87 ± 0.22 ^{A}	0.97
O121:H19 (03:2832)	4.32 ± 0.94 ^{A}	1.91 ± 0.13 ^{AB}	1.08 ± 0.08 ^{AB}	6.95 ± 1.57 ^{A}	0.98
O111:H8 (01387)	4.04 ± 0.31 ^{AB}	2.00 ± 0.23 ^{AB}	1.10 ± 0.13 ^{AB}	7.09 ± 0.55 ^{A}	0.99
O103:H11 (043973)	4.43 ± 0.38 ^{A}	2.10 ± 0.34 ^{AB}	1.19 ± 0.16 ^{A}	7.08 ± 0.86 ^{A}	0.95

[1]Data are mean values ± standard deviation (n=3)
[2]Letters indicate D-values values that are significantly different within each organism at each temperature ($P \leq 0.05$)

Table 2. 2 D-values (min) for 5 strain cocktails of *E. coli* O157 and non-O157 *E. coli* in blended spinach at 56, 58 and 60°C.

Cocktail	$D_{56°C}$[1]	$D_{58°C}$	$D_{60°C}$	z-value	R^2 of z
***E. coli* O157:H7**	7.12±0.32[A,2]	1.95±0.20[A]	0.97±0.33[A]	4.80 ± 0.92[A]	0.98
Non-O157 STEC	5.79±0.77[B]	2.01±0.17[A]	0.93±0.07[A]	5.22 ± 0.20[A]	0.96

[1]Data are mean values ± standard deviation (n=3)
[2]Different letters indicate D-values values that are signifcantly different within each temperature ($p \leq 0.05$)

Table 2. 3 6D reduction projections (in seconds) for 5 strain cocktails of *E. coli* O157 and non-O157 in blended spinach homogenate.

	E. coli O157:H7		*E. coli* non-O157	
Temperature	**D-value [1]**	**6D[4]**	**D-value**	**6D**
56°C	5.55[2]	33.31	5.48	32.89
58°C	2.34	14.05	2.31	13.85
60°C	0.99	5.93	0.97	5.83
70°C	0.79[3]	4.75	0.77	4.62
80°C	0.01	0.06	0.01	0.06
90°C	0.00	0.00	0.00	0.00

[1] D-values were calculated using the linear regression equation.
[2] D-values from 56 to 60 C are expressed in minutes.
[3] D-values from 70 to 90 are expressed in seconds.
[4] 6D is the time at specified temperature required to achieve a 6-log reduction

CHAPTER III
DETERMINATION OF THE THERMAL INACTIVATION KINETICS OF *ESCHERICHIA COLI* O157:H7 AND NON-O157 SHIGA TOXIN-PRODUCING *E. COLI* (STEC) IN GROUND BEEF AND TURKEY DELI MEAT

Abstract

In 2012, the USDA declared six non-O157 Shiga toxin-producing *Escherichia coli* (STEC) as food adulterants and included them in the zero tolerance policy. Their thermal inactivation kinetics in foods needs to be determined in order to design adequate thermal treatment processes. The objective of this study was to determine the thermal inactivation parameters of non-O157 STEC in comparison to O157 STEC on raw (ground beef) and fully-cooked (deli-) meat products. Five-strain cocktails of overnight cultures of O157:H7 or non-O157 STEC at 8 log CFU/ml were either spread onto sliced turkey deli-meat surfaces or mixed with ground beef, held for 30 min and placed into vacuum-sealed polyethylene-nylon bags. Bags were submerged in a circulating water-bath at 56°C, 58°C or 60°C for various times (come-up times were noted) and immediately cooled. Surviving bacteria from three replicate experiments were enumerated by spread-plating on Tryptic Soy Agar plates, and incubating at 24 h at 37°C. D- and z-values were calculated using linear regression. In deli-meat at 56, 58 and 60°C, D-values obtained were 15.42±0.08, 5.23±0.16 and 1.89±0.27 min for O157:H7 STEC, and 15.48±1.0, 7.20±0.56 and 2.86±0.22 min for non-O157 STEC, respectively. In ground beef at 56, 58 and 60°C, D-values were 11.85±0.93, 5.66±0.05, 1.55±0.04 min for O157, and 10.59±1.59, 3.30±0.25,1.39±0.29 min for non-O157 STEC, respectively. For O157 and non-O157 STEC in deli meat, z-values were 4.39±0.31 and 5.46±0.14°C, respectively, and in ground beef were 4.54±0.24 and 4.54±0.15°C, respectively. Food composition influences the thermal inactivation parameters for

STEC. These data will help in designing adequate thermal processes for STEC inactivation in meats.

Introduction

Shiga toxin-producing *Escherichia coli* (STEC) strains are pathogenic food-borne infectious agents, which cause various life-threatening diseases, including post-diarrheal sequelae such as hemorrhagic colitis and hemolytic uremic syndrome *(24, 44)*. It is estimated that more than 70 different serotypes of STEC cause disease in humans. Among these serotypes, several foodborne outbreaks have been associated with non-O157 *E. coli (8)*. Some of the reported non-O157 *E. coli* outbreaks include the following: O111 in Ohio (1990), O104 in Montana (1994), 0121 in Connecticut (1999), O111 in Texas (1999), O103 in Washington (2000), O111 in South Dakota (2001), O26 in Minnesota 2001*(9)*.

STEC are present in different animals as part of their natural gut microflora, as a consequence this is a very heterogeneous group of microorganisms. It is generally acknowledged that food is the main vehicle for *E. coli* O157:H7 transmission and cattle have been identified as the primary reservoir for this microorganism *(2)*. The potential risk of presence and transmission of non-O157 serotypes are presumed to follow the same pathway as O157 serotypes. Consequently one of the food items more frequently associated with *E. coli* outbreaks is undercooked ground beef *(36)*.

In 2010, the first outbreak of non-O157 *E. coli* (O26) was reported in beef and a slaughtering and processing facilitiy in Pennsylvania was identified as the contamination source *(30)*. This was the starting point for the growing concerns about the regulations regarding non-O157 STEC. In 2011, the Food Safety and Inspection Services (US FSIS) declared the top six non-O157 *E. coli* as adulterants in raw meat, non-intact beef

product or components *(37)*. These serotypes are *E. coli* O26, O45, O103, O111, O121, and O145 *(49)*, that are responsible of 26%, 5%, 22%, 19%, 6%, and 4% of reported incidences, respectively *(16)*. Together, the top six non-O157 serotypes are known to cause approximately 70 to 83% of all non-O157:H7 illnesses *(16)*.

Studies revealed that *E. coli* O157:H7 is present in 1.5% to 3.7% of the retail meat sold in groceries stores in the United States and Canada, including beef, pork, poultry and lamb *(11)*. STEC, particularly *E. coli* O157:H7 have been traditionally associated with beef and beef process products. Additionally, Beery *et al. (5)*, also highlighted chickens as potential reservoirs for *E. coli* O157:H7, since this microorganism can easily colonize the ceca of chickens, turkeys and other avian species. Given all of the above, mitigation and control strategies are essential to prevent the spread transmission of food borne illnesses cause by STEC.

Adequate heating regimes are known to inactivate foodborne pathogens. For the heat inactivation of microorganisms, traditionally the thermal tolerance of microorganisms have been calculated based on data obtained from trial testing in a liquid heating menstruum at neutral pH and high a_w *(7)*. However, the pH and a_w among food products is very heterogeneous, that consequently can affect the heat tolerance of microorganisms present in the food matrix *(7)*. Through the literature, it is evident that different types of meats, meat products and formulations can influence the thermal resistance of the tested microorganisms*(2, 11, 14, 18, 19, 25, 27, 31, 39)*.

Therefore, validation of the thermal process on each product is necessary. The thermal resistance of *E. coli* O157:H7 has been studied in several food systems, including ground beef *(10),* chicken, turkey, beef and pork sausage *(2)* lean and fatty

ground beef *(27)* where the reported D-values at 50-64^{o}C ranged from 96.67 to 0.16 min. These data suggest that *E. coli* O157:H7 shows normally expected heat resistance of bacterial pathogens. The majority of thermal resistance studies of *E. coli* O157:H7 are focused on the effect of temperature on different foods, principally meat products such as steaks and hamburger patties due to their epidemiological importance *(46)*. However, the comparison between studies is challenging due to differences between their heating menstruum, heating method, and recovery methods. Thermal inactivation data on the emerging non-O157 STEC are also needed.

Juneja *et al. (20)* studied the thermal inactivation in ground turkey meat, however, it is know that a higher salt concentration can enhance the thermal tolerance of microorganisms *(20)*. Turkey deli meat as other deli products contains a higher level of sodium and other curing salts, used to enhance the color and flavor of the product. To our knowledge the thermal resistance of Shiga toxin-producing *E. coli* in turkey deli meat has not been previously assessed. Thus, the objective of this study was to compare the thermal inactivation parameters (D- and z-values) of *E. coli* O157:H7 with the emerging big six non-O157 *E. coli* serotypes in raw ground beef with 10% fat content and turkey deli meat with 3% fat content as a fully cooked ready-to-eat product, where contamination with Shiga toxin-producing *E. coli* could potentially occur after the product has been thermally processed. The determination of D- and z-values is important when the product is intended to be re-heated by the final consumer or when it is used as an ingredient on other products as part of their product formulation.

Materials and methods

Bacterial strains

Bacterial isolates were either purchased from ATCC (Manassas, VA), provided by Dr. Pina Fratamico, USDA-ARS (Wyndmoor, PA) or obtained from the culture collections of the Animal Science and Food Science and Technology Departments, University of Tennessee, Knoxville, TN. A total of 10 strains were evaluated and divided into 5-strain cocktails of *E. coli* O157:H7 or non-O157 *E. coli*, including: ATCC EDL 933, ATCC 43888, ATCC 43894, ATCC 7927 (cider isolate) and ATCC 2175 for the *E. coli* O157:H7 cocktail and O145:H18 (ATCC 07865), O26:H11 (ATCC 05-6544), O121:H19 (ATCC 03:2832), O111:H8 (ATCC 01387), O103:H11 (ATCC 043973) and O45:H2 (ATCC 5J8) for the non-O157 *E. coli* cocktail.

Growth conditions

Stock cultures were kept frozen at -80^{o}C in mixtures of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.) and glycerol in a 50:50 proportion. Working active cultures for heat inactivation trials were prepared by streaking a loop full of the frozen stock on to a Sorbitol MacConkey Agar (SMAC; Difco Becton Dickinson) plate. Isolated single colonies were collected and transferred into TSB and incubated at 37^{o}C for 18 to 24 h. In order to promote lag-stationary phase cells, the individual strains were sub-cultured twice before use for thermal experiments. The cocktails were prepared by mixing together One-ml aliquots of each of the overnight individual cultures.

The cells were harvested by centrifugation (3 min at 5,000 x *g*), the supernatant was discarded and the pellet was re-suspended in sterile phosphate buffered saline (PBS) (Difco, Becton Dickinson), and washed twice. Cells were re-suspended to a final

concentration of 5 x 10^8 CFU/ml, the cell numbers/counts were assessed by plating the cocktails onto TSA plates and incubating at 37^{o}C for 48 h.

Thermal treatment of spiked ground beef

Freshly ground lean beef meat (3% fat content) was purchased at the local grocery store. Two 5 g samples were aseptically weighed into vacuum packaged plastic bags (polyethylene-nylon bags), one for a negative control (un-inoculated) and the other to place a thermocouple (Omega Engineering, Inc., Stamford, CT) connected to an MMS3000-T6V4 type portable data recorder (Commtest Ins., New Zealand), for monitoring the internal temperature at the geometric center of the bag.

The inoculated ground beef was prepared by mixing 40 g of ground beef samples with 4 ml bacterial culture cocktail, for each cocktail, the inoculated mixture was allowed to attach to the meat by storing at room temperature for 30 min. Bags were aseptically filled with 5 g of the inoculated sample, and sealed using a vacuum packaging machine (Multivac, D4981), the bags were flattened in order to ensure a consistent thickness for even heat distribution. The initial inoculum level prior to the heat treatment was also measured. Sealed bags were loaded into the thermospacer, which was completely immersed in the isothermal water bath (Lauda Eco Silver, Type E 40S) as described before *(32)*. The come-up time was recorded once the samples were immersed, until the target temperature was reached; and then the first bag was pulled from the water bath and the heating time commenced (time = 0). The come-up time ranged from 23 s to 1.5 min. The samples were heated at 56°C, 58°C and 60°C and the bags were removed at different time intervals based on the inactivation temperature used. At 56°C, bags were retrieved at 0, 4, 8, 12, 16 and 20 min; at 58°C after 0, 1.5, 3, 4.5, 6, and 7.5

min; and at 60°C after 0, 1, 2, 3, 4 and 5 min. The bags were immediately immersed in an ice bath in order to stop further cell inactivation.

Bags were opened aseptically by cleaning the outer surface with 70% ethyl alcohol and 135 ml of PBS was added to the bag. Bags were placed in a stomacher in order to homogenize and de-attach the cells from the ground meat. One-ml aliquots were transferred to 9 ml dilution tubes for further ten-fold serial dilutions and spread plated onto TSA plates, which were incubated at 37°C for 48 h. All the thermal inactivation experiments were conducted in triplicate.

Thermal treatment of spiked turkey deli meat

Fresh sliced turkey deli meat without added preservatives containing 1.5% fat was purchased at the local grocery store. Samples were cut in to circular shape units of diameter 3.8 cm (11.34 cm^2, approx. 5 g). Two circular sample samples were aseptically cut and placed into vacuum packaged plastic bags, one for a negative control (un-inoculated) and the other to place a thermocouple (Omega Engineering, Inc., Stamford, CT) for monitoring the internal temperature at the geometric center of the bag as described above.

The inoculated turkey deli meat was prepared by spreading 100 µL of cocktail on the sample surface and stored for 20 min at room temperature inside a BSL2-safety cabinet to allow attachment to the meat. Individual inoculated samples were placed aseptically into the bags using sterile forceps; bags were sealed using a vacuum packaging machine (Multivac, D4981).

Prior the heat treatment the initial inoculum level was determined by spread-plating an unheated sample on TSA. Sealed bags were loaded into the thermospacer,

which was completely immersed in the isothermal water bath at a predetermined temperature. The come-up time was recorded since the samples were immersed, until the target temperature in the product was reach. The samples were heated at 56°C, 58°C and 60°C.

Bags were removed at different time intervals based on the inactivation temperature used. At 56°C, bags were retrieved at 0, 4, 8, 12, 16 and 18 min; at 58°C after 0, 1, 2, 3, 4, and 5 min; and at 60°C after 0, 1, 2, 3, 4 and 5 min. After removal bags were immediately immersed in an ice bath in order to stop further cell inactivation. Bags were opened aseptically by cleaning the outer surface with 70% ethyl alcohol and cutting the bags using sterile scissors as described above for ground beef samples.

Similarly, bags were filled with 135 ml of PBS and then placed in a stomacher in order to homogenize and de-attach the cells from the turkey deli meat. One-ml aliquots were transferred to 9 ml dilution tubes for further serial ten-fold dilutions and spread plated onto TSA plates, which were incubated at 37°C respectively for 48 h. All the thermal inactivation experiments were conducted in triplicate.

D- and z-value calculations

Thermal death curves were constructed by plotting CFU/g or CFU/cm^2 versus heating time for each of the bacterial cocktails at a given temperature. The thermal death kinetics can be explained using a first order kinetics model, described by the following equation *(6)*:

$$Log_{10}\big(N(t)\big) = Log_{10}\big(N(0)\big) - \frac{t}{D} \qquad \text{(Eq. 1)}$$

Where N(t) is the observed population at time t of the process, N(0) is the initial population and the D-value is the time required for a 1-log reduction in the population at

a given temperature. D-value was calculated as the negative reciprocal of the slope of the plotted thermal death curves.

The z-value is described as the change in temperature required to cause a 1-log change in the D-value. The z-values were estimated by calculating the linear regression *(35)* of mean log D-values vs. their corresponding heating temperatures, then calculated as the negative reciprocal of the slope, as the log D-value is a linear expression of temperature:

$$z = \frac{T_2 - T_1}{Log\ D_1 - Log\ D_2} \qquad \text{(Eq. 2)}$$

Where T_1 is the initial temperature, $T_2$is the final temperature either in °C or °F; D_1 is the initial population and D_2 is the final population. 6D projections at 70, 80 and 90°C were calculated using the linear regression equation, and multiplying each D-value by 6.

Statistical analysis

Data was analysed using the statistical analysis software (SAS 9.3), an analysis of variance (ANOVA) was conducted and the statistical significant differences on the thermal resistance among the strains were established by Tukey's test using an alpha of 0.05.

Results and discussion

Thermal inactivation of STEC in ground beef and turkey deli meat over the temperature range of the study (56 to 60^{o}C), indicated that a linear relationship between the Log D-value and temperature existed for both tested cocktails of *E. coli* O157:H7 and non-O157 *E. coli*. Plotting the survivor counts per gram or cm2 versus time

generated thermal-death regression curves. The anticipated behavior was observed, where the recovery of *E. coli* O157 and non-O157 *E. coli* declined as the inactivation temperature increased (Figure 3.1 and 3.2). It is important to highlight that the pH of the 90% lean ground beef and the 1.5% turkey deli meat was around 6.0.

Ground beef results. In our study the D-values (Table 3.1) obtained at 56^{o}C in ground beef were found to be significantly different ($P \leq 0.05$) between the cocktails for *E. coli* O157 and non-O157 *E. coli*, which were 12.58±0.17 min and 10.50±0.17 min, respectively. At 58^{o}C, the D-values in ground beef were significantly different ($P \leq 0.05$) at 5.65±0.05 and 3.29±0.25 min for *E. coli* O157 and non-O157 *E. coli*, respectively. At 60^{o}C, the D-values in ground beef were not statistically significantly different ($P > 0.05$) between 1.55±0.0 and 1.39±0.29 min for *E. coli* O157 and non-O157 *E. coli*, respectively. There was a statistical difference between the O157 and the non-O157 cocktails at lower temperature, however at the highest tested temperature both cocktails exhibit the same thermal tolerance. When comparing the z-values of both cocktails in ground beef there were not statistical significant differences found. The z-values obtained in the present study were in ground beef 4.40±0.08 and 4.57±0.60 for *E. coli* O157:H7 and non-O157 respectively (Figure 2.1).

Turkey deli meat results. In turkey deli meat, at 56^{o}C, the D-values (Table 3.1) obtained were 15.39±0.14 min and 14.55±1.45 min for *E. coli* O157 and non-O157 *E. coli*, respectively, which were not significantly different ($P > 0.05$). At 58^{o}C, the D-values in turkey deli meat, the values were 5.23±0.16 and 7.20±0.55, for *E. coli* O157 and non-O157 *E. coli*, respectively (Figure 2.2) At 60^{o}C there were statistically significant differences ($P \leq 0.05$) between the two cocktails in turkey deli meat where the D-values

were 2.17±0.28 and 2.86±0.22 min for *E. coli* O157 and non-O157 *E. coli*, respectively (Table 3.1). When comparing the z-values between both cocktails in turkey deli meat the non-O157 STEC exhibited a higher z-value of 5.72±0.36°C compared to 4.40±0.37°C for the *E. coli* O157 cocktail.

In order to evaluate the goodness of fit of the log-linear model the R^2 of the linear regression equation was calculated for each of the cocktail in both products. The R^2 values ranged from 0.98 to 0.99, meaning that the log-linear model was able to explain at least 98% of the survival behavior of STEC.

The differences between the D-values obtained in ground beef and turkey for the tested strains could be attributed to the combination of several factors, including: the differences in the composition of the food products tested (Table 3.3). The proximal composition of lean ground beef is approx. 20% protein and 10% fat; while the turkey deli meat used in this study proximal composition was 11% protein and 1.5% fat (Table 3.3). Despite both being meat products one is a fully cooked formulated product, while the other is raw with no additives, different species (beef vs. turkey), different fat content (beef 10% vs. turkey 1.5%). Moreover, the difference in the inoculation method (beef, mixed thoroughly; turkey, surface spread) can play roll having an affect in the heat penetration on the product and the consequent exposure of the inoculated microorganisms. This difference could also be attributed to the heterogeneity of the non-O157 *E. coli* cocktail, since it is a blend of 5 different serotypes, their thermal response is more variable, compared to the *E. coli* O157 cocktail, where all the individual strains are more closely related.

Therefore the difference in thermal resistance of the microorganisms after heat exposure in two different products is expected. Selecting a common temperature is essential to establish comparisons among published data of the thermal inactivation of *E. coli (23)*. In a study by Doyle and Shoeni *(10)*, the D-value at 60°C was 0.75 min for *E. coli* 0157:H7 strain 932 in ground beef with 17-20% fat content. However, this experiment was carried out using 2 ml Pyrex test tubes capped with rubber stopper that may influence the heat transfer and make heating less efficient. Moreover, the D-values at 60^{o}C reported by Ahmed *et al. (2)* were 0.45 and 0.47 min in ground beef with 7% and 20% fat content, respectively, which was carried using (2g/tube) thermal death tubes.

It is also known that product fat content can influence the thermal inactivation kinectic responses and provide thermoprotection of microorganisms such as *E. coli* O157:H7 *(2)*. Juneja *et al. (23)* determined the thermal tolerance of *E. coli* O157:H7 in ground beef with 10% fat and pH 6.0 at 55, 57.5, 60, 62.5 and 65^{o}C with reported D-values of 21.13, 4.95, 3.17, 0.93 and 0.39 min, respectively. Line *et al. (27)* determined the thermal tolerance for *E. coli* O157:H7 in ground beef with two levels of fat content, 30% and 2% and reported D-values at 57.2^{o}C to be 5.3 and 0.47 min, respectively; while at 62.8^{o}C, the D-values were 4.1 and 0.3 min for 30% and 2% fat content, respectively *(27)*.

Moreover, Ahmed *et al. (2)* reported D-values for *E. coli* O157:H7 in different meat products with different fat contents including chicken (3% and 11% fat), turkey (3% and 11%), beef (7%, 10%, and 20%) and pork sausage (7%, 10, and 30% fat), at 50^{o}C and at the higher fat content D-values were 105.5 , 115.0, 92.67, and 80.64 min for

chicken, turkey, beef and pork sausage, respectively; at the same temperature and the lowest fat contents, D-values were 65.24, 70.41, 55.34, and 49.50 min for chicken, turkey , beef and pork sausage, respectively. At 55^{o}C and higher fat content, the reported D-values were 9.74, 9.69, 19.26, and 11.28 min for chicken, turkey, beef and pork sausage, respectively; at the same temperature and the lowest fat contented D-values were 8.76, 6.37, 11.40, and 6.37 min for chicken, turkey, beef and pork sausage, respectively *(2)*.

At 60^{o}C and higher fat content, D-values were 0.55, 0.58, 0.47, and 0.55 min for chicken, turkey, beef and pork sausage, respectively; at the same temperature and the lowest fat contented D-values were 0.38, 0.55, 0.45, and 0.37 min for chicken, turkey, beef and pork sausage, respectively *(2)*. Murphy *et al. (33)* reported D-values at 60 ^{o}C for *E. coli* O157:H7 to be 2.06 and 1.96 min in pre-cooked ground beef and turkey patties (127 mm diameter by 12.7mm thick), which are in agreement with the findings of our study, however the ground beef used in our study was raw and the turkey was already cooked (deli meat).

The D-values from this study were higher than those reported by Ahmed *(2)*, which could be explained by the differences in the methodology; different fat content meat products were used (7% 10% and 20% fat vs. 5% on ground beef and 3% on turkey deli meat), different *E. coli* O157:H7 strains (*E. coli* O157:H7 204P vs. five strain cocktails of *E. coli* O157:H7 and 5 strains cocktail of non-O157 *E. coli*), different heating apparatus (thermal death time (TDT) tubes vs. nylon bags), different sample size (2 g on TDT vs. 5g on bags), different plating method (Spiral plating vs. spread plate) and different recovery medium (phenol red agar with 1% sorbitol vs. tryptic soy agar plates).

It is well documented that meat products with higher fat lever resulted in higher D-values not only for *E. coli* O157:H7, but also for other foodborne pathogens *(11, 17, 27)*. The microorganisms suspended in the aqueous phase are more susceptible than those suspended in the lipid phase of the product, therefor they are harder to kill, due do to a reduction in the water activity, it is know that the water activity of the heating menstruum can influence the heat resistance of microorganisms *(4, 34)*. According to Ahmed at al *(2)*, vegetative bacterial cells are more sensitive to heat inactivation in food products with higher water activity values; in general when the fat content increases the water content of a product decreases, therefore the heat conductivity changes, this may account for the differences observed in recovery of survivors on products with different fat content.

However, in our study, when comparing the two products ground beef with 10% fat and turkey deli meat with 1.5% fat content, despite the higher fat content of ground beef, turkey deli meat had a higher thermal resistance overall. The reduced fat level and other related components such as salt and other additives of those products may diminish the effect of heat, therefore enhancing the thermal tolerance of *E. coli* 0157:H7 and non-O157 *E. coli*, despite the fat content of the products tested. This was in agreement with the results reported by Shipp *et al. (41)*, where products with higher fat content resulted in higher D-values, which is attributed to differences in the rate of heat transfer of lean vs. high fat meats.

In general, differences in the D-values from our study compared with those reported by other authors could be attributed to different strains of *E. coli* O157:H7, as well differences between individual strains vs. cocktails, physiological conditions of the

cells, fat content of the tested product, methodology for detection of survivors, selective vs. non selective medium, aerobic vs. anaerobic incubation, removal of the background microflora prior to inoculation with the target microorganism; all these factors could potentially influence the resulting D- and z-values *(2)*.

It is been suggested that the difference in the heat resistance observed with increased fat content could be attributed to the lower heat conductivities or reduced water activity in the fat portion *(39)*. The comparison between studies is difficult due to the differences in the microbial material, meat species, muscle type, product formulations and other environmental factor *(33)* including experimental errors as well.

The obtained z-values in this study were not statistically significantly different ($p>0.05$) when compared between the two cocktails, with the exception of the non-O157 *E. coli* cocktail in turkey deli meat. The z-values from this study are comparable to those published by Line *et al. (27)* who reported z-values for *E. coli* O157:H7 of 4.6 and 4.78^{o}C for lean and fatty ground beef using glass thermal death tubes.

The z-value can be influenced by the differences in the proximal composition of the food items tested (Table 3.3), also by the differences in the inoculation method between the samples, where the inocula was mixed thoroughly with the ground beef sample, while the inocula was spread on to the surface of one side of the turkey deli meat slice and then used for heating.

The study by Luchansky *et al. (28)* concluded that there were no significant differences in the thermal resistance of *E. coli* O157:H7 and non-O157 *E. coli* after cooking blade-tenderized steaks on a gas grill. As expected in our study using ground beef the thermal resistance of non-O157:H7 did fall within the range of that exhibited by

E. coli O157:H7 due to the fact that the two types of organisms are closely related. They were no significant differences ($p>0.05$) observed in the z-value of *E. coli* O157:H7 and non-O157 *E. coli* in ground beef and turkey deli meat with exception of z-value for the non-O157 *E. coli* cocktail in turkey deli meat (Table 3.1). This behaviour in the z-value has been observed by other reserchers in the past, where the reported z-values were 5.43 and 5.17^{o}C for *E. coli* O157:H7 in beef and turkey patties, respectively *(33)*.

Blackburn *et al. (7)* determined that for *E. coli* O157:H7 increasing the NaCl concentration resulted in a thermo-protectant effect resulting in higher z-values. Kontrola and Conner *(25)* studied the effect of sodium chloride, sodium lactate, polyphosphate, and fat content in the thermal tolerance of *E. coli* O157:H7 in turkey meat and concluded that the use of additives commonly used in the formulation of meat products increases the D- and z-values, however they did not find any unusual heat resistance on *E. coli* O157:H7 in turkey products. At 60^{o}C the D-values reported were 2.4 and 2.7 min for O157:H7, at 3% and 11% fat content, respectively, when using all the combination of additives *(25)*. This is in agreement with the values obtained in our study, were D-values for turkey deli meat with 1% fat were 2.17 and 2.86 min for *E. coli* O157:H7 and non-O157 *E. coli*, respectively.

Aditionally the z-values in turkey meat reported by Kontrola and Conner *(25)*, ranged from 4.08 to 6.09^{o}C and 3% and 11% fat levels with different additives combinations, which also is in agreement with our findings for *E. coli* O157:H7 and non-O157 *E. coli,* where the z-values were 4.40 and 5.72 respectively. Slightly higher z-values were reported by Juneja and Marmer *(22)*, which was 6^{o}C in lean turkey rawmeat for a cocktail of *E. coli* O157:H7, but they did not specify the fat contet of the

raw meat. In general, the higher thermal resistance exhibited by the turkey deli meat compared to the ground beef was expected due to the complexity in the formulation of the turkey deli meat compared to the ground beef; it is known that the food components such as carbohydrates, lipids and salts can provide a thermo-protectant effect*(21)*.

Overall, both cocktails of *E. coli* O157:H7 and non-O157 *E. coli* showed the same behavior in both food products ground beef and turkey deli meat, as the temperature increase resulted in a decrease in the D-value. In general, *E. coli* O157:H7 in ground beef had a higher thermal resistance compared to non-O157 *E. coli* among the tested temperatures; in contrast to turkey deli meat where non-O157 *E. coli* exhibited a higher thermal tolerance compared to *E. coli* O157:H7.

6D estimates are often used by the food industry as a criteria to evaluate the effectiveness of a thermal process and they are usually adopted as a critical control point in HACCP plans to ensure safety as a processing criteria for a specific product at a given temperature targeting a particular pathogen *(48)*. The differences in the thermal resistance among the different tested microorganisms become more obvious when 6D calculations are performed. At 56^{o}C, the time required to kill 6 logs of the microbial populations ranged from 81.93 to 91.32 min with the exception of non-O157 *E. coli* in ground beef, which needed just 59.98 min in order to achieve the same log reduction (Table 3.1).

Abdoul-Raouf *et al. (1)* reported that *E. coli O157*:H7 decreased by 6 log cycles within 14 min in ground roasted beef heated at 56^{o}C. In minced salami at pH 4.7-5, a decrease of 6 to 7 log of three *E. coli* O157:H7 strains was observed, when heated a

55^{o}C for 105-120 min *(12)*. Heating at 60^{o}C for 7 min was reported to result in a reduction of 3 log for one of the strains and a complete reduction of the other two *(12)*.

For the 6D projections increasing the processing temperature form 60 to 70°C has a drastic effect on the processing time required for a 6 log reduction, changing from values in the magnitude of minutes to seconds. Overall in ground beef, non-O157 required less time for inactivation, in contrast the opposite behavior was observed in turkey deli meat, where non-O157 *E. coli* cocktail was shown to be more thermo-tolerant. At higher temperatures, more commonly used for food processing with 6D projections, the same trend was observed. At 70°C for ground beef, 3.25 and 3.03 s appear to be sufficient to kill 6 logs of *E. coli* O157 and non-O157 *E. coli*, respectively; while 3.65 and 18.47 s in turkey deli meats are needed to achieve the same 6 log reduction for *E. coli* O157 and non-O157 *E. coli*, respectively.

The same behaviour was observed for the remaining 6D calculations at 80 and 90^{o}C, where the non-O157 in turkey deli meat required a longer time for a 6-log reduction. This is important for the food industry, where our data suggest that any thermal process that provides an internal product temperature of 70°C for 18.47 seconds would be sufficient enough to provide a >6D kill of *E. coli* 0157:H7 and non-O157 *E. coli* in the tested products.

The growing market for value-added and ready-to-eat (RTE) (products which can be consumed without any additional cooking steps) has raised concerns about their microbial safety. In the past *E. coli* O157:H7 has been found responsible for outbreaks in deli meats *(50, 29)*. The potential of cross-contamination of deli meat are of particular

concern, there are studies that show the potential contamination of RTE specifically deli meats during slicing to at least 20 consecutive slices *(38, 40)*.

Moreover it is know that *E. coli* O157:H7 have the ability to attach, colonize produce Bio-films on several types of surfaces, this ability allows *E. coli* to adhere more efficiently to foods products and also enhance the microbial resistance to environmental stresses such as sanitizing agents *(45, 15)*

Even though the relevance of STEC in meat products is well establish little is known about the cross-contamination prevalence in retail processing facilities and the control strategies. It has been estimated that 83% of the cases of listeria can be attributed to contaminated deli meats sliced at the retail as opposed to the products slice at a USDA inspected facility *(13)*. Therefore contamination with STEC can be expected to be similar in proportion.

The study by Sliagy et at *(42)* showed the *E. coli* O157:H7 forms better biofilms on stainless steel surfaces suspended in chicken, pork, turkey broths compared with glass surfaces. This is important, for the in case of post thermal treatment contamination of deli meats and the potential enhances resistant to further inactivation treatment. Bacterial cell integrated in biofilms might become up to 500 times more resistant to sanitizer compared with free cells *(43)*, it is know that bacterial resistance to one environmental stress can trigger enhanced resistance to other stresses. Therefore the importance of correct characterization of the thermal tolerance of Shiga toxin-producing *E. coli* O157 and non-O157 strains in and fully cooked slice products, given the chance of potential contamination post-processing. Deli meats can be also use as ingredient in other products such as soups, heated or toasted sandwiches, pizzas,

cordon blue *(26)*, where they will undergo through a thermal processing step; therefore knowing the D- and z-value of STEC becomes a valuable piece of information for food processing industry.

Several factors are influencing the increasing risk of microbial safety of meat products, including: consumers demanding minimally processed and more convenient food products, increasing in international food trading, increasing population at risk (infants and elderly), increasing meat and meat products consumption, and emerging of new pathogenic microorganisms *(43)*. Considering all the above there still the need for correct characterization of the thermal inactivation parameters of this microorganisms en every thermal process food, Moreover with the enforcement of the Food Safety Modernization Act, validation of the thermal process as one of the critical control points for the HACCP plans.

Therefore, it is important to determine and validate thermal kinetic parameters for each thermally processed product in order to achieve a specific lethality to prevent foodborne outbreaks *(33)*. The information presented in table 3.1 represents a useful tool for the food processing industry, to calculate the time needed at specific temperatures to achieve a desired logarithmic reduction in the population of potential contamination with either *E. coli* O157:H7 and non-O157 *E. coli* for lean ground beef (10% fat) or lean turkey deli meat (1% fat).

In summary, ground beef (10% fat) contaminated with 6 log *E. coli* O157:H7 will require heating until the internal temperature of the product reaches 70^{o}C for at least for 3.25 s and will require heating at 3.03 s for non-O157 *E. coli.* Similarly, turkey deli meat (1% fat) will require heating until the internal temperature reaches 70^{o}C for at least for

3.65 s and 18.47 s for non-O157 *E. coli* to obtain a 6-log reduction. Based on a 6D kill processing criteria, this data will help food processors in designing thermal process acceptance limits on critical control points in the manufacturing process of ground beef and turkey deli meat products to control against *E. coli* O157:H7 and non-O157 *E. coli* contamination. This data should be useful in designing adequate thermal processes to control STEC in these products.

Despite all the efforts aiming to reduce the contamination of meat products with STEC, the incidence of cases related with this pathogen has increased for both raw and process foods *(42)*. Therefor the elimination of cross-contamination of food products still a challenge and correct thermal inactivation kinetics determination still relevant.

Thermal inactivation date obtained for a specific target pathogenic microorganism in a meat food product cannot be extrapolated to other products directly because of the inherent differences in formulations, the variability of the heat tolerance among the different bacterial strains, and the heating process itself.

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Appendix

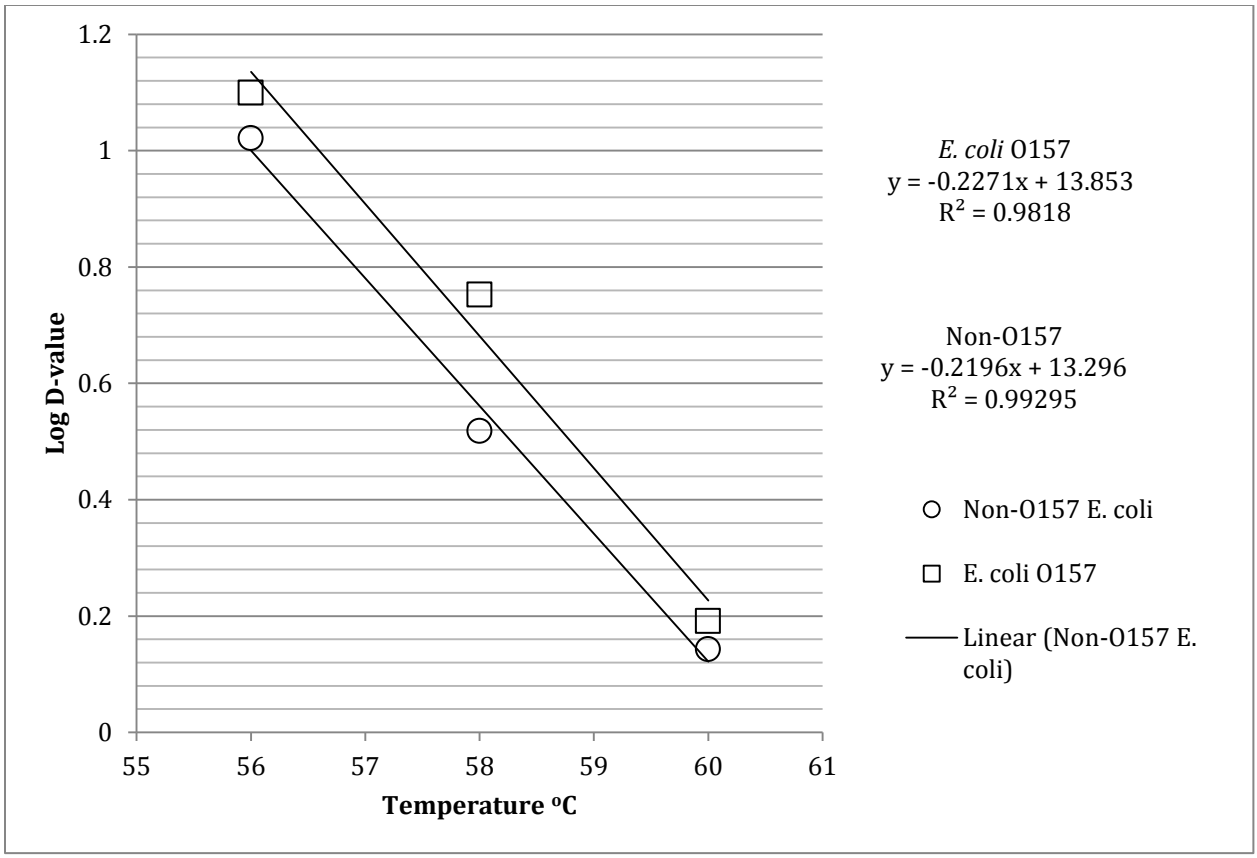

Figure 3. 1 Comparison of logarithm of D-value of cocktails of *E. coli* O157 and non-O156 *E. coli* in ground beef.

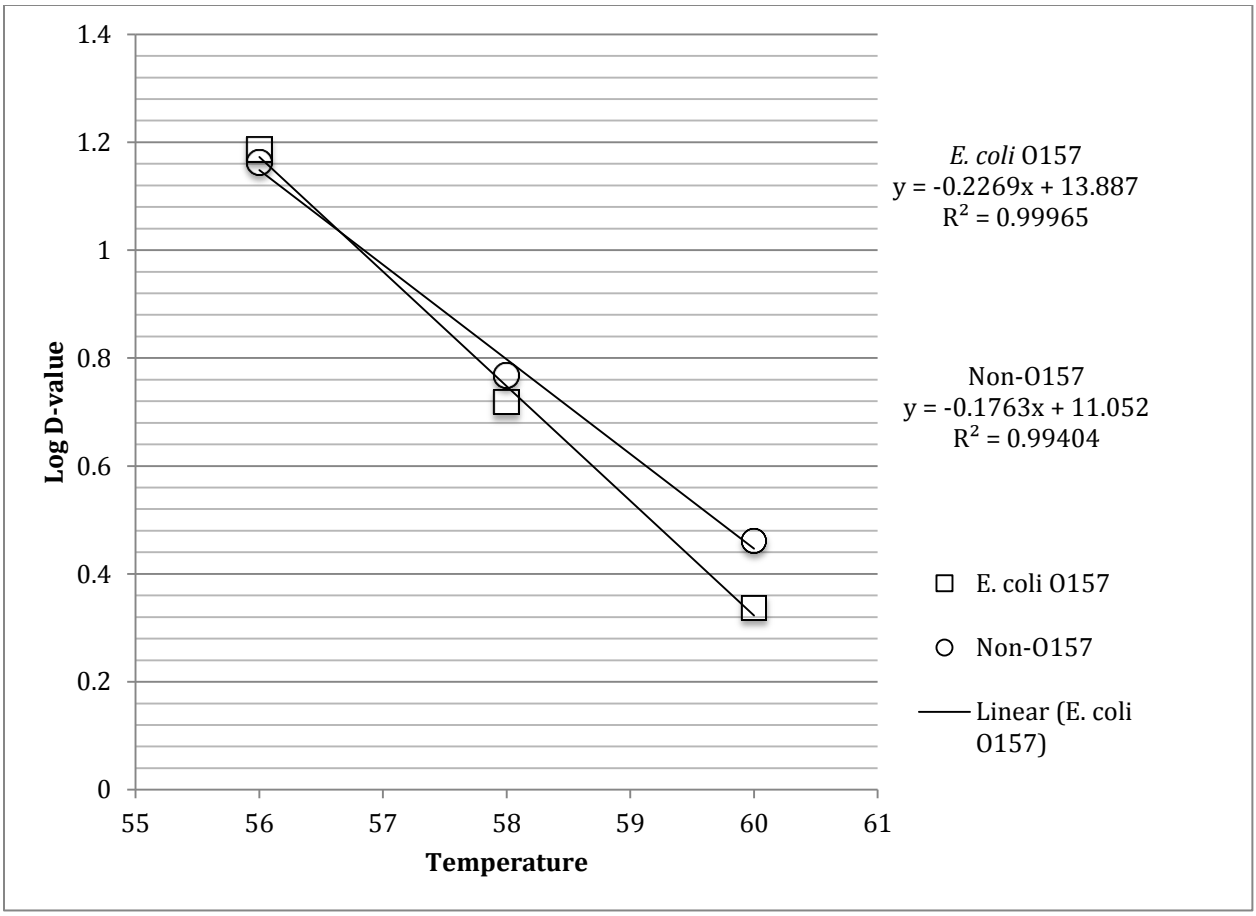

Figure 3. 2 Comparison of D-values of cocktails of *E. coli* O157 and non-O157 *E. coli* in turkey deli meat.

Table 3 1 D-values (min) of 5 cocktails of *E. coli* O157 and non-O57 *E. coli* in ground beef and turkey deli meat at 56, 58 and 60°C.

	Ground beef		Turkey deli meat	
	O157	**Non-O157**	**O157**	**Non-O157**
$D_{56°C}$ (min)	12.58±0.17^{b1}	10.50±0.17^{c}	15.39±0.14^{a}	14.55±1.45^{ab}
$D_{58°C}$ (min)	5.65±0.05^{a2}	3.29±0.25^{b}	5.23±0.16^{a}	7.20±0.55^{a}
$D_{60°C}$ (min)	1.55±0.04^{c}	1.39±0.29^{c}	2.17±0.28^{b}	2.86±0.22^{a}
z-value	4.40±0.08^{b3}	4.57±0.60^{b}	4.40±0.37^{b}	5.72±0.36^{a}
R^2	0.981	0.992	0.996	0.994

[1]Data are mean values ± standard deviation (n=3)
[2] different letters indicate D-values values that are signifcantly different within each temperature ($P \leq 0.05$)
[3]different letters indicate z-values values that are signifcantly different between columns

Table 3. 2 6D[1] reduction projections (in seconds) for 5 strain cocktails of *E. coli* O157 and non-O157 in ground beef and turkey deli meat.

	Ground beef		Turkey deli meat	
	O157:H7	**Non-O157**	**O157:H7**	**Non-O157**
$6D_{56°C}$ (min)[2]	81.93	59.98	91.32	90.55
$6D_{58°C}$ (min)	28.79	21.82	32.12	40.02
$6D_{60°C}$ (min)	10.12	7.93	11.3	17.85
$6D_{70°C}$ (s)[3]	3.25	3.04	3.65	18.47
$6D_{80°C}$ (s)	0.02	0.02	0.02	0.32
$6D_{90°C}$ (s)	0.00	0.00	0.00	0.01

[1] 6D is the time at specified temperature required to achieve a 6-log reduction
[2] D-values from 56 to 60 C are expressed in minutes.
[3] D-values from 70 to 90 are expressed in seconds.

Table 3 3 Comparison of the proximal composition of ground beef and turkey deli meat.

Proximal composition		
	Ground beef[1]	**Turkey deli meat[2]**
Calories (kcal)	175	60
Total fat (g)	10	1.5
Saturated fat (g)	3.92	0.5
Trans fat (g)	0.54	0
Cholesterol (mg)	65	35
Sodium (mg)	66	440
Total carbohydrates (g)	0	0
Dietary fiber (g)	0	0
Sugars (g)	0	0
Protein (g)	19.97	11

[1] Source: The national database for standard reference - Ground beef calculator *(47)*
[2] Source: Sara Lee products nutritional information *(3)*.

CHAPTER IV
COMPARISON OF THE HEAT INACTIVATION PARAMETERS OF *ESCHERICHIA COLI* O157:H7 AND NON-O157 SHIGA-TOXIN PRODUCING *E. COLI* (STEC) IN FETTUCCINE WITH ALFREDO SAUCE

Abstract

Little is known about the thermal inactivation kinetics of Shiga toxin-producing *Escherichia coli* (STEC) in pasta products. Although, most of the studies focus on the survival of *Salmonella* and *Staphylococcus* in pasta, the possibility of STEC contamination and survival in undercooked pasta products exist, especially when mixed with other ingredients. The objective of this study was to compare the thermal resistance of O157:H7 and non-O157 STEC cocktails in fettuccine with Alfredo sauce using linear and Weibull models. Five-strain cocktails of individually grown O157:H7 or non-O157 STEC at 8 log CFU/ml were combined with 40 g of fettuccine pasta mixed with sauce (1:1 ratio) for 30 min, aliquoted in vacuum-sealed bags and immersed in a recirculating water-bath pre-heated at 56°C, 58°C or 60°C (come-up times were monitored) for various time intervals. Serially-diluted stomached samples were spread-plated on Tryptic Soy Agar plates. Surviving bacteria were enumerated after 24 h at 37°C from triplicate experiments. The D-values from the first-order model ranged from 6.40±1.67 to 1.19±0.19 min for *E. coli* O157:H7 and 7.02±0.64 to 1.26±0.24 min for non-O157. t_D values from the Weibull model ranged from 5.28±1.52 to 1.31±0.41 min *E. coli* O157 and 6.00±0.59 to 1.49±0.37 min for non-O157. The z-values for the first-order model were 5.47±0.29 and 5.36±0.79°C for O157:H7 and non-O157, respectively; while for the Weibull model were 6.62±1.43 and 6.60±0.98°C for O157:H7 and non-O157, respectively. The utility of the Weibull model for describing the thermal inactivation of pathogenic *E. coli* was demonstrated, however the results should be interpreted cautiously.

Introduction

Pasta is one of the food products most commonly consumed worldwide *(19)*, due to ease of cooking and nutritional value *(10)*. However, the high moisture and high nutrient content of fresh pasta makes it perishable within a short time period. In the past, pasta dishes have been linked to food borne outbreaks. Where a tomato-meat based sauce was identified as the source of contamination *(6)*. In order to extend shelf-life, pasta has to be thermally processed to ensure microbial safety and biochemical stability *(20)*. Thermal processing is a conventional microbial inactivation practice used by manufacturers for fresh egg pasta products like fettuccine *(3)*. Pasta manufacturers use treatments with varied combinations of time and temperature. These thermal treatments are performed in a two-step process. One-stage pasteurization is achieved by injecting steam and two-stage pasteurization is achieved by the application of second pasteurization stage in static cells after the products are packed *(3)*.

It is known that the intensity of the thermal processing can affect the functional and rheological characteristics of the fresh egg pasta products *(2, 4)*. However, generally pasta manufacturers are not aware of the effect of heat treatments on the structural characteristics of the product and its effects on the cooking properties as a function of the treatment intensity *(3)*. These changes may also have an effect on the thermal inactivation kinetics of pathogenic microorganisms in the pasta products. Structural changes of fresh non-pasteurized pasta products when exposed to heat involve protein denaturation causing a firmer structure compared to the raw product.

This is because the newly denatured protein structure is held by stronger bonds, compared to the one formed by gelatinized starch *(3)*.

The severity of the thermal treatment is directly correlated with the weight increase and matter-loss of pasta. *(3)*. This means that during cooking the protein denaturation contributes to the swelling of starch granules, while at the same time the starch gets embedded in a protein matrix preventing the diffusion of amylose to the heating medium *(3)*. However, rehydration of pasta is known to occur from the outer surface to the center during cooking, therefore the process is not uniform until complete rehydration is accomplished *(5)*.

Regarding the microbial safety of pasta products, Goepfert *et al. (18)*, demonstrated that in general the heat resistance of microorganisms' increases as the water activity of the heating menstruum decreases. This is important because pathogenic microorganisms can potentially survive the thermal treatment in low water activity products. Anderson *et al. (5)* studied the thermal inactivation of *Clostridium botulinum* spores using different rehydration regimes for pasta, and highlighted the importance of complete rehydration of the pasta, before the application of lethal temperatures to ensure the safety of the process.

Anderson *et al. (5)* also showed that 1.2 mm thick fully hydrated pasta sheets inoculated with *C. botulinum* spores when heated at 122°C for 2 min in distilled water could achieve 3.5 log reduction; while dry pasta treated under the same conditions showed only 0.7 log reduction of *C. botulinum* spores. Thus, they illustrated enhanced thermal resistance of microorganisms due to the low water activity heating menstruum. It has also been shown that traditional pasta cooking is not enough to kill toxins

produced by *Bacillus cereus* and *Staphylococcus aureus*, moreover, pasta products have also been associated with *Salmonella* outbreaks, in the past *(12, 25)*.

Lee *et al.* (1975) reported that *S. aureus* cells were nonviable after 180 days, while enterotoxins and *Salmonella* could be recovered after one year of storage at 25°C, from inoculated dough shaped in to noodles that were incubated at 35°C for 24 h and dried to 11% moisture content at 40°C *(20).* Despite these studies described above, very little is known about the survival and thermal inactivation of *E. coli* O157:H7 and non-O157 strains in pasta products.

Also, there is need to determine if the thermal kinetic parameters (namely D- and z-values) of pathogens in fresh and dry pasta products are be similar and if they correspond to the values reported for dry products being rehydrated during the cooking process *(14).* In pasta products, water absorption occurs in two stages: the first is characterized by a rapid uptake of water through the capillaries and cavities near the surface *(16)*; and in the second stage the speed of hydration decreases due to an increment of the extraction rate of soluble materials that obstruct the capillary and inter-micellar space *(1).* These differences between dry pasta and fully rehydrated pasta could poentially influence the thermal kinetics of STEC when they are present in a fully cooked product due to cross-contamination. Many of these pre-made fully cooked pasta dishes with sauce are intended to undergo a final heating step by the consumer.

Therefore, the objective of this study was to determine and compare the thermal inactivation kinetic parameters (D- and z-values) of Shiga toxin-producing *Escherichia coli* (O157:H7 and non-O157 serotypes) when inoculated into fully cooked fettuccine pasta mixed with Alfredo sauce in a 1:1 ratio. This information will be useful to the food

processing industry for establishing appropriate thermal conditions and parameters to inactivate STEC in pasta and pasta products, consequently reducing the risk of foodborne outbreaks.

Materials and methods

Bacterial strains

Bacterial isolates were obtained directly from either ATCC (Manassas, VA), from Dr. Pina Fratamico, USDA-ARS (Wyndmoor, PA), or from the University of Tennessee, Food Science and Technology Department culture collection. Five individual strains of *E. coli* O157:H7 including ATCC EDL 933, ATCC 43888, ATCC 43894, ATCC 7927 and ATCC 2175 were used to prepare the cocktail. The non-O157 *E. coli* cocktail included O145:H18 (ATCC 07865), O26:H11 (ATCC 05-6544), O121:H19 (ATCC 03:2832), O111:H8 (ATCC 01387), O103:H11 (ATCC 043973) and O45:H2 (ATCC 5J8).

Growth conditions

Bacterial cell stock cultures were stored frozen at -80^{o}C in media containing a 1:1 mixture of tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.) and glycerol. The working cultures for the thermal inactivation experiments were made by streaking a loop full of the frozen stock on to a tryptic soy agar (TSA) plates and also on Sorbitol MacConkey (SMAC; Difco Becton Dickinson) plates for confirmation. An Isolated single colony was picked and transferred into test tube with TSB and incubated at 37^{o}C overnight for 18 to 24 h to promote lag-stationary phase cells, the individual strains were

sub-cultured twice before use for thermal experiments. This was performed for each individual strain before they were mixed into cocktails as described before *(21)*.

As described before in earlier studies, the bacterial cocktails were prepared by mixing a 1 ml aliquot of the overnight individual cultures *(21, 23)*. The cells were washed twice by centrifugation (5,000 x *g* for 3 min), the supernatant was discarded and the cell pellet was re-suspended in sterile phosphate buffered saline (PBS) (Difco, Becton Dickinson). The cell cocktail was further diluted in PBS in order to reach the final of 5 x 10^8 CFU/ml; bacterial counts were confirmed by spread plating both cocktails on to TSA plates and further incubation at 37^{o}C for 24 h.

Pasta preparation

Dry Fettuccine pasta and Bertolli Alfredo sauce were purchased at the local grocery store. The dry fettuccine was cooked in boiling water for about 10 min (al dente). The cooked noodles were cut into 3.81 cm (1.5 in) length pieces and mixed with Bertolli Alfredo sauce in a 1:1 proportion. Two 5 g samples (to mimic the experimental treatment conditions described below) were aseptically weighed into vacuum package plastic bags (polyethylene-nylon bags), one for a negative control (un-inoculated) and the other to place a thermocouple (Omega Engineering, Inc., Stamford, CT) connected to an MMS3000-T6V4 type portable data recorder (Commtest Ins., New Zealand), for monitoring the internal temperature at the geometric center of the bag.

Thermal treatment

The inoculated pasta-sauce was prepared by mixing 40 g of blended sample with 4 ml individual cocktails of the *E. coli* O157 or non-O157 *E. coli*. The inoculated blend was allowed to rest for 30 min at room temperature, in order to allow the

microorganisms to attach and adapt to the conditions present in the mixture. The experimental units of nylon-polypropylene-nylon vacuum bags were aseptically filled with five-grams of the inoculated pasta sauce blend in the center of the bag, and the noodles were aligned to ensure even thickness of the sample for uniform heat distribution. Bags were sealed using a vacuum packaging machine (Multivac, D4981).

The initial inoculum level of the inoculated blend prior to the heat treatment was measured. Sealed bags were placed into the thermospacer (a structure that helps to ensure proper water circulation for efficient heat distribution along all the samples/bags). The loaded thermospacer was completely immersed into the isothermal water bath (Lauda Eco Silver, Type E 40S). The come-up time was recorded, beginning immediately upon samples immersion, until the internal target temperature was reached.

Immediately after the target temperature was reached, the first bag was removed from the water bath and labeled as the time zero. The come-up time ranged from 23 to 35 s. The samples were heated at 56°C, 58°C and 60°C and the bags were removed at different time intervals based on the inactivation temperature used. At 56°C, bags were retrieved at 0, 2, 4, 5, 8 and 10 min; at 58°C after 0, 1, 2, 3, 4, and 5 min; and at 60°C after 0, 0.5, 1, 1.5, 2 and 3 min. after retrieval the bags were immediately placed in an ice bath in order to heat inactivation.

Enumeration of survivors

The outer surfaces of the bags were cleaned with 70% ethyl alcohol and aseptically opened using sterile scissors as described before *(21)*. The bags were filled with 135 ml PBS and then placed in a stomacher in order to homogenize and de-attach

the cells from food samples. One-ml aliquots were transferred to 9 ml dilution tubes for further ten-fold serial dilutions and spread plated onto TSA plates, followed by incubation at 37°C for 48 h. Each thermal inactivation trial was performed/replicated thrice.

D- and z- value calculations

Heat inactivation curves were constructed by plotting the surviving bacterial counts (CFU/g) against the heating time at each of the temperatures; this was done for both of bacterial cocktails. The thermal inactivation parameters were described by fitting a linear model using the following equation *(7)*:

$$Log_{10}\big(N(t)\big) = Log_{10}\big(N(0)\big) - \frac{t}{D} \qquad \text{(Eq. 1)}$$

Where *N(t)* is the observed population at time t of the process, *N(0)* is the initial population and the D-value is the time for a 1-log reduction in the population at a given temperature. D-value was calculated as the negative reciprocal of the slope of the plotted thermal death curves. The z-value is explained as the change in temperature to cause a 1-log change in the D-value. The z-values were calculated using the negative reciprocal of the slope:

$$z = \frac{T_2 - T_1}{Log\ D_1 - Log\ D_2} \qquad \text{(Eq. 2)}$$

Where T_1 is the initial temperature, T_2 is the final temperature either in °C or °F; D_1 is the initial population and D_2 is the final population. 6D projections at 70, 80 and 90°C were calculated using the linear regression equation and multiplying each D-value by 6 as described earlier by Bozkurt *et al. (9)*.

Weibull calculations

The Weibull model is based on the assumption that the survival of microorganisms can be described by a cumulative distribution of the lethal effects of heat damage to the cells *(13)*. It can be calculated using the following equation:

$$S(t) = \exp\left(-\left(\frac{t}{\alpha}\right)^{\beta}\right) \quad \text{(Eq. 3)}$$

Which is the equivalent to equation (Eq. 4)

$$S(t) = \frac{1}{2.303}\left(\frac{t}{\alpha}\right)^{\beta} \quad \text{(Eq. 4)}$$

It al so can be re written in the form of equation (Eq. 5)

$$logS(t) = -bt^{\beta} \quad \text{(Eq. 5)}$$

Where b is equivalent of equation (Eq. 6)

$$b = \left(\frac{1}{2.303}\right)\alpha^{-\beta} \quad \text{(Eq. 6)}$$

The time required to achieve a specific logarithmic reduction using the Weibull model can be calculated using the following equation.

$$t_d = \alpha\left(-ln\left(10^{-d}\right)^{1/\beta}\right) \quad \text{(Eq. 7)}$$

Where d is the number of decimal reductions desired. For the calculations in this article t_d was calculated using $d = 1$ (meaning one logarithmic reduction).

Statistical analysis

Data were analysed using the statistical analysis software (SAS 9.3), an analysis of variance (ANOVA) and the mean separations were determined using a Tukey's test using an alpha of 0.05. For the non-linear regression analysis of the Weibull model SPSS release 21.0.0.1 statistical package (SPSS, IBM Armonk, NY) was used.

To evaluate the goodness of the fit between the two models, three statistical parameters have to be considered: higher regression coefficient R^2, lower chi-square valueχ^2, and lower root mean square error (RMSE) *(9)*. χ^2 and the RMSE values were calculated by comparing the experimental and predicted survival ratio values for each time data point, for all the tested temperatures, using the following equations:

$$\chi^2 = \frac{\sum_{i=1}^{N}\left(S_{exp,i}=S_{pred,i}\right)^2}{N-n} \quad \text{(Eq. 8)}$$

$$RMSE = \left[\frac{1}{N}\sum_{i=1}^{N}\left(S_{exp,i} - S_{pred,i}\right)^2\right]^{1/2} \quad \text{(Eq. 9)}$$

Where *Sexp,i* is the i^{th}experimentally observed survival ratio, *Spred,i* is the i^{th} predicted survival ratio, N is the number of observations and n is the number of constants.

Results and discussion

In order to explain the thermal inactivation of the tested microorganisms, first order kinetic and Weibull models were evaluated. Thermal death curves were determined graphically by plotting survivor counts recovered per gram against time of the thermal treatment at a given temperature (Figure 4.1). As expected, the surviving bacterial counts decreased as the temperature increased, for both *E. coli* O157 and non-O157 STEC cocktails.

For the first order kinetics model, the D-value is defined as the time necessary to inactivate 1 log cycle of the initial population at a given temperature. However when the Weibull model is used T_d (a pseudo D-value) can be used to establish the time required

for 1-log reduction, this allow for direct comparison between the linear and the Weibull model. D-values were determined for all the tested temperatures using the first-order model and the T_d -values for the Weibull model at all the tested temperatures. For the Weibull model, there is some additional thermal inactivation parameters used for describing the thermal inactivation behaviour of the data. These parameter are α and β, where, α is the mean of the cumulative distribution or scale factor while β is the shape parameter *(24).*

Table 4.1 shows the summary of the thermal inactivation parameters, namely D- and z-values for the first order kinetics and the Weibull model. There was no statistically significant differences ($p<0.05$) observed between the mean D-values of the two cocktails, *E. coli* O157 and non-O157 *E. coli* within each of the tested temperatures 56, 58 and 60°C. The $D_{56°C}$ values were 7.66±0.04 and 7.47±0.17min for *E. coli* O157:H7 and non-O157 *E. coli*, respectively; $D_{58°C}$ values were 3.40±0.14 and 3.29±0.25 min for *E. coli* O157:H7 and non-O157 *E. coli*, respectively and $D_{60°C}$ values were 1.20±0.19 and 1.39±0.29 min for *E. coli* O157:H7 and non-O157 *E. coli*, respectively.

The results of the thermal inactivation experiment using fully cooked fettuccine pasta with Alfredo sauce inoculated with O157 or non-O157 STEC in general follows a linear behavior between the number of survivors recovered and temperatures, over the tested temperature range of this study (56 to 60°C). However, the Weibull model does an overall better job at explaining the thermal inactivation behaviour of both O157 and non-O157 STEC presented by the experimental data (Table 4.2).

For the Weibull model, t_d values comparable to D-value from the first order model were obtained (Eq. 7), the t_d values at 56^{o}C were 5.28±1.52 and 6.00±0.59 min for *E.*

coli O157:H7 and non-O157 *E. coli*, respectively; at 58^{o}C the values were 3.46±0.80 and 4.88±0.61 min for *E. coli* O157:H7 and non-O157 *E. coli*, respectively; and at 60^{o}C values were 1.31±0.41 and 1.49±0.37 min for *E. coli* O157:H7 and non-O157 *E. coli*, respectively. There was not statistical difference (P<0.05) between the D-values and the t_d values within each of the tested temperatures,

The calculated z-values for the Weibull model were 6.62±1.43 and 6.60±0.98^{o}C for *E. coli* O157:H7 and non-O157 *E. coli*, respectively. These values were higher compared to those calculated from the first order model, which were, 5.47±0.29 and 5.36±0.79^{o}C for *E. coli* O157:H7 and non-O157 *E. coli*, respectively, however they were not statistically different (P>0.05).

For the Weibull model, parameter α and β were estimated using non linear regression, the α or scale parameter is an indicative of the effect of the heating environment on the inactivation *(8)*; while the β parameter can be used to interpret the thermal inactivation behavior of the microorganisms, this parameter is indicative of shouldering or tailing effect.

At 56^{o}C, β were <1 (0.56±0.15 and 0.60±0.44, respectively) suggesting that the cells at any point in the inactivation curve were capable to adapt to the applied stress. At 58^{o}C the β parameter was close to 1(0.98±0.19 and 1.06±0.12, for *E. coli* O157 and non O157 respectively), making the Weibull parameter follow a linear behavior; while at 60^{o}C β was >1(1.23±0.22 and 1.01±0.29, for *E. coli* O157 and non O157 respectively), which can be interpreted as the remaining cells during the heat treatment as becoming increasingly damaged.

Table 4.2 summarizes the different statistical parameters used to establish the goodness of fit of the two models. For the Weibull model, the regression coefficient R^2, which can be understood as the ability of the model to explain the data values, ranged from 0.95 to 0.99; while for the first order model the R^2 ranged from 0.90 to 0.98, meaning that the Weibull model was better at explaining the thermal inactivation data of the pasta with sauce system, when compared with the linear model. Moreover, the Weibull model exhibited lower RMSE values compared with the linear model (the smaller the value the better). For the first order model, RMSE values ranged from 0.293 to 0.819, while for the Weibull model the values ranged from 0.070 to 0.275 meaning that the Weibull predicted model, on average deviated less from the experimental data compared to the first order model.

Additionally χ^2 is used to compare the individual observation of each category and if it significantly differs from the expected value calculated from the predicted model, the lower the value the better. χ^2 values for the linear model ranged from 0.054 to 0.785, while for the Weibull model the values ranged from 0.008 o 0.026. Given that the Weibull model exhibits lower values compared to the first-order model, the Weibull model was more adequate at explaining the variation of the observed values compared to the predicted values.

Overall, the Weibull model was better at explaining the thermal inactivation data obtained from the inoculated fettuccine Alfredo with cocktails of *E. coli* O157 and non-O157 at the tested temperatures. This better fit is expected since the Weibull model uses/optimizes two parameters (α and β) in order to explain the thermal inactivation

behavior of the data, while the linear model just explains the thermal inactivation data in terms of one parameter (heating time).

It is acknowledged that Weibull model exhibits a better fit compared to the first-order kinetics model. However this is only true when the target is one log reduction, for industry applications the target is usually 6 or 7 decimal reductions (6D or 7D) as required for pasteurization. The calculation for a 6D reduction using the linear model is easy, however, while using the Weibull model the 6D becomes a more complex calculation. It is not $6t_{d=1}$ is actually $t_{d=6}$ (Eq. 7), due to the non-linearity of the model, where the slope of the curve changes as function of time *(11)*. Weibull also has another pitfall whenever deviations from the linear behavior occur (either shouldering or tailing) 6D projections can lead to inaccuracies of the thermal process incurring either in over- or under-processing, which can consequently result in food quality or safety problems respectively *(11)*.

6D projections were calculated for both cocktails (Table 4.3) and small differences were obtained between the cocktails, but they are negligible since there is no statistically significant difference between the z-values of both cocktails ($p>0.05$). For example, at lower processing temperatures $6D_{56°C}$ meaning that after the cold spot of the product reached 56°C, 40.78 and 47.78 min are required for 6 logs reduction of the initial population, for *E. coli* O157 and non-O157 *E. coli*, respectively. Moreover at $6D_{70°C}$ after the cold spot of the product reaches 70°C, 6.78 and 7.05 s are required to achieve 6 log reduction for *E. coli* O157 and non-O157 *E. coli*, respectively. 6D projections were calculated only for the linear model. However, If the t_6 was used for the calculation of the 6D reduction it will result in a shorter processing time, less than is

accurately required, resulting in potential survivors due to under-processing (data not shown) *(11)*.

There are some studies that determined the thermal resistance of microorganisms in pasta products. However, they are not specific either to fettuccine with Alfredo sauce or pathogenic *E. coli*, therefore a direct comparison is difficult. In a study using *Bacillus stearothermophilus* spores at approximately 10^5 CFU/g pasta, Cunningham *et al. (14)*, determined the D-values at 121°C with different moisture content ranging from 70 to 145% dry basis; the reported $D_{121°C}$ values ranged from 4.6 to 6.5 min, respectively to lower to higher moisture content. When the moisture content increased, the thermal resistance of the spore decreased. In the same study by Cunningham *et al. (14)*, z-values were determined to evaluate the thermal sensitivity of the spores within a temperature range 110 to 125°C; the z-values ranged from 15.5 to 10.7°C at corresponding moisture contents, 70 to 145%, respectively.

Earnshawn *et al. (15)* attributed the increased thermal stability of spores, when heated in low water products to the formation of strong complexes by the dipoles of the proteins inside the cells that stabilize the protein in the absence of water. In a similar way, the high concentration of carbohydrates, a major constituent of pasta products is known to affect the heat tolerance of microorganisms, large amounts of starch decrease the water activity of the product, therefore increasing the thermal tolerance of the microorganisms *(19)*.

Pasta products usually are flavored with other ingredients such as spinach, tomato sauce, Alfredo sauce etc. or stuffed with dairy or meat products, which could affect the thermal tolerance of the present microorganism. It is known that pathogenic *E.*

coli could be present in pasta dishes. In a study by Giannuzzi *(17)*, *E. coli* was detected in 20% one out of five samples of the raw materials used to make the pasta, moreover *E. coli* was present in 20% of the ricotta cheese used to prepare stuffed ravioli; suggesting that the raw materials could be potentially hazardous, if they are not cooked properly.

Therefore, it is importance to determine the thermal inactivation kinetic parameters of the final pasta product once already mixed with all the ingredients, including the sauce and stuffing. As stated by Pittia *(22)* the raw material quality as well as the formulations have a direct effect on the microbial quality of the food products before cooking and also if additional thermal process is expected from the final consumer. It is also important to evaluate different thermal inactivation models and compare the thermal inactivation parameters obtained from each model, in order to design more appropriate thermal processes, without underestimating the potential survival of pathogenic microorganisms or over applying the heat treatment that can diminish the organoleptic and nutritional characteristics of the product.

The majority of reported thermal tolerance studies are performed using a synthetic liquid medium. Therefore, their results are not very applicable for more realistic scenarios using food product exposed to different heat and processing treatments such as extrusion or drying. The results of this study therefore provide the necessary information required to the fill the existing data gaps for thermal processing parameters of mixed pasta products to ensure food safety of the consumers and for the food processing industry in establishing appropriate thermal parameters to inactivate STEC in pasta and pasta products.

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Appendix

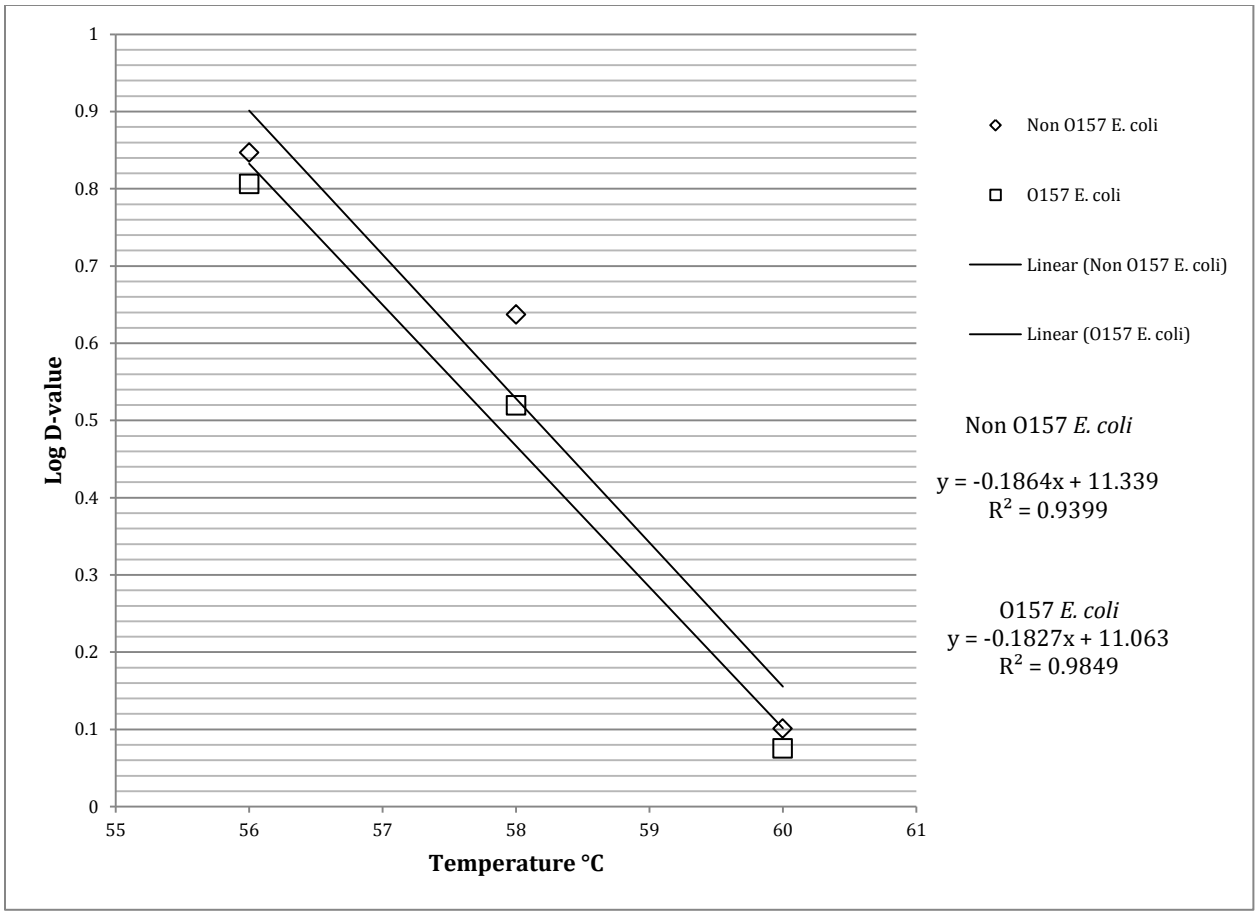

Figure 4. 1 Logarithm of D-values of cocktails of *E. coli* O157 and non-O157 *E. coli* in fettuccine with Alfredo sauce.

Table 4. 1 D-values (min) of 5 strain cocktails of *E. coli* O157 and non-O157 *E. coli* in fettuccine with Alfredo sauce at 56, 58 and 60°C.

		First order kinetics		Weibull model			
Strain	**Temp °C**	**D-value**	**z-value**	***β***	***α* (min)**	**$T_{D=1}$**	**z-value**
***E. coli* O157**	$D_{56°C}$ (min)	6.40±1.67^{A}	5.52±0.29^{A,3}	0.56±0.15	1.12±0.52	5.28±1.52^{AB,1}	6.62±1.43^{A}
	$D_{58°C}$ (min)	3.30±0.14^{B}		0.98±0.19	1.49±0.59	3.46±0.80^{BC,2}	
	$D_{60°C}$ (min)	1.19±0.19^{C}		1.23±0.22	0.67±0.28	1.31±0.41^{C}	
Non-O157	$D_{56°C}$ (min)	7.02±0.64^{A}	5.41±0.79^{A}	0.60±0.44	1.46±0.9	6.00±0.59^{A}	6.60±0.98^{A}
	$D_{58°C}$ (min)	4.33±0.27^{B}		1.06±0.12	2.23±0.48	4.88±0.61^{AB}	
	$D_{60°C}$ (min)	1.26±0.24^{C}		1.01±0.29	0.66±0.32	1.49±0.37^{C}	

[1]Data are mean values ± standard deviation (n=3)
[2] Different letters indicate D-values values that are signifcantly different within each column ($P \leq 0.05$)
[3] Different letters indicate z-values values that are signifcantly different between models and strains.

Table 4. 2 Statistical comparison of the first-order kinetics and Weibull models for the survivor curves of *E. coli* O157 and non-O157 *E. coli* cocktails.

		Weibull distribution			First Order Kinetics		
Strain	Temp (^{o}C)	R2	RMSE	X2	R2	RMSE	X2
E. coli O157	56	0.9801	0.193[2]	0.0253	0.900	0.723	0.390
	58	0.986	0.053	0.008	0.959	0.348	0.125
	60	0.995	0.060	0.009	0.976	0.338	0.201
Non-O157	56	0.984	0.275	0.026	0.919	0.819	0.785
	58	0.948	0.070	0.012	0.965	0.293	0.054
	60	0.981	0.312	0.026	0.964	0.637	0.485

[1]The higher the bvalue the better the fit
[2] the lower the value the better the fit
[3] the lower the value the better the fit

Table 4. 3 6D projections for thermal inactivation of *E. coli* O157 and non-O157 cocktails in pasta fettuccine with Alfredo sauce.

	Weibull distribution		First Order Kinetics	
	E. coli O157	Non-O157	*E. coli* O157	Non-O157
$6D_{56}$ (min)	73.87	44.79	40.78	47.78
$6D_{58}$ (min)	22.59	26.78	17.58	20.25
$6D_{60}$ (min)	5.77	9.51	7.58	8.58
$6D_{70}$ (s)	N/A[2]	N/A	6.78	7.05
$6D_{80}$ (s)	N/A	N/A	0.10	0.10
$6D_{90}$ (s)	N/A	N/A	0.00[1]	0.00

[1]value less than 0.00 s
[2]Not applicable

CHAPTER V
CHAPERON DNAK PROTEIN PRODUCTION BY THE SHIGA TOXIN PRODUCING *ESCHERICHIA COLI* (STEC) UNDER HEAT AND ACID-SHOCK CONDITIONS AND SUBSEQUENT THERMAL INACTIVATION

ABSTRACT

Emerging non-O157 Shiga toxin-producing *E. coli* (STEC) were recently added to the zero tolerance policy by the USDA-FSIS. Therefore, precise characterization of their stress response to common processing conditions and its effects on their thermal tolerance is needed. The cellular mechanism for stress adaptation involves production of chaperon proteins including DnaK, which helps to re-fold damaged proteins. The objective of this study was to compare the DnaK concentration among eight different STEC serotypes subjected to heat- and acid-shock and subsequent effects on their D-values. Six non-O157 STEC, *E. coli* O157:H7 and non-pathogenic *E. coli* K12 strains were used. For the heat-shock treatment, overnight cultures were held at 46°C for 15 min and cells were held in an acidified tryptic soy broth (TSB, pH 6.5) for 1 h at 37°C for acid-shock treatment, prior to protein extraction. DnaK concentration was determined using competitive ELISA. Significant differences ($p<0.05$) were found in DnaK concentrations between strains; heat-shocked *E. coli* O111 and O121 showed the highest concentration of DnaK molecules/cell (18,182.72 and 17086.91, respectively). Acid-shocked *E. coli* O103 had the highest D-value (4.09±0.29 min) among all the tested strains. Monitoring the concentration of DnaK revealed that higher levels were detected after sub-lethal injury either with heat- or acid-shock, enhancing their thermal tolerance. D-values for un-shocked cells ranged from 1.49±0.35 to 2.21±0.17 min; heat-shocked from 2.04±0.35 to 2.83±0.35 min and acid-shocked from 2.32±0.29 to 4.09±0.29 min. This should be taken in consideration when acid- or heat-shock conditions might occur during food processing in order to design proper thermal inactivation processes.

Introduction

There are more than 250 serotypes of Shiga toxin-producing *E. coli* (STEC) and over 100 have been related to sporadic outbreaks and development of hemolytic uremic syndrome (HUS) *(12, 39)*. Like *E. coli* O157:H7, non-O157 STEC are known to cause infection infections in humans that can lead to the same gastrointestinal diseases, including HUS and potential death *(12)*. Non-O157 STEC are responsible of a large number of food related outbreaks in the US, Europe, Australia and other countries worldwide *(15)*. It has been estimated that *E. coli* O157:H7 causes around 63,153 cases of domestic food borne illness per year, while non-O157 STEC are responsible for 112,752 cases, almost 49,600 cases more than O157:H7 *(40)*. According to the Brooks *et al. (4)*, about 75% of the non-O157 STEC cases reported to the CDC, belong to "the big six" serogroups, including: O26, 045, O111, O121 and O145. This has generated an increasing concern about their regulation and control strategies. In 2011 the USDA Food Safety and Inspection Service *(8)* declared these top six serogroups of non-O157 STEC as adulterants in raw beef products, by including them in the zero tolerance policy *(8)*.

Non-O157 STEC has been found responsible for several outbreaks in diverse food items. Some of these implicated food include raw and pasteurized milk, raw and pasteurized goat cheese, fermented sausage, cider, vegetables and drinking water *(15,28)*. Since the declaration of the big six non-O157 STEC as adulterants, there is a need to characterize their stress response to common food processing conditions. However a very small amount of studies have been conducted in this regard *(5,42)* but

the results obtained at this point suggest that heat and acid treatments designed for *E. coli*O157:H7 will also be effective killing non-O157 STEC *(42)*.

However, the non-O157 STEC groups is very heterogeneous, and there is variability in their stress tolerance, for example it has been shown that non-O157 STEC can survive in fermented products such as sausage and cheese, in which other treatments are needed for their inactivation *(31, 32, 36)*. The stress response of pathogenic microorganisms and *E. coli* O157:H7 to environmental factors such as pH, $a_{w,}$ heat-shock and cold shock stress has been widely studied *(9,2,29)*. In contrast, little is known about the growth and survival of non-O157 STEC under stress in food products and water. Therefore, it cannot be assumed that they will behave in a similar way to *E. coli* O157:H7 when they are exposed to environmental stress or in food systems

Heat treatment is one of the most common traditional food preservation processes to enhance the shelf life and ensure the safety of the food products. Cells respond to the exposure to supra-optimal temperature or heat-shock in several ways, severe exposures can lead to cytotoxicity and cell death *(19)*. The heat treatment can induce important changes in the internal cell structure, leading to damage, such as protein denaturation and degradation and consequently resulting in cell apoptosis *(11, 18, 43)*. Sub-lethal thermal injury can result in sensitivity to other stresses and/or the production of chaperon proteins, which can enhance the cell tolerance to heat *(16)*. These specific proteins can alleviate or reduce damage incurred by the cell *(27)*.

The main heat-shock protein is DnaK, which is highly conserved during evolution sharing 50% of it amino acid sequence with its eukaryotic homologous HSP70 protein

(21). DnaK is known to work synergistically with DnaJ and GrpE, which together form the “chaperone machine” *(10)*. Considering the crucial role of DnaK in the heat-shock response and the changes in concentration upon heat stress, Enzyme Linked Immunosorbent Assays (ELISA) represent a rapid way to detect and measure the intracellular levels of DnaK in *E. coli*.

Therefore the objectives of this study are (1) to determine the effects of two environmental stresses, heat and acid-shock on the concentration of intracellular DnaK among the different STEC strains and (2) to understand the effect of the concentration of intracellular DnaK on the thermal tolerance of the different STEC strains after exposure to sub-lethal injury by either heat or acid-shock treatments.

Materials and methods

Bacterial strains

Bacterial strains were obtained from ATCC (Manassas, VA), Dr. Pina Fratamico, USDA-ARS (Wyndmoor, PA), and from the culture collections of the Animal Science and Food Science and Technology Departments, University of Tennessee, Knoxville, TN. A total of eight strains, *E. coli* K12, *E. coli* O157:H7 ATCC EDL 933, and six non-O157 strains included *E. coli* O145:H18 (ATCC 07865), *E. coli* O26:H11 (ATCC 05-6544), *E. coli* O121:H19 (ATCC 03:2832), *E. coli* O111:H8 (ATCC 01387), *E. coli* O103:H11 (ATCC 043973) and *E. coli* O45:H2 (ATCC 5J8) were used.

Growth conditions

Stock cultures were maintained in a mixture of tryptic soy broth (TSB) medium with 15% (vol/vol) glycerol and stored at -80°C. Active cultures for thermal inactivation experiments were prepared by streaking material from the frozen cultures onto tryptic soy agar (TSA; Difco, Becton Dickinson) plates. Single colonies were transferred to TSB media and incubated at 37°C for 18-24h. These growing cultures were sub-cultured overnight twice prior to the application of sub-lethal stress treatments

The heat or acid-shock treatments were applied to the cells only after the cells reached the exponential growth phase to a final concentration of approximately 8 logs. This was verified by measuring the optical density at 600 nm (values were expected to be approx. 0.5). Cells were harvested and the intracellular concentration of DnaK was determined.

Acid-shock treatment

Twenty-ml of overnight cultures were centrifuged at 3000 x g for 3 min and the pellet was washed twice with phosphate buffered saline (PBS pH 7.0). The pellet was re-suspended in 5 ml of acidified tryptic soy broth (TSB) (pH 6.5 with 0.5M acetic acid) and incubated for 1h at 37°C.

Heat-shock treatment

Twenty-ml of overnight cultures were centrifuged at 3000 x g for 3 min and the pellet was washed twice with PBS, re-suspended in 5 ml of TSB (pH 7.0) and heated at 46°C for 15 min in a re-circulating water bath. After the heat treatment, the cells were cooled down in an ice-bath.

Protein extraction

After treatment with either with acid or heat-shock, the cells were centrifuged at 5000xg for 10 min). The pellets were treated with B-PER Complete Bacteria Protein Extraction Reagent according to the manufacturer's instructions (250 mL of B-PER is sufficient for lysis of 50g of bacterial biomass). Two- μL of 0.5 EDTA per mL of BPER was added to improve the cell lysis. The treated pellets were incubated for 15 min at room temperature with a gentle rocking movement, followed by centrifugation of the lysate at 16,000xg for 20 min.

The lysated pellet was re-suspended in 5 ml of 0.1 M $NaHPO_4$ buffer at pH 7.0, containing PMSF (phenyl methyl sufonyl fluoride from pierce; at final concentration of 0.1mM per mL of total bacterial protein extract), PMSF was added in order to inhibit degradation by endogenous serine proteases including: trypsin, chemotrypsin, thrombin and papain. The resulting protein extract was kept at -20^{o}C until further use.

Total protein determination by the Bicinchoninic Acid (BCA) method

The total protein content of the bacterial protein extract from was measured using a commercial BCA kit (Sigma-Aldrich) and the manufacturers recommended procedure was followed. The resulting absorbance was measured at 562 nm and the standard curve was constructed using bovine serum albumin (BSA) as a protein standard.

Competitive Enzyme Linked Immunosorbent Assay (ELISA) method for DnaK measurement.

The bacterial protein extracts obtained above from the different treated and untreated strains were used to measure the DnaK concentration with a competitive ELISA method using a modified protocol published by Seyer *et al.* (2003). A stock DnaK solution (1 mg/ml) was prepared in 0.1 M NaHCO3 at pH 9.6 and microplates (Plate

MaxiSorp surfaces; Nunc-Immuno, Roskilde, Denmark) were coated with 100 µl DnaK (0.25 µg/ml; StressGen Biotechnologies Corp., Victoria, British Columbia, Canada) per well, followed by an overnight incubation at 4°C.

The plate was washed four times using a washing solution (phosphate-buffered saline (PBS) supplemented with 0.05% Tween 20(from AlfaAesar®), blocked with 200 µl per well of the block solution (1%PBS-bovine serum albumin solution) and incubated for 1 h at room temperature (22°C) and washed (4 times) with the washing solution. The competition step was performed using a mouse anti-DnaK monoclonal antibody (1 mg/ml; from MyBiosource) that was diluted to 1:10,000 in PBS-1% BSA-0.05% Tween 20. The diluted anti-DnaK and the bacterial protein extract were mixed in a 1:1 ratio using a micro-centrifuge tube and incubated for 1 h at room temperature with constant agitation and 100 µl of this mixture were added to the coated wells and incubated for 2 h at 37°C. The plate was washed (4 times), and 100 µl of the sheep polyclonal antimouse immunoglobulin G-peroxidase conjugate HRP labeled (1mg/ml; Bethyl laboratories Inc.) per well were added. The secondary antibody was prepared using a 1:50,000 dilution in PBS-1% BSA-0.05% tween 20.

The microplate was then incubated for 30 min at 37°C, followed by a washing step (4 times) and addition of 100 µl of the substrate, tetramethyl benzene (Thermo Scientific) for 15 min at room temperature with a gentle rocking movement followed by the addition of 100 µl of 2M H_2SO_4 to each well. The optical density was read at 450nm using a spectrophotometer (Synergy HT from Bio-tek Instruments, Inc.).The standard curve was performed with purified DnaK diluted in a PBS-1% BSA-0.05% Tween 20 solution (detection limit, 269 molecules/cell).

Thermal tolerance method

Two ml aliquots of the treated and untreated (control) eight cultures were washed twice and re-suspended in 18 ml of PBS to reach a final concentration of approx. 7 logs. Two-ml aliquots were placed into a 2 ml glass vial for each of the time points (a total of 6 vials) that were placed in an isothermal water bath (Lauda Eco Silver, Type E 40S, Karlsruhe, Germany) using a plastic rack to ensure the proper water circulation in between the glass vials. In order to monitor the vials internal temperature, an extra vial was filled with PBS and a type-T thermocouple (Omega Engineering, Inc., Stamford, CT) was placed in the geometric center of the vial and connected to an MMS3000-T6V4 type portable data recorder (Commtest Inc., New Zealand).

Another vial was also placed at the geometric center of the water bath. In addition, the water-bath temperature was verified using a mercury-in-glass (MIG) thermometer (Fisher Scientific, Pittsburgh, PA). The come up time, defined as the time required for the vials internal temperature to reach the target inactivation temperature, was recorded for all the inactivation experiments. The come-up time ranged from 1.15 to 1.25 min. Once the target temperature was reached, the vial tube was immediately removed from the water bath and place in and iced-bath and the sample was labeled as time zero. Samples were heated at 56°C and the vials were retrieved every 2 min (time interval was determined by preliminary studies) at 0, 2, 4, 6, 8, and 10 min. After removal from the water bath, the vials were placed in an ice water bath for at least 5 min to stop the thermal inactivation process. The samples were then sprayed with 70% ethyl alcohol prior to aseptically opening.

Determination of thermal inactivation kinetics.

For determining the number of surviving bacteria, ten-fold serial dilutions of the samples in PBS were spread plated onto tryptic soy agar (TSA) plates and incubated at 37°C for 48 h before colonies were enumerated. The initial counts were determined for the untreated cells and also for the heat and acid-shock treated cells prior to the thermal inactivation test. All the experiments were performed in triplicate, and untreated cells were used as controls. The recovery counts were then used for the determination of the thermal inactivation kinetics for each individual strain.

The thermal death kinetics for a first order model according to Bigelow and Esty (1920) using survivor curves, were constructed by plotting CFU/ml against the heating time for each of the strains. The mathematical equation of the model was expressed as:

$$log_{10}\frac{N_{(t)}}{N_o} = -\frac{t}{D} \qquad \text{(Eq. 1)}$$

Where $N_{(t)}$ is the number of survivors after exposure time t in CFU/ml or g, N_o is the initial population (CFU/ml or g) and the D-value is the time for a 90% population reduction at the isothermal process temperature. D- value was determined as the negative reciprocal of the slope of the plotted survivor curves.

Statistical methods

An analysis of variance (ANOVA) test using Bonferroni's correction was conducted to establish the differences of the means and to determine if there was a statistical difference between the thermal resistance of the different strains after being treated either with acid or heat-shock, as well as to determine if there was a statistical difference in the concentration of DnaK, among the different strains and also among the heat or acid-shock treatments. Correlation analysis was calculated using the Pearson's

correlations coefficients. The data was analysed using the statistical analysis software (SAS) (version 9.3, SAS Institute, Cary, NC, USA). Differences were considered to be significant at P >0.05.

Results and discussion

Cell counts results

Results indicate that there was a statistical difference (P>0.05) between the initial counts of each heat-shocked strains and their un-shocked cells for all the tested strains (Table 5.1). In general lower values were obtained for the heat-shocked cells compared with the un-shocked control. However (Table 5.2) for the acid-shocked cells there was not statistical difference (P>0.05) between the initial counts of each of the acid-shocked cells and the un-shocked cells within each strain for all the tested strains, with exception of *E. coli* O26 where the values were 7.05±0.15 and 7.91±0.01 log CFU/ml, for the acid-shocked and the control cells, respectively.

Total Protein results

For the heat-shocked cells results (Table 5.1) indicate that there were no statistical differences (P>0.05) between the levels of total amount of bacterial protein of the heat-shocked cells and the un-shocked cells within each strain for all the tested strains. However, *E. coli* K12 and *E. coli* O121 exhibited the highest levels of total bacterial protein. For *E. coli* K12 the protein concentrations values were 535.61±54.01and 568.59±48.07 **μg/ml** for the heat-shocked and control cells, respectively; while for *E. coli* O121 the protein concentrations values were 737.77±63.19 and 680.43±64.29 **μg/ml** for the heat-shock and control cells, respectively. *E. coli* O157:H7 showed the lowest protein concentration the values were

409.93±47.04 and 455.24±49.19 **μg/ml** for the heat-shock and control cells, respectively.

For the acid-shocked cells results (Table 5.2) indicate that there was a statistical difference (P>0.05) between the levels of total amount of bacterial protein of the acid-shocked cells and the un-shocked cells within each strain for all the tested strains. The levels of total bacterial protein were lower after exposing the cells to the acid-shock treatment. The most drastic changes in concentration of protein were observed for *E. coli* O121 and *E. coli* O145. For *E. coli* O121 the values were 403.92±50.50 and 680.43±64.29 **μg/ml** for the acid-shock and control cells, respectively; while for *E. coli* O145 the values were 358.20±39.82and 520.79±51.95 **μg/ml** for the acid-shock and control cells, respectively.

These results showed that the cell protein synthesis remains un-altered after the cell was exposed to the sub-lethal heat-shock treatment. However, for the acid-shock cells the total proteins level decreased after the exposure to sub-lethal acid-shock treatment. This suggests that the *E. coli* cells uses different mechanisms to handle acid and heat stress and the response to these stresses is strain dependent in terms of the total amount of bacterial synthesis.

DnaK results

For the heat-shocked cells results (Table 5.1) indicate that there was no statistical difference (P>0.05) between the levels of total amount of DnaK protein of the heat-shock treated cells and the un-shocked cells within each strain for all the tested strains, except for *E. coli* O121. The highest DnaK concentrations were obtained for the

E. coli K12 and *E. coli* O121. For *E. coli* K12 19.89±3.01 and 19.59±3.01 ng/ml; while for *E. coli* O121 the values were 20.57±4.89 and 17.44±3.39 ng/ml.

For the acid-shocked cells, results (Table 5.2) indicate that there was not statistical difference (P>0.05) between the levels of DnaK protein of the acid-shock treated cells and the un shocked cells within each strain for all the tested strains, except for *E. coli* O121. For *E. coli* O121 the levels of DnaK protein were significantly (P<0.05) higher after exposing the cells to the acid-shock treatment. The DnaK values were 22.62±4.39 and 17.44±3.39 ng/ml for the acid-shocked and the control cell of *E. coli* O121, respectively. This represents a 30% increment in the DnaK concentration of *E. coli* O121 of the treated cells compared to their control.

DnaK molecules per cell results

Once the DnaK values are normalized by the cell counts; for the heat-shock cells there were statistical differences in the values of DnaK molecules per cell. This was influenced by the variability of the initial count values. The highest DnaK molecules/cell values were obtained for *E. coli* O111 with 18182.72 molecules/cell. The lowest concentration of DnaK molecules/cell was obtained for *E. coli* O26 and O121 un-treated cells with values of 1821 and 2035.76 molecules/cell, respectively. However, *E. coli* O121 after heat-shock had increased concentration of 17086.091 molecules/cell. Similarly for *E. coli* O26 DnaK molecules per cell increased after heat treatment, reaching a value of 6867.5 molecules/cell. For the acid-shock cells there was no statistical difference in the values of DnaK molecules per cell. This was influenced by the initial bacterial counts values, since they have no statistical difference (P<0.05)

within each strain after acid-shock and control cells and also among all the tested strains.

D-value results

There was no statistical difference (P>0.05) between the D-values of the heat-shocked cells and the un-shocked cells within each strain for all the tested strains, except for *E. coli* O145 (Table 5.1). The D-values at 56°C ranged from 1.49±0.35 to 2.83±0.35 min and the highest value was obtained for un-treated *E. coli* O103 cells. For *E. coli* O145 there was a negative correlation between D-value and the initial bacterial counts number (r=0.99, P=0.04 n=3), meaning that the higher the initial count number of cells, the lower the D-value.

There was no statistical difference (P>0.05) between the D-values of the acid-shocked cells and the un-shocked cells within each strain for the tested strains with exception of strains *E. coli* 026, O103, and O121 (Table 5.2). For these strains the D-value increased after being exposed to a sub-lethal acid-shock treatment. The D-values for *E. coli* O26 were 2.32±0.29 and 1.81±0.20 min for the acid-shock and the control cells, respectively; while for *E. coli* O103 the values were 4.09±0.29 and 2.11±0.29 min for the acid-shock and the control cells, respectively; and for *E. coli* O121 values were 3.52±0.35 and 1.92±0.29 min for the acid-shock and the control cells, respectively.

For *E. coli* O26 there was a positive correlation between D-value and the initial counts number (r=0.80, P=0.05 n=3), meaning that the higher the initial cell count number the higher the D-value. Also there was a negative correlation between the total amount of bacterial protein and the molecules/cell (r=-0.82, P=0.04, n=3), meaning that the increase in DnaK concentration was correlated with a decrease in the synthesis of

total bacterial protein, the cell produces more chaperon proteins to help to re-fold any damaged proteins instead of producing new proteins.

For acid-shocked *E. coli* O103, there was a significant positive correlation between the amount of total bacterial protein and the amount of molecules per cell ($r=0.99$, $P=0.01$, $n=3$), meaning that the total bacterial protein present highly correlated with the concentration of DnaK molecules/cell. For *E. coli* O121, there was a positive correlation between total amount of bacterial protein and D-value ($r=-0.96$, $P=0.002$, $n=3$), meaning the higher D-value is correlated with a higher amount of total bacterial protein.

From these data we can conclude that for *E. coli* 0103 and O121 the drastic decrease in the total protein production with the simultaneous increment in the production of DnaK, can be held responsible for the enhanced thermal tolerance after acid-shock. The same behavior was observed for all the tested strains after acid-shock though, on a smaller scale. In contrast, *E. coli* O157 exhibited a small reduction in the concentration of DnaK after heat-shock, however its heat tolerance was not affected, suggesting that the interaction of other chaperon proteins in different proportions, possibly helped in the protection of this strain against further thermal inactivation.

Our results are in agreement with the study carried by Juneja *(13)*, that used ground beef inoculated with *E. coli* O157:H7 pre-treated with a heat-shock at 46°C for 15 min to 30 min, and had increased thermal tolerance when exposed to 60°C, compared with non pre-treated samples *(13)*. They correlated the enhanced thermal tolerance in the heat-shocked samples with the increased concentration of two proteins of 60 and 69 kDa immunologically related to GroEl and DnaK, respectively *(13)*.

The effect of acetic acid on different STEC serotypes including O26:H11, O111:H2, O91:H2, O145:H2, and O157:H7 in Luria Broth (LB) at different pH was also reported by others *(30)*. No growth at pH 4.5 and 5.0 in the presence of acetic acid was reported; while at pH 5.5 all strains were reported to grow with the exception of O111:H2 and O145; at pH of 6.5 almost 50% of the samples reportedly showed growth, determined by monitoring turbidity over a 72 h period *(30)*. Thus, substantial variability in the tolerance of both *E. coli* O157:H7 and non-O157 *E. coli,* when exposed to acetic acid was shown *(30)*. Another study comparing the survival rates of *E. coli* O157:H7 against non-O157 STEC (O26, O111 and O121:H19), using minimal medium at pH 2.0 and 2.5, indicated that the survival rates of O157:H7 were less than those for non-O157 STEC *(17)*. This is important for potential acid adaptation and enhanced thermal tolerance against further thermal processing. This clearly shows the importance of considering the food product characteristics before designing an appropriate thermal process. This data gathered in this study supports this need.

It is known that *E. coli* O157 and non O157 STEC have similar mechanisms to respond to acid environment, including the acid tolerance response, the oxidative system, and the glutamate and arginine decarboxylase systems *(6)*. However some non-O157 STEC strains have an extra acid stress response not present on *E. coli* O157 strains*(6)*, which is based on chaperon proteins, HdeA and HdeB *(6)*. Even tough *E. coli* O157 and non O157 STEC can cause the same diseases; the study of their genome reveals that they have different evolutionary histories *(35)*. Carter *et al. (6)* demonstrated that the genes hdeA and hdeB were necessary for acid tolerance of STEC serotypes O145:NM and K-12 but not for serotypes O157:H7 and O55:H7;

despite the genes being present in all serotypes, the HdeB protein was not produced in the O157:H7 serotype. An additional single nucleotide polymorphism study found that a G→A transition in the putative start codon of the *hdeB* gene explained the absence of the HdeB protein on the O157 serotype *(6)*. Moreover, this G→A transition in the +3 position was found in 20 strains of the O157:H7 serotype but not on 12 of the non-O157 STEC including: O26:H11, STEC O111:H8, O111:H11, STEC O111:HN, and STEC O45:H2). The study concluded that the acid resistant mechanisms of *E. coli* O157:H7 and non-O157 STEC follow different evolutionary paths; *E. coli* O157:H7 acid survival is not HdeB/A dependent since the *hdeB* gene is silenced.

Given the heterogeneity of the non-STEC group and their variability in terms of virulence stress response, it makes it difficult to predict their behavior under environmental stress, in food systems and during processing, that emphasizes the importance of studying their kinetic behavior*(42)*. Traditional heat inactivation methods are based on a first order kinetic model, which has some limitations especially when bacteria exhibit either a more sensitive or resistant and/or adaptation to a particular stress *(41)*, in some cases sub-lethal exposure to stress can enhance the tolerance to subsequent severe heat treatments *(3, 25, 26, 34)*. Therefore, prediction of thermal inactivation becomes a challenge especially at moderate processing temperatures (50 to 65^{o}C) like those used in *sous vide* cooking style, which allows stress adaptation *(41)*.

There are several studies that assessed the thermal tolerance parameter for *E. coli* O157:H7, that indicate that the organism does not exhibit an unusual heat resistance *(1, 7, 22, 37)*. However, very little is known about the thermal resistance of non-O157 STEC. Studies have shown that environmental factors can enhance the

thermal resistance of microorganisms *(23, 33)*. The stress induced by sub-lethal temperature triggers the production of heat-shock proteins that can enhance the thermal tolerance of the microorganism considerably. Such conditions can be found in products minimally processed, refrigerated foods of extended shelf life such as *sous-vide* foods, where heating temperature allows the come up time to be long enough for the microorganism to produce heat-shock proteins to enhance their thermal stability and therefore their survival in the food product *(14)*. This should be also considered for products that are kept in warming trays before the final heating step or products that are intended to be reheated by the final consumer, also if there are interruptions In the cooking process due to failures, the thermal process should be designed with enough safety to ensure the inactivation of potential thermo-tolerant enhanced microorganisms *(14)*. In this regard, all the different stresses should be considered for the design of proper thermal process, against *E. coli* or any other food borne pathogens to ensure the safety of the products.

Although, the thermal inactivation process is normally studied by using different cultural methods, to evaluate the impact of temperature on the conformation of the different cell structures *(24)*, the exact mechanism by which temperature kills bacterial cells is still not fully understood. However, studies have shown that there is a correlation between the thermal denaturation of critical components in the cell and thermal cell death *(20, 38)*.

Besides expanding the body of knowledge, this research will help to provide better insights on the differences of the thermal activation of Shiga-toxin producing *E. coli* O157:H7 and non-O157 STEC, as well understand how sub-lethal injury after

exposure to acid conditions or a heat-shock can potentially enhance the thermal stability of the bacterial cells. This will help the industry to create more suitable processing conditions when the target is the destruction of the potential pathogenic *E. coli* using mild heat treatments.

Since the addition of the non-O157 STEC to the zero tolerance policy, the interest on this serogroup has increased. Smith and Fratamico *(42)*, expressed clearly that “Clinicians, food scientists and regulatory agencies cannot and should not assume that the non-O157 STEC will behave similarly to *E. coli* O157:H7”. Therefore, research is needed in order to draw proper conclusions about the “big six” strains responsible for the majority of reported cases of non-O157 STEC infections in the US.

In conclusion, this study showed that for some STEC strains, the thermal tolerance can be enhanced either by acid or heat-shock treatment with the increase in the production of the chaperon protein DnaK; while the total protein synthesis decreased. Overall, the application of a heat or acid-shock stress resulted in a higher thermal tolerance of the STEC when heated in buffer, however the magnitude of this effect was strain dependent. These results represent useful insights on the effects of processing conditions on the survival of pathogenic microorganisms in foods and to help mitigate the risk of food borne outbreaks when STEC is a potential hazard.

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Appendix

Table 5.1 Total protein and DnaK levels in comparison to thermal tolerance for heat-shock treated and control (untreated) cells

E. coli serogroup	Treatment	Initial Counts (Log)	Total Protein (μg/ml)	% Variation*	DnaK (ng/ml)	% Variation **	Dnak (molecules/cell)	D_{56}-value (min)
O157:H7	Heat-shock	7.05±0.05^{c,1}	409.93±47.04^{g}	0.9	14.58±2.13^{de}	0.94	11493.42^{abcd}	2.71±0.35^{ab}
	Un-treated	7.77±0.07^{ab}	455.24±49.19^{fg}	1	15.47±2.76^{cde}	1	2323.21^{cd}	2.04±0.29^{abcd}
K12	Heat-shock	7.08±0.07^{c}	535.61±54.01^{a}	0.94	19.89±3.01^{ab}	1.02	15030.53^{abc}	2.42±0.35^{abcd}
	Un-treated	7.45±0.05^{abc}	568.59±48.07^{a}	1	19.59±3.01^{ab}	1	6204.02^{abcd}	2.15±0.22^{abcd}
O26	Heat-shock	7.41±0.19^{abc}	486.06±45.71^{def}	0.99	19.30±3.46^{abc}	1.15	6867.5^{abcd}	2.04±0.35^{abcd}
	Un-treated	7.91±0.01^{a}	492.60±40.91^{def}	1	16.80±3.38^{abcde}	1	1821^{d}	1.81±0.29^{cd}
O45	Heat-shock	7.19±0.37^{c}	598.27±50.65^{b}	1.03	19.29±2.68^{abc}	1.04	13188.8^{abcd}	2.75±0.35^{ab}
	Un-treated	7.77±0.07^{ab}	578.47±46.85^{bc}	1	18.56±2.62^{abcd}	1	2795.05^{cd}	2.21±0.17^{abcd}
O103	Heat-shock	7.26±0.42^{bc}	534.56±47.13^{bcd}	1.01	16.50±4.22^{bcde}	1.16	9627.57^{abcd}	2.83±0.35^{abcd}
	Un-treated	7.52±0.20^{abc}	529.94±38.09^{bcde}	1	14.18±3.713^{e}	1	4348.8^{bcd}	2.11±0.29^{a}
O111	Heat-shock	6.98±0.19^{c}	460.45±32.80^{efg}	0.95	19.83±4.72^{ab}	1.1	18182.72^{a}	2.32±0.35^{abcd}
	Un-treated	7.77±0.07^{ab}	482.71±31.84^{def}	1	17.98±3.30^{abcde}	1	2702.98^{cd}	2.01±0.29^{abcd}
O121	Heat-shock	7.04±0.22^{c}	737.77±63.19^{a}	1.08	20.57±4.89^{a}	1.18	17086.91^{ab}	2.73±0.35^{ab}
	Un-treated	7.88±0.01^{a}	680.43±64.29^{a}	1	17.44±3.39^{abcde}	1	2035.76^{d}	1.92±0.29^{bcd}
O145	Heat-shock	7.10±0.06^{c}	486.36±41.13^{def}	0.93	17.03±2.77^{abcde}	1.07	12154.39^{abcd}	2.67±0.35A^{abc}
	Un-treated	7.77±0.07^{ab}	520.79±51.95^{cdef}	1	15.90±2.46^{bcde}	1	2389.26^{cd}	1.49±0.35^{d}

*% Variation of total protein was calculated using the untreated total protein concentration as the base within each strain.

**% Variation of DnaK protein was calculated using the untreated total protein concentration as the base within each strain.

[1]Different letters mean significant differences between the means (P>0.05).

Table 5. 2 Total protein and DnaK levels in comparison to thermal tolerance for acid-shock treated and control (untreated) cells

E. coli serogroup	Treatment	Initial Counts (Log)	Total Protein (μg)	% Variation*	DnaK (ng/ml)	% Variation **	Dnak (molecules/cell)	D_{56}-value (min)
O157	Acid-shock	7.34±0.27^{ab,1}	373.88±45.56^{g}	0.82	17.50±2.68^{bcd}	1.13	10138.56^{a}	2.41±0.29^{bdc}
	Un-treated	7.77±0.07^{ab}	455.24±49.19^{def}	1	15.47±2.76^{cd}	1	2323.21^{a}	2.04±0.29^{bcd}
K12	Acid-shock	7.42±0.36^{ab}	451.35±59.71^{ef}	0.79	20.26±3.27^{abc}	1.03	9005.73^{a}	3.34±0.35^{abc}
	Un-treated	7.45±0.05^{ab}	568.59±48.07^{b}	1	19.59±3.01^{ab}	1	6204.02^{a}	2.15±0.22^{bcd}
O26	Acid-shock	7.05±0.15^{b}	407.64±61.93^{fg}	0.83	19.37±2.44^{abc}	1.15	15919.5^{a}	2.32±0.29^{a}
	Un-treated	7.91±0.01^{a}	492.60±40.91^{cde}	1	16.80±3.38^{bcd}	1	1821^{a}	1.81±0.20^{bcd}
O45	Acid-shock	7.31±0.19^{ab}	440.37±57.98^{ef}	0.76	19.84±2.83^{abc}	1.07	9412.27^{a}	3.12±0.29^{abc}
	Un-treated	7.77±0.07^{ab}	578.47±46.85^{b}	1	18.56±2.62^{abc}	1	2795.05^{a}	2.21±0.17^{bcd}
O103	Acid-shock	7.36±0.40^{ab}	397.12±43.23^{fg}	0.75	17.52±2.18^{bcd}	1.24	9545.63^{a}	4.09±0.29^{a}
	Un-treated	7.52±0.20^{ab}	529.94±38.09^{bc}	1	14.18±3.71^{d}	1	4348.8^{a}	2.11±0.29^{bcd}
O111	Acid-shock	7.04±0.11^{b}	408.09±33.44^{fg}	0.85	18.06±2.73^{bcd}	1	15247.31^{a}	2.63±0.29^{abcd}
	Un-treated	7.77±0.07^{ab}	482.71±31.84^{cde}	1	17.98±3.30^{abcd}	1	2702.98^{a}	2.01±0.29^{bcd}
O121	Acid-shock	7.46±0.41^{ab}	403.92±50.50^{fg}	0.59	22.62±4.39^{a}	1.3	9947.43^{a}	3.52±0.35^{ab}
	Un-treated	7.88±0.01^{b}	680.43±64.29^{a}	1	17.44±3.39^{bcd}	1	2035.76^{a}	1.92±0.29^{cd}
O145	Acid-shock	7.66±0.40^{a}	358.20±39.82^{g}	0.69	18.81±2.92^{abcd}	1.18	5032.51^{a}	2.42±0.29^{bcd}
	Un-treated	7.77±0.07^{ab}	520.79±51.95^{bcd}	1	15.90±2.46^{bcd}	1	2389.26^{a}	1.49±0.35^{d}

*% Variation of total protein was calculated using the untreated total protein concentration as the base within each strain.
**% Variation of DnaK protein was calculated using the untreated total protein concentration as the base within each strain.
[1]Different letters mean significant differences between the means (P>0.05).

CONCLUSION

Given the increase in the number of outbreaks related to non-O157 Shiga toxin-producing *E. coli* and their recent addition the zero tolerance policy, awareness for the big six non-O157 STEC serogroups has increased. Therefore, there is an urgent need for effective control strategies and their validation. Thermal inactivation treatments are combination of time and temperature designed to reduce microbial load, reduce the risk of foodborne outbreaks and also extend shelf-life. This study determined the D- and z-values for individuals strains of *E coli* O157 and non-O157 STEC in phosphate saline buffered (PBS), as well as the D- and z-values of five cocktails of *E. coli* O157 and non-O157 STEC in different food matrices including frozen chopped spinach, ground beef, turkey deli meat, and fettuccine with Alfredo sauce. This study evaluated two thermal inactivation models, a first-order kinetic model and a Weibull model for the inactivation of STEC in the pasta with Alfredo sauce product only. Finally, the mechanism of thermal inactivation after sub-lethal of eight different STEC strains either by acid- or heat-shock based on chaperon protein DnaK levels and subsequent effects on thermal resistance was explored.

The emerging non-O157 STEC in these studies were found to be similar in their thermal resistance compared to the more widely know *E. coli* O157 strains, in all the food products tested. The D-values in PBS were found to be lower when compared to all the food products tested; therefore values obtained from synthetic media should not be extrapolated to food products. Validation of each thermally processed food is necessary against potential pathogen contamination. When comparing the different models in the pasta product for the inactivation of STEC, the Weibull model was found

to be more appropriate in describing the thermal inactivation behavior of both STEC cocktails, compared to the first order kinetic model in terms of goodness of fit. However, the D-and z-values obtained for the linear model were not different from the values obtained from the Weibull model. Despite the better fitness and the similarities in the calculated kinetic parameters with the first order model, the Weibull model has some pitfalls when severe deviations from the linear behavior are observed and 6D projections are needed. This can result in over or under processing of the food product, with potential consequences on food quality and food safety issues. Although, the thermal inactivation parameters, D- and z-values for the non-O157 STEC were found to be similar to the O157 STEC in buffer and in food matrices, when exposed to sub-lethal stress either by heat or acid, the thermal resistance of these microorganisms increased (although varied in a strain dependent manner). Where *E. coli* O145 exhibited the highest D-value after heat-shock; while *E. coli O26,* O103, and O121 exhibited the highest D-value after acid-shock.

The chaperon DnaK protein is considered crucial for the cell heat-shock response mechanism. The increase in concentration of intracellular DnaK can enhance the thermal tolerance of STEC to further heat treatments. The thermal processing recommendations (combinations of time and temperature) used to eliminate potential contamination of *E. coli* O157 appear to be sufficient to inactivate the emerging non-O157 pathogenic strains. However, caution and special attention is needed when there is a possibility of sub-lethal injury due to the processing conditions. This study provides useful information that will aid the food processors and food policy makers to design

appropriate thermal processes to ensure the safety of the food products to decrease the risk of foodborne outbreaks related to STEC.

VITA

Malcond D. Valladares was born on August 26th of 1984 in Tegucigalpa, Honduras. He is the older son of Isabel Cristina Herrera. He attended Elementary school in his native city. He Attended Zamorano University In Honduras and received a B.S. in Food Science and Technology in 2005. In 2009 he obtained a M.Sc. in Food Science and Technology from the University Of Nebraska-Lincoln and In 2015 Malcond D. Valladares will obtain a doctorate degree in Food Science and Technology with emphasis in food microbiology from the University of Tennessee-Knoxville