THERMAL INACTIVATION OF ACID ADAPTED AND NON-ADAPTED STATIONARY PHASE SHIGA TOXIN-PRODUCING *ESCHERICHIA COLI* (STEC), *SALMONELLA* SPP., AND *LISTERIA MONOCYTOGENES* IN ORANGE JUICE

By

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To my mother Mrs. Guler Topalcengiz, my father Mr. Muhsin Topalcengiz, my brother, Mr. Muharrem Yekta Topalcengiz, my sister-in-law, Mrs. Medine Topalcengiz, and my lovely nephew, Mr. Hasan Topalcengiz, for their endless support

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Thermal inactivation parameters of stationary phase, non-adapted and acid adapted pathogens, primarily as cocktails of multiple strains, have been studied in various juice products. All *D*-values for STEC, *Salmonella,* and *L. monocytogenes* in orange juice were obtained using strain cocktails. The objective of this study was to evaluate the heat resistance of individual strains of stationary phase non-adapted and acid adapted Shiga toxin-producing *Escherichia coli* (STEC), *Salmonella* spp., and *L. monocytogenes in* orange juice.

Three STEC, *L. monocytogenes,* and *Salmonella* strains/serotypes were evaluated. STEC and *Salmonella* isolates were grown in TSB, and *L. monocytogenes* strains grown in BHI, supplemented with 1% glucose for acid adaption, and inoculated into single-strength pasteurized orange juice without pulp. Inoculated juice was sealed into microcapillary tubes. Microtubes were immersed into water baths at 56, 58, and 60°C for STEC and *L. monocytogenes* strains and at 55, 58, and 60°C for *Salmonella* serotypes, removed at predetermined time intervals, and placed immediately onto ice. Thermally treated and sterilized tubes were crushed in 0.1% peptone using a sterile glass rod for microbiological analysis. Populations of STEC and *Salmonella* were enumerated on TSA supplemented with 0.1% sodium pyruvate; BHI agar supplemented with 0.1% sodium pyruvate was used for *L. monocytogenes* strains.

Different strains in the same species responded to heat differently. Thermal tolerance was increased significantly ($P < 0.05$) for acid adapted STEC strains, however, acid adaptation did not improve heat resistance for *Salmonella* spp., and *L. monocytogenes* strains at most temperatures tested. *Salmonella* serotypes are less heat resistant, at all temperatures tested, than *L. monocytogenes* and STEC. Shiga toxin-producing *Escherichia coli*, especially strain O111, are the most heat resistant at 56 and 58°C; *L. monocytogenes* strains are the most thermal tolerance at 60°C. Combining individual results of all pathogens tested, the formula of log D = 8.2 – 0.14*T* (°C) was used to calculate a general process for orange juice at 71.1°C. Using this equation, a 5-log reduction of all three pathogens in single strength orange juice requires 5.29 s at 71.1°C, with a *z*-value of 7.1°C.

CHAPTER 1
INTRODUCTION

Pathogenic, foodborne microorganisms remain a significant food safety concern in food production. To inactivate these pathogens, thermal pasteurization is one of the most commonly applied and effective technique (MacGregor and Farish, 2000). The thermal inactivation parameters of acid adapted and non-adapted stationary phase *Escherichia coli* O157:H7, *Salmonella* spp, and *Listeria monocytogenes,* primarily as cocktails of multiple strains, has been studied in various food products; most of these studies were conducted a decade ago (Mazzotta, 2001; Mak et al., 2001; Singh et al., 2008). Fruit juices are widely consumed product with a pasteurization step during process (Ngadi et al., 2010). In thermal fruit juice pasteurization, the success of the process depends on the establishment of appropriate heat application times and temperatures. Validation of these parameters through elaborated studies with appropriate methodologies is essential.

Several outbreaks related to fruit juices occurred in 1990s, threatening public health (Vojdani et al., 2008). The Centers for Disease Control and Prevention (CDC) report a total of 21 outbreaks of *E. coli* O157:H7, *Salmonella* spp., and *Crytosporidium,* associated with the consumption of unpasteurized fruit juice between 1995 and 2005 (Vojdani et al., 2008). Outbreaks of *E. coli* have been associated with the consumption of unpasteurized apple cider and apple juice. Outbreaks of *Salmonella* serotypes have been linked mainly to unpasteurized orange juice consumption. *Listeria monocytogenes* has not been implicated in any outbreak related to fruit juice consumption (FDA, 2001). The routes of contamination of these pathogens have not been completely identified in any of the juice outbreaks. The use of dropped fruit, non-potable water, and the presence of cattle and wildlife in, or close to, the production or processing environment are included as likely sources of juice contamination (Harris et al., 2003).

Inactivation of pathogens before consumption of fruit juices has become mandatory as a result of outbreaks associated with fruit juice. Food and Drug Administration (FDA) published a juice final rule (66 FR 6137) in January, 2001, requiring that all the juice processors must comply with the Hazard Analysis Critical Control Points (HACCP), and achieve a 5-log reduction of the pertinent pathogenic microorganism (FDA, 2001).

Pasteurization in the juice industry can be achieved by many different technologies; however thermal pasteurization is the most common. High temperature short time (HTST) pasteurization is commonly used as a heat treatment technique with optimum temperatures and time parameters. Current thermal treatment of fruit juices and apple cider appears to be efficient in the prevention of microbial spoilage and elimination of pathogens. Pasteurization parameters for fruit juice with the use of cocktails of target microorganisms have been studied by numerous researchers (Mazzotta, 2001; Mak et al., 2001; Singh et al., 2008), and appropriate time and temperature parameters in pasteurization have been recommended as 3 s at 71.1º C by FDA. The inactivation of "pertinent microorganism" is targeted by processors in the determination of pasteurization parameters. Pasteurization may also cause unavoidable change of physicochemical, nutritious, and sensory properties of juice (Lee and Coates, 2002; Moshonas et al., 1993; Moshonas and Shaw, 1995). Determination of the appropriate thermal inactivation parameters constitute essential variables for the designation of juice pasteurization to produce safe, stable, and quality juices. In the absence of pasteurization, spoilage microorganism can take advantage of favorable environment of fruit juices, grow, and spoil products. However, foodborne pathogens do not normally grow in fruit juices.

Acidic foods, like most fruit juices, were not recognized as a vehicle of foodborne pathogens until the first confirmed outbreak of *E. coli* O157:H7 related to unpasteurized apple

cider in 1991 (Besser et al., 1993). Some foodborne pathogens can develop acid adaptation systems that induce cross-protection, and makes them more resistant against other environmental stresses (Leyer and Johnson, 1993; Bearson et al., 1997; Ryu et al., 1998; Ryu and Beuchat, 1998), thus, increasing their ability to survive in juice long enough to cause an outbreak. *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella* spp., and *Cryptosporidium parvum*, can tolerate low pH values and survive in fruit juices and juice concentrates longer than non-adapted cells (Hsin-Yi and Chou, 2001; Oyarzabal et al., 2003; Gahan et al., 1996). The acid adaptation of *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* spp, also increases the heat resistance of these bacteria in apple, orange, white grape juices, apple cider, cantaloupe, and watermelon juice (Mazotta, 2001; Ryu and Beuchat, 1998; Sharma et al., 2005). Research comparing acid adapted and non-adapted strains used various strains and serotypes of each microorganism as cocktails with similar temperature values (52, 56, 57, 60, 62, 64°C).

Fruit juice pasteurization parameters should be studied in more detail due to differences among the methodologies applied and the use of cocktails rather than individual strains in currently published studies. The number of current studies regarding thermal inactivation of acid-adapted microorganisms in the fruit juices is limited, and compounded by the use of strain cocktails. In this master thesis, we evaluate the effect of acid adaptation on the heat resistance of individual isolates of Shiga toxin-producing *E. coli* (STEC), *Salmonella* spp. and *Listeria monocytogenes* in single strength orange juice. The purpose of this study is to evaluate the thermal inactivation responses of acid adapted and non-adapted stationary phase Shiga toxin-producing *E. coli* (STEC), *Salmonella* spp., and *L. monocytogenes* by using individual strains of each organism in single strength orange juice, respectively. We inoculated each microorganism separately for a respective determination of thermal inactivation values, rather than inoculating as cocktail where

strain to strain variability may be lost or masked, as previous studies have done. Also, the specific objectives of this study are to determine how acid adaptation of each pathogen affects the thermal inactivation values in the absence of potential microbial interactions and metabolic end products among different species, to estimate thermal death time parameters (*D*- and *z*- value) for acid adapted and non-adapted stationary phase of these pathogens, and to validate current studies about thermal death time parameters (*D*- and z- *value*) for acid adapted and non-adapted stationary phase pathogens in orange juices. Results of this project should provide more detailed and clarified information about the effect of acid adaptation of stationary phase microorganisms on thermal inactivation of bacterial survival, and should lead to be a better understanding of the determination of pasteurization parameters for processors.

CHAPTER 2
LITERATURE REVIEW

Fruit Juice, Pathogens, and Process

Legally, a commercial product that is "100% juice" is called juice in the United States, while other carbonated or noncarbonated beverages that contains less than 100 % juice are called "beverages", "cocktails", or "drinks" under 21 CFR 102 (FDA, 2010) . "Juice" is defined under 21 CFR 120.1(a) as "the aqueous liquid expressed or extracted from one or more fruits or vegetables, purees of the edible portions of one or more fruits or vegetables, or any concentrates of such liquid or puree" (FDA, 2009). Lozano (2006) describes fruit juices as the products that are ready for direct consumption, and are obtained by the extraction of cellular juice from fruit tissues.

Fruit juices are significant commodities in the global market with the consumers from all age groups. Health advantages, freshness and preferable taste and flavor properties, are some of the reasons for the popularity of fruit juices. In 2009, the U.S. juice industry in had a value over $22 billion, volume in excess of 16 billion liters, and accounted for 30.8% of the global juices market value (Datamonitor, 2010). According to the U.S. Department of Agriculture/Economic Research Service in 2008, out of the 25.7 gallons of beverages consumed per capita, 6.9 gallons were fruit juice (USDA-ERS, 2010).

Fruit Juice Pathogens

Fruit juices can be spoiled by microorganisms; particularly yeasts, molds, and acid tolerant bacteria, due to their high water activity and sugar content. Foodborne pathogens do not normally grow to levels of spoilage in fruit juices, primarily due to the high acid content of many of these juices. Nonetheless outbreaks, including the first confirmed outbreak of *Escherichia coli* O157:H7 associated with apple cider in 1991 (Besser et al., 1993), led researchers to reevaluate

the ability of foodborne pathogens to survive for extended periods in high-acid food products (Moody, 2003). Shiga toxigenic *E. coli* (STEC), *Listeria monocytogenes*, *Salmonella* spp., and *Cryptosporidium parvum*, can tolerate the acidic environment and survive in fruit juices and juice concentrates longer than initially expected. The ability of some of these pathogens to adapt to the acidic environment increases the risk (Hsin-Yi and Chou, 2001; Oyarzabal et al., 2003; Gahan et al., 1996). With the exception of *L. monocytogenes*, STEC *Salmonella* spp and *C. parvum* are implicated as the primary cause of many fruit juice associated outbreaks. Although not linked to any fruit juice associated outbreaks; *Listeria* species have been isolated from unpasteurized apple and apple raspberry blends in a survey of fifty juices (Sado et al. 1998).

Escherichia coli

The genus *Escherichia* is a typical member of enterobacteriaceae that live in the intestinal system of humans and warm-blood animals. *Escherichia coli* are gram-negative, rod-shaped facultative anaerobes, some species of which may causes a large variety of diseases including diarrhea, urinary tract infections, sepsis and meningitis (Meng et al., 2007). The optimum temperature for growth is 37°C, but growth has been observed between 8°C and 45°C (Meng et al., 2007). Generic *E. coli* is commonly used as an indicator of fecal contamination indicator in water supplies as up to 1% of the fecal bacterial flora of mammals is compromised of *E.coli* (Windfield and Groisman, 2003).

The serotypes of *E. coli* are separated according to three major antigens: The "O" antigen on lipopolysaccharide of the outer membranes, "H" antigens on the flagella, and "K" antigens on the capsules. A total of 173 O antigens, 56 H antigens, and 103 K antigens have been identified (Orskov and Orskov, 1992). Based on virulence properties, mechanisms of pathogenicity, clinical symptoms, and different O:H serotypes, *E. coli* isolates that induce diarrheal diseases have been classified into groups called pathotypes (Meng et al., 2007). Enterotoxigenic *E. coli*

(ETEC), Enteroinvasive *E. coli* (EIEC), Enterohemorrhagic *E. coli* (EHEC), Enteropathogenic *E. coli* (EPEC), diffuse-adhering *E. coli*, and Enteroaggregative *E. coli* (EAEC) represent the six pathotypes of *E. coli* that are responsible for outbreaks due to the consumption of contaminated food and beverages.

Escherichia coli O157:H7, (EHEC; STEC) were first recognized as human pathogens following two outbreaks of bloody diarrhea (hemorrhagic colitis; Riley et al. 1983). Although numerous serotypes of EHEC including O26, O111, Sorbitol–fermenting O157:NM have been implicated as the cause of hemorrhagic colitis, *E. coli* O157:H7 remains the primary cause of hemorrhagic colitis in the U.S., which may be followed by the fatal hemolytic uremic syndrome (HUS) complication, especially among children (Karch et al., 2005). The cause of the bloody diarrhea and HUS is the ability of EHEC to produce a called Shiga-toxin (or verotoxin, STEC, or verotoxin-producing *E. coli* (VTEC); Griffin, 1998). The name of toxin comes from its similarity to toxin produced by *Shigella dysenteriae* type 1 (O'Brien et al., 1992).

STEC strains are not part of the natural microflora of fresh juice or the fruit used in the production of fresh juice. It is believed that the presence of *E. coli* in fruit juice is a consequence of fecal contamination emanated from other reservoirs, such as cattle, wild birds, deer, rodents, goats, sheep, cats, dogs (Nielsen et al., 2004). In the light of numerous juice outbreaks, STEC can tolerate the low pH of juice and survive to cause illnesses. The effect of environmental stresses on the resistance of *E. coli* O157:H7 have been studied in different types of buffer, media, models, and food. Acid-adapted *E. coli* O157:H7, grown in medium supplemented with 1 % glucose, increased the thermal resistance in apple cider and orange juice; however the strain, type of organic acid, acid adaptation, and acid shocking affected the acid resistance (Ryu and Beuchat 1998). Acid-adapted *E. coli* O157:H7 survive longer than non-adapted cells in fruit

juices under refrigeration (Hsing-Yi and Chou, 2001). *Escherichia coli* O157:H7 survived at detectable levels in apple, orange, pineapple, and write grape concentrates in 12 weeks period storing at -23°C (Oyarbazal et al., 2003) and at levels of 10^3 CFU/100 mL from orange juice concentrates stored for 147 day at -12.2°C with 10^7 CFU/100 mL initial concentrations (Larkin et al., 1995). In apple juice, *E. coli* O157:H7 survives up to 24 days at 4°C. (Miller and Kaspar, 1994; Fratamico et al., 1997; Splittstoesser et al., 1996).

Salmonella

Although *Salmonella* Typhi was discovered in 1880 by Karl Joseph Eberth, and isolated in 1884 by Georg Theodor August Gaffky, the name of this microorganism comes from the name of one of the two researchers, Daniel Elmer Salmon, a U.S. veterinary surgeon and Theobald Smith, who isolated *Salmonella cholera-suis* from ill hogs in 1885 (ICMSF, 1996).

Salmonella spp. are gram-negative, facultative anaerobic, non-spore forming, rod-shaped bacterium in the family Enterobacteriaceae. Non-typhoidal *Salmonella* infections cause salmonellosis, whose symptoms include diarrhea, fever, vomiting, nausea, and abdominal pain. The most vulnerable groups to *Salmonella* infection are young children, the elderly, and immunocompromsied individuals. Although some members of this family are nonflagellated, member of *Salmonella* genus predominantly have peritrichous flagella, and are motile (Maure and D'Aoust, 2007). *Salmonella bongori* and *Salmonella enterica* represent the two species of the genius *Salmonella*. *Salmonella enterica* are subdivided into seven subspecies: *Salmonella enterica* subsp. *enterica* (I), *Salmonella enterica* subsp. *salmonae* (II), *Salmonella enterica* subsp. *arizonae* (IIIa), *Salmonella enterica* subsp. *diarizonae* (IIIb), *Salmonella enterica* subsp. *houtenae* (IV), *Salmonella bongori* (V), and *Salmonella enterica* subsp. *indica* (VI), that induces the majority of *Salmonella*-causing diseases among human and the warm-blooded animals

(Cooke et al., 2007).According to Kauffmann-White scheme, an antigenic formula for *Salmonella* serotypes, there are over 2,600 *Salmonella* serotypes (Popoff et al., 2004).

Salmonella can be easily cultured in the laboratory, and it is easy to see visible colonies after 24 h incubation at about 37°C. They can survive a wide range of pH, between 4.5 and 9.0; with the optimal pH for growth around neutrality (Rovira et al., 2006). The maximum growth temperature is just above body temperature, and most serotypes do not grow below 7°C, however some serotypes can grow as low as 2°C and as high as 54°C (Baker et al., 1986; Droffner and Yamamoto, 1992). Recent outbreaks of *Salmonella* have been associated with consumption of fresh tomatoes, raw/undercooked shell eggs, lettuce, unpasteurized orange juice, cantaloupes, chocolate, alfalfa sprouts, and poultry (Yaun, 2002; CDC, 2012). *Salmonella* can tolerate and adapt to acidic conditions, and high acidic foods, such as unpasteurized orange juice, and tomatoes have caused outbreaks (Yuk and Schneider, 2006; Leyer and Johnson, 1992).

There are a number of ways *Salmonella* may be introduced to fruit juices from harvest to the final product. Although pathogen contamination routes have not been definitively confirmed in any juice outbreak, the use of dropped fruit, nonpotable water, and the presence of cattle, deer, or, in one case, amphibians, in or near production of fruit appear to be a reoccurring theme (Harris et al., 2003). Danyluk et al. (2010) concluded that under typical postharvest handling practices *Salmonella* population on the orange peel surface did not grow or penetration in to juice for intact fruit, even in the presence of minor wounds or natural-light labeling; survival on the peel surface exceeded 45 days. The long term survival on fruit surfaces may be a potential contamination risk for processing equipment. However, microbial levels detected in fresh juice were 90–99% lower than levels found on corresponding fruit surface, indicating that much of the surface contamination on fruit can be eliminated by commercial citrus juice extraction. Valero et

al. (2010) indicate that high initial microbial population level (10^5 to 10^6 CFU/mL) in citrus juice may occur as a consequence of insufficient sanitation, poor hygiene practices, deteriorated fruits, and poor equipment sanitation, potentially increasing the risks of foodborne pathogens in juice. *Salmonella* can adapt to extreme acid environments in a similar manner as *E. coli*, and can survive in apple juice with a pH of 3.6 for 30 day at 22°C (Goverd et al., 1979). In orange juice, Parish et al. (1997), describe the survival of *Salmonella* serotypes Gaminara, Hartford, Rubislaw, and Typhimurium in pH 3.5, 3.8, 4.1, and 4.4, and stored at 0 and 4°C with 106 CFU/mL initial concentration survived in detectable numbers more than 26 days at pH 3.5, 46 days at pH 3.8, 60 days at pH 4.1, and 73 days at pH 4.4.

According to CDC (2010), an estimated 1.4 million cases of salmonellosis occur annually in the U.S. resulting in approximately 400 fatalities. Historically, a number of *Salmonella* outbreaks were linked to consumption of unpasteurized juices.

Listeria monocytogenes

Listeria monocytogenes differs from *E. coli* and *Salmonella* due to severity of illness it causes, the high case-fatality rate, long incubation period prior to consumption, unique growth capabilities, and ability to survive.

Listeria monocytogenes is a gram positive, rod-shaped, non-sporeforming, motile, facultative anaerobe, and the causes a disease called listeriosis. The onset time for serious listeriosis symptoms may be five weeks, complicating traceback investigations. Severe listeriosis symptoms may include meningitis, septicemia, and spontaneous miscarriage or still birth following early influenza-like symptoms including nausea, vomiting, persistent fever, and diarrhea. Newborns, immunocompromised people, elderly, and pregnant are the high risk population of listeriosis. *Listeria monocytogenes* is a critical public health concern due to the 20-30% mortality rate (Ramaswamy et al., 2007). Each year an estimated 500 deaths out of 2,500

infections are linked to *Listeria* in the U.S., making it the leading cause of death due to foodborne bacterial pathogens (CDC, 2000).

Various changes in social patters with complex interaction have resulted in the emergence of listeriosis as an important public health concern (Swaminathan et al., 2007). These factors include: increase of life expectancy; increase of immunocompromised population due to AIDS and organ transplantation; centralization and consolidation of food production and processing; increase of demand for Ready-to- eat, refrigerated, frozen, and short time cooking foods (Swaminathan et al., 2007).

Within the six *Listeria* species, (*L. monocytogenes*, *L. inanovii*, *L. welshimeri*, *L. seeligeri*,*L.murayyi and L. grayi*) in the genus *Listeria*, only *L. monocytogenes* and *L. ivanovii*, are considered pathogenic. An estimated 90% of human *L. monocytogenes* isolates belong to serotype 1/2a, 1/2b, and 4b in total of 13 serotypes of *L. monocytogenes* (Dalgaard, 2006). Serotype 4b is an epidemic strain that causes sporadic human cases worldwide. The U.S. has a zero-tolerance policy regarding the detection of *L. monocytogenes* in foods and beverages.

In 1981, the first confirmed outbreak of *Listeria* with 41 cases and 8 deaths was associated with consumption of contaminated coleslaw Nova Scotia, Canada (Montville and Mathews, 2005). Following this outbreak, investigations demonstrated that the primary transmission of listeriosis was from the consumption of contaminated food (Swaminathan et al., 2007).

Listeria monocytogenes can be found in soil, mammalian feces, sewage, water, and decaying vegetation, *and* can grow temperature range of 2 to 45°C (Swaminathan et al., 2007). Also it is a pschrotroph that can multiply at temperatures as low as -0.4°C (Dalgaard, 2006). *Listeria* also has the ability to survive in the high salinity conditions, and grow between pH 4.4

and 9.6, depending on type of acid and incubation temperature (Swaminathan et al., 2007). These survival capabilities make *L. monocytogenes* very durable under extreme environmental conditions.

Listeria monocytogenes is commonly associated with foods such as ready-to-eat foods, meat and poultry, milk products (particularly cheese), and seafood. Although *L. monocytogenes* has not been implicated in any juice outbreaks it has been reported that acid adaptation causes *Listeria* become more resistant to other environmental stresses as a result of cross-protection (Mazotta, 2001). *Listeria monocytogenes* survives in white grape juice for 12 days, in apple cider for 24 days, and in orange juice for 61 days, respectively in different storage temperatures (Piotrowski, 2003). Acid adapted *L. monocytogenes* cells grew in orange juice (pH 2.6) stored at 37°C, by almost 10^4 CFU/mL in 6 h (Caggia et al., 2007). Also, Parish and Higgins (1989) demonstrated that population of *Listeria* cells decreased 10^6 CFU/mL in orange juice stored at 4°C in 25 days at pH 3.6 and in 43 days at pH 4.0.

Cryptosporidium parvum

Cryptosporidium is a single-celled, protozoan parasite that was first recognized by Ernest Edward Tyzzeras in the gastric glands of laboratory mice in 1907 (Fayer, 2008). It was not identified until 1976 when two distinct research groups reported separate cases of cryptosporidiosis in humans (Nime et al., 1976; Meisel et al, 1976). The taxonomic classification of *Cryptosporidium* only became clear in 1990s when studies based on molecular taxonomy indicated the genus is much more complicated than morphology and host specify can define. Thirteen *Cryptosporidium* species are identified, including *C. parvum, C. muris, C. felis* and *C. wrairi,* that infect mammals; *C. baileyi* and *C. meleagridis* that infect birds; *C. serpentis* and *C. saurophilum* linked to the infection of reptiles, and *C. nasorum* tropical fish infections (WHO,

2006). The most common human isolate is *C. parvum*, which has been linked to consumption of unpasteurized fruit juices.

The symptoms of infection include diarrhea; abdominal cramps, headaches, nausea, and vomiting. In individuals with weakened immune system, such as immunocompromised people (AIDS), elderly, and the very young children symptoms can be more severe. Onset of symptoms takes up to twelve days after consumption of contaminated food or water. *Cryptosporidium* is transmitted through the fecal-oral route, beginning with ingestion of the *Cryptosporidium* oocycst that survives for a long time in the environmental conditions (Robertson et al., 1992).

History of Fruit Juice Outbreaks

High acid foods, such as fruit juices, with pH 4.6 or below are generally considered as safe to consume directly by researchers and agencies like Food and Drug Administration (FDA) (FDA, 2010). The assumption is that while fruit juices provide favorable environments for spoilage by yeasts, molds and acid tolerant bacteria due to the high water activity and sugar content; the high acidity would prevent pathogenic bacteria from being a problem. However, outbreaks associated with the consumption of fruit juice increased in the 1990s (Mazotta, 2001).

In 1991, fresh-pressed apple cider contaminated with *E. coli* O157:H7 caused four children in southern Massachusetts to be hospitalized with HUS (Besser et al., 1993). A total of 23 HUS cases were reported. Cattle raised by the cider press operator and grazed in the field adjacent to the mill were identified as the possible source of contamination resulting in apples being contaminated with manure from equipment, handling of workers, contaminated water, or contaminated storage areas. In 1996, the Seattle-King county Department of Public Health reported an outbreak of *E. coli* O157:H7 associated with Odwalla brand unpasteurized apple juice and Odwalla juice mixtures containing apple juice, resulting in a voluntary nationwide

recall that ended with 70 cases (14 HUS) and one death due to the use of dropped fruits and the localization of apple orchard near cattle farm (CDC, 1996).

Since 1990, a number of outbreaks involving salmonellosis have been reported mostly associated with fresh (unpasteurized) orange juice. An outbreak of *Salmonella* enteric serotype Hartford resulted in 62 case patients from 21 states during May and June 1995 because of unpasteurized locally produced orange juices (Cook et al.1998). An outbreak of *Salmonella* Muenchen causing 423 cases and 1 death in Canada was due to consumption of unpasteurized orange juice, produced by a single producer and distributed widely in the United States and Canada (CDC, 1999; CCDR, 1999).

A total of 21 juice associated outbreaks – 10 related to apple juice and cider, 8 linked to orange juice, and 3 caused by other kind of juices – occurred from 1995 through 2005, with 1366 total illnesses (Vojdani et al., 2008). As a result of these outbreaks, the U.S. Food and Drug Administration published a juice rule (66 FR 6137) in January, 2001, requiring all processors of 100% juice must comply with Hazard Analysis Critical Control Points (HACCP) to achieve 5-log reduction of pathogenic microorganisms (U.S. FDA, 2001).

This regulation made HACCP mandatory in juice processing and packaging plants. The potential hazards for juice fall into three categories a) chemical including pesticide residues, food allergens, toxins and heavy metal contamination; b) Physical including glass and metal fragments; and c) biological including pathogen microorganisms, such as *Salmonella,* STEC, and *Cryptosporidium*. The US FDA has also declared patulin, a mold toxin, as a potential hazard in apple juice that must be controlled. The control of these hazards is explained in guidance for industry regarding juice HACCP hazards and controls published by FDA in March 3, 2004 (FDA, 2004).

Prior to the development of a HACCP plan, juice processors must have Good Manufacturing Practices (GMP) requirements and Sanitation Standard Operating Procedures (SSOPs) in place as prerequisite programs. If juice is not pasteurized, the U.S. FDA requires a warning statement to inform consumers about potential food safety risks related to the product (FDA, 1998) that reads

> THIS PRODUCT HAS NOT BEEN PASTEURIZED AND THEREFORE MAY CONTAIN HARMFUL BACTERIA THAT CAN CAUSE SERIOUS ILLNESS IN CHILDREN, THE ELDERLY AND PERSONS WITH WEAKENED IMMUNE SYSTEMS.

Under HACCP, fruit juices must be processed to achieve a 5-log pathogen reduction (21 CFR 120.24) of the "pertinent microorganism". This is most commonly achieved through a thermal pasteurization treatment. However, citrus juice processors who produce fresh juice have an option to apply a cumulative 5-log reduction plan to fruit surfaces. After HACCP has been made mandatory to juice processors, no outbreak associated with the consumption of pasteurized juice has occurred.

Orange Juice

Originating in Southeast Asia, orange trees are grown in China, Brazil, U.S., Spain, Mexico, and Turkey (Orange book, 1998). Approximately 55 million tones of oranges are produced throughout world annually with 40% of this production processed and consumed as orange juice. Brazil and Florida in the U.S. represent the highest global orange juice producers (USDA, 2011). One thousand kilograms of Florida 'Valencia' oranges yields on average 553 kg juice, 413 kg peel, rag, and seeds, 30 kg pulp (Orange book, 1998). Orange juices can be classified as ready to drink (RTD) orange juices, fortified orange juices, concentrated orange juices, and RTD orange products which may not be called as orange juice, such as orange nectars, orange juice drinks, and orange flavor drinks (Orange book, 1998).

Orange Juice Process

The process of juice roughly includes four main steps: washing, sorting, extraction, and pasteurization. The first step of juice production is to wash the harvested fruits. The purpose of the washing is to reduce microbial population on fruit, eliminate pesticide residues, and remove the foreign matters such as soil, sand, and mud. Spoiled fruit should be discarded before washing in order to prevent other fruits from contamination. Washing efficiency is described by the total number of microorganisms present on fruit surface before and after washing (Dauthy, 1995).

Removal of partially or completely decayed fruit and any foreign substance is the most significant factor in the preparation of fruit for production of fruit juice. Determination of variety and maturity of fruits is also part of initial sorting. Sorting can be done automatically or by hands.

The purpose of extraction process is to separate the liquid phase of fruit from solid particles. Mainly, two types of extractors manufactured by different companies are common in citrus processing (Ramaswamy, 2005). The Brown extractor (automatic machinery corporation) and the rotary press involve cutting the fruit in half and reaming the each half of fruit. The reaming extraction methods results with separation of white skin lining, segment walls, cell tissue, piece of skin, and seeds in the juice and these residues must be taken away from juice (Rutledge, 2001). The John Bean Technologies Corporation (JBT) citrus juice extractors do not require fruit to be halved prior to extraction, and are used by more than 75% of the world's citrus juice processors (Lozano, 2006). The principle of JBT machines depends on squeezing the fruit through hole from the center of bottom cup shaped set of fingers. After extraction of juice, pasteurization is applied to obtain safe and more stable final products.

Thermal Orange Juice Pasteurization and Alternatives

Pasteurization is the main step of orange juice production that eliminates foodborne pathogens, and inactivates spoilage microorganisms. Pasteurization also helps to stabilize cloudy appearance by inactivating enzymes, especially pectin methyl esterase (PME) to prevent cloud loss. Pasteurization parameters chosen must reduce the number of pertinent microorganism at least 5-log in accordance with FDA regulations. Pasteurization may also cause unavoidable changes in of physicochemical, nutritious, and sensory properties of juice (Lee and Coates, 2002; Moshonas et al., 1993; Moshonas and Shaw, 1995).

Currently, high temperature short time (HTST) pasteurization is used as a heat treatment for citrus pasteurization. In HTST, the optimal temperature is 76.6–87.7°C with a holding time between 25 and 30 s (Moyer and Aitken, 1980), and specifically for orange juice, conventional HTST treatment is 98°C, 21 s (Rivas et al., 2005).

Alternatives to the thermal pasteurization of orange juice include microwave processing, ohmic heating, stem and hot water treatments, high pressure processing, UV radiation, electromagnetic fields, ozone, irradiation, chemical treatment, and pulsed field electric. (NACMCF, 2006). Pulsed electrical field (PEF), high-pressure treatment microwave heating, and gamma radiation are some of the last developments in juice processing (Kulwant and Kuldip, 2006). PEF is a commonly studied non-thermal processing technology, based on the use of an external electric field to destabilize cell membranes by applying high voltage pulses (generally 20–80 kV/cm) for very short time (μs to ms), producing PEFs between two electrodes (Cserhalmi, 2006). As a non-thermal pasteurization technique, UV radiation pasteurization is an FDA approved technique to reduce pertinent microorganism population by 5-log in some juices (FDA, 2011). This technique also doubles shelf life of refrigerated juice compare to untreated or unpasteurized juice (Piotrowski, 2003).

Acid Adaptation and Thermal Resistance

Acid Adaptation

Foodborne pathogens are exposed to various kinds of stresses in the environments and in the gastrointestinal system of their hosts. During all steps of food production foodborne pathogens encounter to different stresses, including pH, temperature, and osmotic stress. To endure these stressful conditions, pathogens develop protective mechanisms to survive.

Some bacteria species, including *E. coli* O157:H7, *Salmonella spp.*, and *L. monocytogenes* are able to protect themselves from acidic conditions as a result of acid adaptation. As a result of acid adaptation of *E. coli* O157:H7, *Salmonella* spp, and *L. monocytogenes*, not only enhanced the survival capability of these pathogens, but also increased their heat resistance in apple, orange, and white grape juices (Mazzotta, 2001). Acid adaptation of *E. coli* O157:H7 increased Decimal reduction times (*D*- values) in apple cider and orange juice (Ryu and Beuchat, 1998) and also significantly increased *D*-values of *Salmonella* and *E. coli* O157:H7 in watermelon and cantaloupe juice (Sharma et al., 2005). Detailed descriptions of acid adaptation mechanisms employed by these pathogens, especially in stationary phase, are available.

Factors influencing the acid tolerance response (ATR)

The acid tolerance response (ATR) is one of survival protection mechanisms used by microorganisms to survive in low pH environments after its induction as a result of growth in moderately low pH or exposure to mild acidic conditions (Álvarez-Ordóñez et al., 2012). There are a number of factors influencing the ATR system. The presence of inhibitory and other chemicals may also affect the development of ATR (Rowbury 1995), as may the type of acid for *E. coli* O157:H7 (Beales, 2004).

The induction of acid tolerance differs with environmental factors and the physiological state of the cells. The effectiveness of ATR directly depends on the pH of the adaptation conditions and the duration of adaptive period (Davis et al., 1996), and is also dependent on the state of the cell for some organisms. *L. monocytogenes* achieved greater acid resistance in stationary phase than in log phase (Davis et al., 1996). Habituation to acidic environments at different pH ranges affects the increase of acid tolerance differently for *L. monocytogenes* (increase at pH 5.0 – 6.0), *E. coli* O157:H7 (increase at pH 4.0 – 5.5), and *S.* Typhimurium (increase at pH 4.0 – 5.0) (Koutsoumanis and Sofos, 2004). *Salmonella* Typhimurium cells in log phase may reach their maximum acid tolerance capacities when they are habituated at pH around 5.5, however, in stationary phase lower pH values, around 4.5, are required (Álvarez-Ordóñez et al.(2012). In the log phase, *S.* Typhimurium in pH 4.3 for 10 to 20 min developed less effective acid tolerance than cells in pH 3.3 for 30 to 40 min (Foster, 1993) and log phase cells that were exposed to different pH values between pH 5.0 and 8.0 for 1 hour before acid challenge varied in their acid resistance (Kwon and Rickie 1998).

Escherichia coli and *Salmonella enteritidis* incubated at 42 - 45°C were more acid-resistant than cells grown at37°C; growth at 20-25°C decreased the acid tolerance beyond that developed at 37°C (Humprey et al., 1993). Temperature is another factor that influences ATR development. At 10°C, *S.* Typhimurium lower acid tolerance developed compare to the acid tolerance of adapted cells at 25, 37, and 45°C (Álvarez-Ordóñez et al., 2010). Under different acidic conditions and incubation temperatures between 5 and 35°C, the acid tolerance of *L. monocytogenes* increased with increases in temperature (USDA- ERRC, 2003).

The ATR system of bacteria can be induced by exposure to various acids including citric, acetic, lactic, malic, hydrocholoric, ascorbic (Álvarez-Ordóñez et al. 2009). Gluconic acid

(pentahydroxycaproic acid) is a final production of enzyme glucose oxidase in fungi and enzyme glucose dehyrogenase in bacteria. When glucose is oxidized by one of these enzymes, gluconic acid is formed after hydrolization step of glucono delta lactone (Ramachandran et al., 2006). Gluconic acid induces the ATR system as a result of glucose fermentation during bacterial growth (Buchanan and Edelson, 1996).

The ATR of *E. coli*

Resistance against low pH can provide a competitive advantage for *E. coli* O157:H7 in acidic foods, heat treatment and passing through the stomach of a host. Generally, ATR is induced by exposing bacteria to moderately low pH (5.5 or lower for *E. coli*) that promote expression of acid-shock proteins. Acid shock proteins prevent protein denaturation and refold proteins that have already been denatured (Diez-Gonzalez and Kuruc, 2009). Several compounds, including glucose, glutamate, aspartate, FeCl3, KCl, and L-proline can induce habituation at neutral pH (Rowbury et al., 1989). The mechanisms of acid adaptation of *E. coli* O157:H7 has been studied in different types of acidic condition and three ATR systems are commonly accepted (Foster 2000). These systems include: acid-induced oxidative system, an acid-induced arginine-dependent system, and a glutamate-dependent system (Lin et al., 1996).

Acid resistance system 1, called oxidative or glucose-repressed system, is one of the characterized ATR systems in stationary phase. This glucose-repressed system is different from the other ATR systems as it does not require exogenous amino acids in acid adaptation process. It does however require the RpoS sigma factor (Ma, 2003). The key regulators of the acid-induced oxidative system include the alternative sigma factor σs, cyclic AMP (cAMP), and cAMP receptor protein (CRP) (Castanie-Cornet et al., 1999). RpoS is critical for the gene expression transition period from log phase to stationary phase, and for survival under acid stress. However, its role in type 1 ATR is questioned (Richard and Foster, 2003). While the

structural components of ATR 1 remain to be completely defined, it is a known component of *E. coli* O157:H7 survival capability under acidic conditions at pH values above 3 (Audia et al., 2001).

Acid resistance system 2, a glutamate-dependent system, occurs during stationary phase, and acts independent of RpoS. This system includes several proteins that are located in different part of cells and extracellular glutamate .The system is located at the cell membrane and glutamate decarboxylase isoenzymes (GadA and Gad B), structural components of this system that uses intracellular glutamate-aminobutyric acid (GABA), present in the cytoplasm (Hersh et al., 1996). In glutamate induced system, Glutamate decarboxylase isoenzymes protects cell from acid stress by consuming one proton then releasing CO2 in acid resistance process. Finally, glutamate-aminobutyric acid (GABA) is carried out of cell by simultaneous uptake of glutamate.

The last resistance system ATR 3, the arginine dependent system, is similar to ATR 2, where the decarboxylation of arginine to agmatine and the interchange of arginine and agmatine form the basic mechanisms of this system. ATR 3 system is induced at low pH numbers under anaerobic conditions in rich medium. When grown under acidic conditions, *E. coli* O157:H7 cells have an internal pH between 4 to 5, and the AdiA enzyme, which is one of two enzymes synthesized by two arginine decarboxylase genes starts to function. AdiA, PLP-dependent enzyme, is the enzyme that catalyzes the exchange of arginine to agmatine (Foster 2004).

The ATR of *Salmonella*

In the last two decade, a number of studies, most using *S.* Typhimurium, have described *Salmonellas* ATR mechanisms. While most experiments have focused on the serotype *S.* Typhimurium, similar adaptive responses have been reported for other serotypes (Álvarez-Ordóñez et al., 2012). *Salmonella* including Agona, Anatum, Gaminara, Mbandaka, Michigan, Montevideo, Poona, Reading and Saintpaul have all been readily adapted in apple, orange, and

tomato juice or broth supplemented with glucose (Yuk and Schneider, 2006; Bacon et al., 2003) After acid adaptation in apple or tomato juice, cells were the most acid tolerant in simulated gastric fluid (Yuk and Schneider, 2006).

Salmonella also use three ATR responses to survive in acidic conditions including the general sigma factor (RpoS) response induced in log or stationary phase, and an arginine dependent system (Diez-Gonzalez and Kuruc, 2009).

The induction of ATR during the log phase leads to the production of more than 50 proteins, and time to development is generally faster than in stationary phase. It is dependent on the RpoS system and gene the *fur* (Hall and Foster, 1996). RpoS is responsible for controlling the expression of at least 10 acid shock proteins (ASPs; Baik et al., 1996; Lee et al., 1995), while *Fur* (Ferric uptake regulation) is a regulatory gene that works as a transcriptional repressor and a controller of ASPs (Hall and Foster, 1996).

The stationary phase dependent ATR is activated when cells are exposed to low pH for a long time. The adaptation period is slower during gradual decrease of pH (Diez-Gonzalez and Kuruc, 2009). In both cases, the ATR is ompR dependent. OmpR (Osmolarity Response Regulator) is a regulator that is responsible for the control of the expression of acid induced virulence operon *ssrAB* and required for the OmpR/EnvZ regulation system (Bang et al., 2002).

In addition to the log and stationary ATR, *S.* Typhimurium has an arginine dependent ATR, what includes an arginine decarboxylase (AdiA) and antiporter, that is only activated under anaerobic conditions (Kim et al., 2002). AdiA consumes the acidic proton by converting arginine into agmatine in the cytoplasm (Álvarez-Ordóñez et al., 2012).

The ATR of *Listeria*

Listeria monocytogenes develops one of the most advanced ATR in mildly acidic conditions (pH 5 -6; Barak et al., 2005). At least four different acid stress response mechanisms

can be activated in *L. monocytogenes* (Becker et al., 1998). The activation of one system only occurs in log phase, and is induced by low pH (Ferreira et al., 2003). Davis et al. (1996) showed that maximum acid resistance of *Listeria* in log phase was observed when the cells were exposed to pH 5.0 for 1 h prior to challenge at pH 3.0, even though medium levels of protection were developed by exposure to pH values ranging from 4.0 to 6.0.When *L. monocytogenes* enters the stationary phase, the σ^{β}-dependent ATR system develops (Becker et al., 1998). The third system is a σ^{β}-independent mechanism that may also develop in stationary phase (Becker et al., 1998). A GAD-based ATR system has also been described (Diez-Gonzalez and Kuruc, 2009) and consists of three glutamate decarboxylase genes (*gadD1, gadD2* and *gadD3*) similar to that of *E. coli* (Cotter et al., 2001).

Thermal Tolerance and Experimental Thermal Treatments

A number of experimental methodologies have been used to conduct thermal tolerance experiments. The use of a water bath being the most common method to hold the inoculated medium or food at constant temperature for a give time. An alternative method is the inoculation of an already heated medium or food on a heating mantle or hot plate stirrer. For liquid food samples, including fruit juices, three variations on these methods are common. Microcapillary tubes are a very effective method, and preferred by several researchers (Sharma et al., 2005; Splittstoesser et al., 1996). In this method, low volumes of inoculated juice in microcapillary tubes are held in waterbaths for predetermined time intervals, following the set time, tubes are cooled immedieately on ice. Alternatively, juice can be heated in advance with a heating mantle priot to inoculation, inoculated with microorganisms, and samples pulled at predetermined time intervals from th larger inoculated sample(Mazotta, 2001). Finally, a submerged coil heating apparatus, consisting of a narrow internal diameter (~2.0828 mm) bore stainless steel coil that is completely submerged in a heat controlled water bath can be used (Enache et al., 2011). This

device is the newest technology for thermal tolerance experiments, and is gaining popularity (Enache et al., 2011).

Thermal tolerance experiments may use either cocktail of multiple strains or single strains, depending on the purpose of study. The use of single strain allows researchers to see variability among the strains, and to have more precise conclusions. For example, *L. monocytogenes* strains from Brie cheese (Brie-1), a cabbage outbreak (LCDC), a human isolate from milk related outbreak (Scott A), and another human isolate have significantly different *D*-values at 56°C (16.0, 10.4, 7.4, 5.7 min, respectively; Golden et al., 1988); and STEC *E. coli* serogroups O26, O45, O103, O111, O121, O145, and O157:H7 have *D*-values at 56°C ranging between 2.14 min (O26) 8.37min (O157:H7) in apple juice (Enache et al., 2011). The use of cocktail of strains mimics what may occur in practice, and as all strains are used at once, these studies are often much quicker to complete. The use of cocktails also allows insight into the effect of possible strain interaction on results. However, the most or least resistant strain in a cocktail may cause misleading result due to masking.

Heat treatments that are used by food processors to eliminate pathogens are often based on experimental data. Heat resistance of distinct *Salmonella* serotypes, *E. coli* and *L. monocytogenes* strains have been studied in numerous types of food products and laboratory media. The thermal tolerance of these pathogens varies depending on the formulation of media and characteristic of foods including total solids, acidity, and water activity (Doyle and Mazotta, 2000).

Thermal resistance of *E. coli*

Escherichia coli is one of the most commonly studied microorganisms in thermal tolerance researches due to its high potential risk of outbreak in different type of foods, high thermal resistance response, and ease of study. Survival of *E. coli* O157:H7 isolate 204P is

increased by fat presence in ground beef (7%, 10%, and 20% fat), pork sausage (7%, 10%, and 30% fat), chicken and turkey (3% and 11% fat) from *D*-values at generated at 60°C (Ahmed et al., 2006). The *D*-values (min) varied in different meat samples as follows: 0.45-0.47 in beef, 0.37-0.55 in pork sausage, 0.38-0.55 in chicken and 0.55-0.58 in turkey. When heat resistance of *E. coli* was tested by using low (3%) and high fat (11%) turkey with mixture of 8% NaCl, 4% sodium lactate, and 0.5% polyphosphate at 52, 55, 57, and 60°C. the addition of additives enhanced survival of *E. coli* (Kotrola and Conner, 1997).

In fruit juices, the *D*-values provided in literature are primarily for *E. coli*. The heat resistance of *E. coli* O157:H7was not affected by increasing the soluble solid of apple juice from 11.8° to 16.5°Brix; but was altered by the reduction of pH from 4.4 to 3.6 by addition of malic acid and the addition of sorbic and benzoic acid at 52°C (Splittstoesser et al., 1996). The acid adaption of stationary phase *E. coli* O157:H7 increased the thermal inactivation activation time compared to non-adapted stationary phase cells in apple, orange, and white grape juices, where *D*-values at 60°C for acid adapted and non adapted *E. coli* O157:H7 were 1.5 and 0.8 min in apple juice, 1.7 and 1.1 min in orange juice, and 1.2 and 0.7 min in white grape juice, respectively (Mazotta, 2001). Serogroup variability of *E. coli* can affect the thermal resistance, where *D*-values of stationary-phase and acid-adapted cells of *E. coli* strains from serogroups O26, O45, O103, O111, O121, O145, and O157:H7 were investigated by using an immersed coil apparatus at various temperature. At 60C *E. coli* O157:H7 and O103 showed the highest *D*-values, 1.37 and 1.07 min, respectively (Enache et al., 2011).

Thermal resistance of *Salmonella*

The thermal inactivation parameters of *Salmonella* may be affected by the type of experiment, methodology, culture conditions (e.g. acid or none adapted), strains and heating mechanism. The thermal resistance of *Salmonella* in whole eggs, egg yolks, and whites has been

widely studied under conditions of variable pH, water activity (including salt and sugar concentrations), age and incubation temperature of *Salmonella* inoculum, previous heat shock, storage conditions of eggs, the presence of lactic acid, hydrogen peroxide, EDTA, nisin, and polyphosphate (Doyle and Mazotta, 2000). *Salmonella* survives in egg yolk better than in egg white, and heat resistance differs according to serotype. In milk and dairy products, an increase in concentration of total solid in milk enhances the heat resistance of *Salmonella*, where *S.* Typhimurium at 55°C have 4.7 min *D*-value at 10% solids but when the solid content increases to 42%, the *D*-value; increased to 18.3 min.

Salmonella Seftenberg is often considered one of the most heat tolerant *Salmonella* serotypes. *D*-values for *S.* Seftenberg in ground beef at 55, 60 and 65°C were 211.4, 13.2, and 3.4 min, respectively, and are higher than reported *D*-values for other *Salmonella* serotypes in the ground beef and *E. coli* O157:H7 in the same conditions (Doyle and Mazotta, 2000). Chocolate, wheat and corn flour, corn-soy milk blends, shellfish, coconuts and pecans, alfalfa seeds are some of the other foods that heat resistance of *Salmonella* have been studied.

The number of studies evaluating thermal inactivation of *Salmonella* in fruit juices is limited. The acid adaptation of *Salmonella* increases its thermal resistance in apple, orange and white grape juice at different temperatures (Mazotta, 2001). *D*-values of *Salmonella* in phosphate-buffered saline (PBS) and apple juice at 55°C are similar at 0.51 and 0.49 min, respectively (Gabriel and Nakano, 2009).

Thermal resistance of *Listeria*

Similar to *Salmonella*, thermal resistance of *L. monocytogenes* is affected by strain variability, heating mechanism, previous growth conditions of cells, and other factors, such as acid and heat shock. The effectiveness of thermal inactivation on *L. monocytogenes* in meat and poultry is affected directly by fat content of meat, age of inoculums, heat source, and exposure of

the bacteria to acid, heat shock and preservatives (Doyle et al., 2001). When inoculated on fresh pork, *L. monocytogenes* have greater *D*-values at 62°C compare to cells inoculated into three month old ground pork (Kim et al., 1998). In high fat (30.5%) ground beef, *L. monocytogenes* survives better at 57.2 and 62.8°C than cell in low fat (2%) ground beef (Fain et al., 1992). Slow heating time (1.3°C/min) also leads to increase *Listeria* survival compared to fast heating (8°C/min) in ground pork (Kim et al., 1994).

Thermal tolerance studies on *L. monocytogenes* have been conducted in various kinds of milk and dairy products including raw milk, sterile milk, whole milk, skim milk, cream, and butter. *Listeria monocytogenes* survives better in raw milk than in sterile milk and in skim milk than in whole milk at temperatures below 63°C (Bradshaw et al., 1995). During cheese production, the heat tolerance of *L .monocytogenes* varies by type of cheese and applied temperatures.

In egg and egg products, *Listeria* is more thermal resistant than *Salmonella* serotypes tested under the same experimental conditions (McKenna et al., 1991; Muriana et al., 1996). Addition of 10% sodium chlorine or 10% sucrose increased the thermal tolerance of *Listeria* (Palumbo et al., 1995). *Listeria monocytogenes* strain Scott A was more durability to heat than strain LCDC, and was more heat tolerance at pH 5.6 than at pH 4.6 (Beuchat et al., 1986). Thermal tolerance of *Listeria* has also been extensively studied fish and shellfish, vegetables and other meat products.

CHAPTER 3
MATERIAL AND METHODS

Juice

One brand of 100% pure and single-strength pasteurized orange juice without preservatives and pulp was purchased from a local supermarket (Publix, Winter Haven, FL). The pH of juice was obtained via Denver model UB-5 pH meter with a combination electrode (Denver Instrument Inc., Arvada, CO, USA). The soluble solid content of juice was measured by using The *Leica* Mark II plus digital refractometer (Buffalo, NY, USA). Juice was filled into 50 mL conical centrifuge tubes (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) aseptically, and stored at -20 °C until use. One tube of juice was used for each experimental trial.

Strains Used

All strains used in this study are listed in Table 3-1. Three strains of shiga-toxin producing *Escherichia coli*, three serotypes of *Salmonella*, and three strains of *Listeria monocytogenes* were used. All strains are available in Dr. Danyluk's culture collection. Three shiga-toxin producing *E. coli* strains including *E. coli* O111 (MDD339; clinical isolate from an apple cider outbreak of 2004, New York), *E. coli* O157:H7 (MDD338; clinical isolate from an apple juice outbreak of 1991, Massachusetts), and *E. coli* O157:H7 (F4546; clinical isolate from a sprout outbreak of 1997) were obtained from Dr. Danyluk's culture collection.

The *Salmonella* serotypes include Typhimurium (ATCC 14028; orange juice outbreak of 1999), Gaminara (CDC H0622); orange juice outbreak of 1995), and Muenchen (MDD30; orange juice outbreak of 1999).

The three *L. monocytogenes* strains from Dr. Danyluk's culture collection include *L. monocytogenes* (LCDC 81-861; raw cabbage outbreak of 1981), *L. monocytogenes* (Scott A; human milk outbreak of 1983), and *L. monocytogenes* (v7; milk associated outbreak of 1985).

Inoculum Preparation

All strains, stored at -80°C, were converted to working cultures by streaking on non-selective Tryptic Soy Agar (TSA; Becton, Dickinson and Company, Sparks, MD, USA) plates for *E. coli and Salmonella*, and on Brain Heart Infusion (BHI) agar (Becton, Dickinson and Company, Sparks, MD, USA) plates for *L. monocytogenes*. Plates were incubated at 37 ± 2°C for 24 ± 2 h.

Inoculum preparation was different for non-adapted and acid adapted cells. For non-adapted stationary phase inoculum preparation, one isolated colony from each strain of *E. coli* O157:H7, and *Salmonella* were grown in Tryptic Soy Broth (TSB;Becton, Dickinson and Company) at 37 ± 2°C for 18 h. One isolated colony from each *L. monocytogenes* strain was incubated in BHI broth at 37 ± 2°C for 18 h. One loopful of overnight growth was transferred to a new tube of broth and incubated at 37 ± 2°C for 18 h. For acid adapted stationary phase inoculum preparation, strains of *E. coli* and *Salmonella* were grown in TSB supplemented with 1% glucose (10 g/l), TSBG; Fisher Scientific, Lawn, NJ, USA) at 37 ± 2°C for 18 h as described by Buchanan and Edelson (1996). For strains of *L. monocytogenes*, BHI supplemented with 1% glucose (10 g/l) BHIG was used at 37 ± 2°C for 18 h. One loopful (10µL) of overnight growth was transferred to a new tube of broth and incubated at 37 ± 2°C for 18 h. The addition of glucose to the broth induces acid production by the *E. coli*, *Salmonella,* and *L. monocytogenes* strains, resulting in an acidic environment and the development of acid adaptation.

Following incubation, cells were collected by centrifugation at 3000 x g for 10 min (Allegra X-12, Beckman Coulter, Fullerton, CA) The supernatant was removed and 10 mL of 0.1% peptone (Becton, Dickinson and Company) water was vortexed with the pellet to wash cells. Cells were centrifuged and the washing step was repeated. After the cells had been

washed three times, the pellets were resuspended in 5 mL orange juice to obtain the desired concentration of cells ($10^8 – 10^{10}$ CFU/mL).

Thermal Treatment of Inoculated Juice

Each strain of STEC, *Salmonella,* and *L. monocytogenes* was inoculated into orange juice as described above. Two sterile microcapillary tubes (1.5–1.8 (ID) 90 mm; Kimble-Kontes, Vineland, NJ, USA) with one head heat sealed, were each injected with 50 µL of inoculated orange juice. The aseptic injection was achieved using a sterile 20 gauge 4 inch deflected-point needle (Popper and Sons, Inc., Hyde Park, NJ, USA), and 1 mL syringe (Luer-Lok Tip, Franklin Lakes, NJ, USA). The open end of the micro capillary tubes was sealed with a Bunsen burner flame. To prevent cracking of the tubes from sudden temperature change before thermal treatment, the microcapillary tubes were held at room temperature for 30 min prior to the beginning of the experiment.

Inoculated, sealed, microcapillary tubes of each strain were immersed into water baths (LAUDA Brinkmann, ECO-Line RE120, Germany) at desired temperatures. Two sealed microcapillary tubes filled with inoculated orange juice were placed in a preheated culture tubes in water bath during heat treatment. Time and temperature values were chosen according to preliminary tests. Briefly, to determine each time interval, the time required for a one log reduction on average was used as reference according to result of preliminary experiments. To estimate *D*-values, the inoculated juice was exposed to the selected temperature until an average five-log reduction was achieved. Last time interval used was determined based on total of five-log reduction of cells from initial concentration. Total of at least seven time intervals were used. Three temperatures: 55, 58, and 60°C for *Salmonella*; 56, 58, and 60°C for *E. coli,* and *L. monocytogenes* were tested. Temperature and time intervals for each strain are available in Tables 3-2, 3-3, and 3-4. After heat treatment, microcapillary tubes were poured immediately

onto ice, and then further cooled and sterilized by immersing 70% ethanol at room temperature for one minute. Excessive alcohol was removed by rinsing microcapillary tubes in sterile deionized water at room temperature. Thermally treated and sterilized microcapillary tubes (0.1 mL inoculated juice) were added into 10 mL of sterile 0.1% peptone water in 15 mL conical centrifuge tubes (Becton, Dickson and Company), resulting in an initial 1:100 dilution. Microcapillary tubes were crushed using a sterile glass rod, and centrifuge tubes were vortexed prior to microbiological analysis. Six replicates were used for each time interval at all temperatures; duplicate samples of each time interval were examined in triplicate.

Microbiological Analysis

Populations of *E. coli*, *Salmonella*, and *L. monocytogenes* in thermally treated juice were determined by spread plating samples and dilutions (0.1 mL) onto TSA for *E. coli* and *Salmonella*, and BHI agar for *L. monocytogenes* supplemented. All media was supplemented with 0.1% sodium pyruvate (Sigma-Aldrich, St Louis, MO, USA; TSAP or BHIP) to enhance the recovery of injured cells (Knudsen et al., 2001; Yamamoto and Harris, 2001). Plates were incubated at 37± 2°C for 24 – 48 h depending on strain to achieve full recovery of survived and injured cells prior to counting. To increase the sensitivity and lower the limit of detection to 2 log CFU/mL, 1 mL of the initial dilution (10^{-2}) was spread over 4 plates (0.25 mL/plate).

Statistical Analysis

The number of enumerated pathogens (log CFU/mL) after heat treatment was plotted on the y-axis versus heating time (min) on the x-axis. Linear regression was used to determine best-fit lines, and to calculate *D*-values of each strain by analyzing data with JMP software (Version 9.0.2 SAS® Institute Inc., Cary, NC, USA 2010). *z*-values (°C) were determined by calculating inverse slope of regression line from the plot of log *D*-value versus the temperature for the *D*-value. *D*-values of acid adapted and non-adapted strains at the same temperature were compared

statistically ($P < 0.05$). *D*-values of *E. coli, Salmonella*, and *L. monocytogenes* strains at the same temperature were compared statistically ($P < 0.05$) in the same specie group. Mean *z*-values of each species were compared statistically by using Analysis of Variance (ANOVA) ($P < 0.05$). To find out statistical significances among the *D*-values, the Analysis of Covariance (ANCOVA - a combination of ANOVA and linear regression) was used via JMP software (SAS® Institute Inc.).

Table 3-1. Isolates used for thermal processing experiments.

Strain or serotype	Strain designation	Year of outbreak	Sources
E. coli O157:H7	F4546	1997	Clinical alfalfa sprout outbreak
E. coli O157:H7	MDD338	1991	Clinical apple juice outbreak Massachusetts
E. coli O111	MDD339	2004	Clinical apple cider outbreak New York
S. Typhimurium	ATCC 14028	1999	Orange juice outbreak
S. Gaminara	CDC HO662	1995	Orange juice outbreak
S. Muenchen	MDD30	1999	Orange juice outbreak
L. monocytogenes	LCDC 81-861	1981	Raw cabbage outbreak
L. monocytogenes	Scott A	1983	Human milk outbreak
L. monocytogenes	v7	1985	Milk associated outbreak

Table 3-2. Time intervals used in heat treatment experiments of STEC strains to plot regression lines in determination of *D*-values.

	Temperature (˚C)					
	56		58		60	
Time intervals (min)	Non	Acid	Non	Acid	Non	Acid
T1	0[a,b,c]	0[a,b,c]	0[a,b,c]	0[a,b,c]	0[a,b,c]	0[a,b,c]
T2	1.5[a,b,c]	1.5[a,b,c]	1[a,b,c]	1[a,b,c]	0.5[a,b,c]	0.5[a,b,c]
T3	3[a,b,c]	3[a,b,c]	2[a,b,c]	2[a,b,c]	1[a,b,c]	1[a,b,c]
T4	4.5[b]	4.5	3[a,b,c]	3[a,b,c]	1.5[a,b,c]	1.5[a,b,c]
T5	6[a,b,c]	6[a,b,c]	4[a,b,c]	4[a,b,c]	2[a,b,c]	2[a,b,c]
T6	7.5[b]	7.5	5[a,b,c]	5[a,b,c]	2.5[a,b,c]	2.5[a,b,c]
T7	9[a,b,c]	9[a,b,c]	6[b]	6[b]	3.0[a,b,c]	3.0[a,b,c]
T8	12[a,b,c]	12[a,b,c]	7[a,c]	7[a,b,c]	3.5[a,c]	3.5[a,c]
T9	15[a,c]	15[a,b,c]				4[a]
T10		18[c]				

[a]Time intervals for *E. coli* O157:H7 from human feces-sprout outbreak (F4546)
[b]Time intervals for *E. coli* O157:H7 from apple juice outbreak Massachusetts (MDD338)
[c]Time intervals for *E. coli* O111from apple cider outbreak New York (MDD339)

Table 3-3. Time intervals used in heat treatment experiments of serotypes to plot regression lines in determination of *D*-values.

	Temperature (˚C)					
	56		58		60	
Time intervals (min)	Non	Acid	Non	Acid	Non	Acid
T1	$0^{a,b,c}$	$0^{a,b,c}$	$0^{a,b,c}$	$0^{a,b,c}$	$0^{a,b,c}$	$0^{a,b,c}$
T2	$0.5^{a,b,c}$	$0.5^{a,b,c}$	$0.17^{a,b,c}$	$0.17^{a,b,c}$	$0.17^{a,b,c}$	$0.17^{a,b,c}$
T3	$1^{a,b,c}$	$1^{a,b,c}$	$0.33^{a,b,c}$	$0.33^{a,b,c}$	$0.33^{a,b,c}$	$0.33^{a,b,c}$
T4	$1.5^{a,b,c}$	$1.5^{a,b,c}$	0.5^{a}	0.5^{a}	$0.5^{a,b,c}$	$0.5^{a,b,c}$
T5	$2^{a,b,c}$	$2^{a,b,c}$	$0.67^{a,b,c}$	$0.67^{a,b,c}$	$0.67^{a,b,c}$	$0.67^{a,b,c}$
T6	$2.5^{a,b,c}$	$2.5^{a,b,c}$	$1^{a,b,c}$	$1^{a,b,c}$	$0.83^{a,b,c}$	$0.83^{a,b,c}$
T7	$3^{b,c}$	$3^{b,c}$	$1.33^{b,c}$	$1.33^{b,c}$	$1^{a,b,c}$	$1^{a,b,c}$
T8	3.5^{a}	3.5^{a}	$1.67^{a,b,c}$	$1.67^{b,c}$		
T9	4^{b}	$4^{b,c}$	$2^{b,c}$	$2^{b,c}$		
T10	5^{a}	5^{a}				

[a]Time intervals for *S.* Typhimurium from orange juice outbreak (ATCC 14028)
[b]Time intervals for *S.* Gaminara from orange juice outbreak (CDC H0622)
[c]Time intervals for *S.* Muenchen from orange juice outbreak (MDD30)

Table 3-4. Time intervals used in heat treatment experiments of *L. monocytogenes* strains to plot regression lines in determination of *D*-values.

	Temperature (˚C)					
	56		58		60	
Time intervals (min)	Non	Acid	Non	Acid	Non	Acid
T1	0[a,b,c]	0[a,b,c]	0[a,b,c]	0[a,b,c]	0[a,b,c]	0[a,b,c]
T2	1[a,b,c]	1[a,b,c]	0.5[a,b,,c]	0.5[a,b,c]	0.25[a,b,c]	0.25[a,b,c]
T3	2[a,b,c]	2[a,b,c]	1[a,b,c]	1[b,c]	0.5[a,b,c]	0.5[a,b,c]
T4	3[b]	3[a,b]	1.5	1.5[a,b]	0.75	0.75[a]
T5	4[a,b,c]	4[a,b,c]	2[a,b,c]	2[a,b,c]	1[a,b,c]	1[a,b,c]
T6	5[a,b,c]	5[a,b]	2.5[a,b]	2.5[a,b]	1.25[a]	1.25[a]
T7	6[a,b]	6[a,b,c]	3[a,b,c]	3[a,b,c]	1.5[a,b,c]	1.5[a,b,c]
T8	7	7[a]	3.5	3.5[a]	1.75	1.75[a]
T9	8[a,b,c]	8[b,c]	4[a,b,c]	4[b,c]	2[a,b,c]	2[b,c]
T10			5[c]	5[c]	2.5[b,c]	2.5[b,c]

[a]Time intervals for *L. monocytogenes* from raw cabbage outbreak (LCDC 81-861)
[b]Time intervals for *L. monocytogenes* from human milk outbreak (Scott A)
[c]Time intervals for *L. monocytogenes* V7 from milk associated outbreak (v7)

CHAPTER 4
RESULTS

Time Interval Determination

The most appropriate time intervals for sampling each strain and serotype at a particular temperature were determined during preliminary thermal death time experiments. Time intervals used to determine *D*-values of each strain varied both among the species and the strains or serotypes of each species. Acid adaptation also influenced the use of different time intervals, even for the same strain at the same temperature. The maximum applicable time intervals, by pathogen, were: *Salmonella* serotypes at 55°C, 5 min; STEC and *L. monocytogenes* strains at 56°C; 18 and 8 min, and STEC, *Salmonella* and *L. monocytogenes* at 58 and 60°C: 7, 5, 2 min and 4, 2.5, 1 min, respectively.

D and *z*-value Determination for Strains and Serotypes of Pathogens Used

To estimate *D*-values of each strain and serotypes of pathogens tested, population of survived cells (log CFU/mL) from thermal destruction treatment versus time (min) were plotted to obtain linear curve at desired temperatures. Linear regression lines for both acid adapted and non-adapted STEC strains were plotted on the same plots to compare regression trends at all temperatures tested, and shown in Figures 4-1, 4-2, 4-3. Similar plots were prepared for *Salmonella* serotypes and *L. monocytogenes* strains to observe linear curves as shown Figures 4-4, 4-5, 4-6 and Figures 4-7, 4-8, 4-9, respectively. The equations of these linear regression lines were used to calculate *D*-values. All equations of thermal death curves used to calculate *D*-values of STEC strains, *Salmonella* serotypes, and *L. monocytogenes* strains were listed in Tables 4-1, 4-2, and 4-3, respectively.

Similar to *D*-value determination, *z*-values were calculated by using the linear regression equations of plots of log *D*-values versus temperature (°C) for each strain. Linear regression

lines of *z*-values for STEC strains, *Salmonella* serotypes, and *L. monocytogenes* strains were shown in Figures 4-10, 4-11, 4-12, respectively. Also, equations of these linear curves to calculate *z*-values were listed in Table 4-4.

D and *z*-values of STEC Strains

D and *z*-value of STEC strains grown in TSB and TSBG and heated at 56, 58, and 60°C were shown in Table 4-5. *D*-values for acid adapted and non-adapted *E. coli* O157:H7 (F4546) at 56, 58, and 60°C are 2.81 ± 0.06 and 2.72 ± 0.07 min, 1.35 ± 0.07 and 1.28 ± 0.07 min, and 0.61 ± 0.06 and 0.52 ± 0.05 min, respectively. A similar trend was seen for *E. coli* O111 (MDD339), where *D*-values for acid adapted and non-adapted cells at 56, 58, and 60°C are 3.41 ± 0.06 and 3.05 ± 0.06 min, 1.60 ± 0.05 and 1.39 ± 0.04 min, and 0.61 ± 0.05 and 0.54 ± 0.05 min, respectively. *D*-values for acid adapted *E. coli* O157:H7(MDD338) were similar to the other two STEC strains at 56, 58, and 60°C, and they are 3.21 ± 0.08 and 1.31 ± 0.04 and 0.61 ± 0.08, respectively. However, *D*-values for non-adapted strain MDD338, are significantly lower than as for the other two STEC strains at 56, 58°C ($P < 0.05$), where *D*-values for non-adapted form of this strain at 56, 58, 60°C are 1.93 ± 0.07, 1.04 ± 0.12, and similar to others 0.52 ± 0.13, respectively. *z*-values for acid adapted and non-adapted STEC strain F4546, MDD338, and MDD339 were, 6.0 and 5.6, 5.5 and 7.0, and 5.4 and 5.3, respectively.

Acid adapted STEC have higher *D*-values than non-adapted cells, at all temperatures for all strains with the exception of strain F4546 at 56, 58°C ($P < 0.05$). *D*-values for *E. coli* O111 were higher at 56, 58°C evaluated than those of both *E. coli* O157:H7 strains ($P < 0.05$). *D*-value differences between acid adapted and non-adapted strain MDD338 cells were greater than in strain MDD339 and strain F4546 at 56 and 58°C. With the exception of *D*-values for strain F4546 at 56 and 58°C, all non-adapted STEC strains have significantly lower heat resistance compare to acid adapted STEC strains ($P < 0.05$). Both acid adapted and non-adapted strain *E.*

coli O111 had the highest *D*-values at all temperatures, where the *D*-values for acid adapted and non-adapted strain *E. coli* O111 at 56, 58, and 60°C are 3.41 ± 0.06 and 3.05 ± 0.06 min, 1.60 ± 0.05 and 1.39 ± 0.04 min, and 0.61 ± 0.05 and 0.54 ± 0.05 min, respectively (Table 4-5). Although *D*-values for both acid adapted and non-adapted STEC strains were significantly different from each other in the same adaptation group at 56 and 58°C ($P < 0.05$), no difference exists among strains for both acid adapted and non-adapted groups at 60°C ($P > 0.05$) in the same adaptation group. Particularly, *D*-values for acid adapted STEC strains at 60°C were the same (0.61 ± 08) ($P > 0.05$) (Table 4-5).

D-values of *Salmonella* Serotypes

D and *z*-values of acid and non-acid adapted *Salmonella* serotypes, heated at 55, 58, and 60°C were shown in Table 4-6. All *Salmonella* species demonstrated similar responses to heat at all temperatures. *D*-values for acid adapted and non-adapted *S.* Typhimurium at 55, 58, and 60°C are 1.03 ± 0.08 and 0.98 ± 0.09 min, 0.28 ± 0.10and 0.30 ± 0.10 min, and 0.17 ± 0.09 and 0.17 ± 0.09 min, respectively. A similar trend was seen for *S.* Gaminara, where *D*-values for acid adapted and non-adapted cells at 55, 58, and 60°C are 0.89 ± 0.06 and 0.80 ± 0.10 min, 0.36 ± 0.09 and 0.36 ± 0.07, and 0.20 ± 0.08 and 0.17 ± 0.017 min, respectively. *D*-value for acid adapted *S.* Muenchen at 55°C temperatures is lower than *D*-values of *S.* Typhimurium ($P < 0.05$), and equal to those *D*-values of *S.* Gaminara. However, *S.* Muenchen had the highest heat resistance for both acid adapted and non-adapted cells at 58 and 60°C. *D*-values for acid adapted and non-adapted *S.* Muenchen at 55, 58, and 60°C are 0.89 ± 0.09 and 0.80 ± 0.12 min, 0.36 ± 0.09 and 0.36 ± 0.07 min, and 0.20 ± 0.08 and 0.17 ± 0.09 min. *z*-values for acid adapted and non-adapted *S.* Typhimurium, *S.* Gaminara, and *S.* Muenchen are 6.3 and 6.5, 7.5 and 7.1, and 6.5 and 6.7, respectively (Table 4-6).

With the exception of *D*-values for acid-adapted and non-adapted *S.* Muenchen at 60°C ($P < 0.05$), there was not a significant difference between *D*-values for acid adapted and non-adapted *Salmonella* serotypes at all temperatures ($P > 0.05$). *D*-value differences among serotypes for both acid adapted and non-adapted groups showed different trends according to temperature. *D*-values for both acid and non-adapted *S.* Typhimurium at 55 and 58°C were significantly different from *D*-values for both acid and non-adapted *S.* Gaminara and *S.* Muenchen in the same adaptation group ($P > 0.05$). However, *D*-values for both acid and non-adapted *S.* Muenchen at 60°C are significantly different from *D*-values for both acid and non-adapted *S.* Typhimurium and *S.* Muenchen in the same adaptation group ($P < 0.05$).

D-values of *L. monocytogenes* Strains

In Table 4-7, *D* and *z*-value of *L. monocytogenes* strains grown in BHI and BHIG and heated at 56, 58, and 60°C are shown. Non-adapted *L. monocytogenes* strains always had higher *D*-values than acid adapted strains except for strain LCDC 81-861 at 55°C. *D*-values for acid adapted and non-adapted *L. monocytogenes* LCDC 81-861 at 56, 58, and 60°C were 1.34 ± 0.08 and 1.59 ± 0.07 min, 0.92 ± 0.09 and 0.91 ± 0.08 min, and 0.48 ± 0.14 and 0.55 ± 0.06 min, respectively. A similar trend was seen for other *L. monocytogenes* strain Scott A, where *D*-values for acid adapted and non-adapted cells at 56, 58, and 60°C were 1.73 ± 0.06 and 1.82 ± 0.05 min, 0.99 ± 0.06 and 1.05 ± 0.07 min, and 0.62 ± 0.07 and 0.65 ± 0.06 min, respectively. Also, *D*-values for acid adapted and non-adapted *L. monocytogenes* v7 resembled to other two *L. monocytogenes* strains at 56°C, but lower at 58, and 60°C. They were 1.47 ± 0.10 and 1.65 ± 0.07 min, 0.85 ± 0.10 and 0.90 ± 0.09 min, and 0.49 ± 0.08 and 0.52 ± 0.05 min, respectively. *z*-values for acid adapted and non-adapted *L. monocytogens* strain LCDC 81-861, Scott A, and v7 were, 9.0 and 8.7, 9.0 and 8.9, and 8.4 and 8.0, respectively.

With the exception of *D*-values for acid-adapted and non-adapted strain LCDC 81-861 at 56°C ($P < 0.05$), there was not a significant difference between *D*-values for acid adapted and non-adapted *L. monocytogenes strains* at all temperatures ($P > 0.05$). *D*-values for non-adapted *L. monocytogenes* strains were significantly different from each other in the same adaptation group at 56°C; however, only strain *D*-values of non-adapted strain Scott A had significantly different *D*-values at 58 and 60°C in the non adapted strain groups ($P < 0.05$). For acid-adapted strain group, different trend was seen. *D*-values for acid adapted *L. monocytogenes* strains were significantly different from each other in the same adaptation group at 56 and 60°C ($P < 0.05$), whereas they were not significant at 58°C ($P > 0.05$).

Heat Resistance of All Pathogens

D-values of STEC strains, *Salmonella* serotypes, and *L. monocytogenes* strains were calculated by using linear regression equations as presented in Tables 4-1, 4-2, and 4-3, respectively. A total of three STEC strains, three *Salmonella* serotypes, and three *L. monocytogenes* strains were tested to determine the heat resistance of the pathogens in single strength orange juice (pH 3.87 ± 0.01; Brix° corrected 12.24). *Salmonella* serotypes were less heat resistance than STEC and *L. monocytogenes* strains. All acid adapted and non-adapted STEC strains had the highest *D*-values at 56 and 58°C; one of acid adapted and non-adapted *L. monocytogenes* strain Scott A was the most heat tolerant strain at 60°C. Acid adaptation caused change of heat resistance for all STEC strains at all temperatures except for strain F4546 at 55 and 58°C ($P < 0.05$). However, it had almost no significant effect on *Salmonella* serotypes and *L. monocytogenes* strains except for *S.* Muenchen at 60°C and *L. monocytogenes* strain LCDC 81-861 at 56°C ($P > 0.05$). An analysis of variance showed that average *z*-values of three *L. monocytogenes* strain are significantly different from average *z*-values of three STEC strains and three *Salmonella* serotypes ($P < 0.05$) (Table 4-8).

A semilogaritmic plot of *D*-value versus temperature for STEC strains, *Salmonella* serotypes and *L. monocytogenes* strains was used to evaluate the heat resistance of these pathogens on the same plot (Figure 4-13). The highest *D*-value for acid-adapted and non-adapted strains and serotypes were plotted to calculate minimum process time at 71.1°C. A linear regression line for each acid adapted and non adapted pathogens were obtained. These linear regression lines were extrapolated to higher temperatures to cover higher processing temperatures including milk pasteurization temperature (71.1°C). Extrapolated regression lines from the highest *D*-value for STEC and *L. monocytogenes* strains crossed around 59°C. To determine process parameters that would provided the thermal inactivation of all three pathogens, a regression line covering all regression lines for all three pathogens was drawn above all regression lines for strains and serotypes with the highest *D*-values. The equation of this cover line ($\log D = 8.2 - 0.14T$ (°C)) was used to calculate a general process for orange juice at 71.1°C. The calculations via this equation yielded that achievement of 5 log reductions of all three pathogens in orange juice requires 5.29 seconds at 71.1°C with a *z*-value of 7.1°C

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Table 4-1. Linear regression equations and R^2 of STEC strains used in *D*-value calculations at 56, 58, 60°C. Non adapted pathogens were grown in TSB and acid adapted pathogens were grown in TSB supplemented with 1% glucose (TSBG) (n = 6).

		Linear regression equations of strains and R^2 at temperatures					
E. coli		56°C		58°C		60°C	
Strain	Adaptation	Equations	R^2	Equations	R^2	Equations	R^2
O157:H7	Non	y = -0.3681x + 9.3693	0.95	y = -0.7841x + 9.6966	0.95	y = -1.9080x + 9.8733	0.97
(F4546)	Acid	y = -0.3554x + 9.6283	0.97	y = -0.7392x + 9.7800	0.97	y = -1.6454x + 9.7084	0.95
O157:H7	Non	y = -0.5187x + 8.9160	0.95	y = -0.9576x + 8.5780	0.88	y = -1.9387x + 8.5726	0.85
(MDD338)	Acid	y = -0.3117x + 9.1629	0.94	y = -0.7612x + 9.1664	0.92	y = -1.6411x + 9.0879	0.93
O111	Non	y = -0.3272x + 9.2080	0.97	y = -0.7216x + 9.3120	0.93	y = -1.8423x + 9.6380	0.97
(MDD339)	Acid	y = -0.2930x + 9.2772	0.97	y = -0.6269x + 9.3018	0.92	y = -1.6338x + 9.5688	0.97

Table 4-2. Linear regression equations and R^2 of *Salmonella* serotypes used in *D*-value calculations at 55, 58, 60°C. Non adapted pathogens were grown in TSB and acid adapted pathogens were grown in TSB supplemented with 1% glucose (TSBG) (n = 6).

		Linear regression equations of strains and R^2 at temperatures					
Salmonella		55°C		58°C		60°C	
Serotype	Adaptation	Equations	R^2	Equations	R^2	Equations	R^2
Typhimurium	Non	y = -1.0203x + 8.3253	0.91	y = -3.3286x + 8.6029	0.91	y = -5.8235x + 8.8061	0.93
(ATCC 14028)	Acid	y = -0.9721x + 8.4866	0.94	y = -3.5334x + 9.0032	0.93	y = -5.8561x + 9.0074	0.93
Gaminara (CDC	Non	y = -1.2465x + 8.9191	0.90	y = -3.0724x + 8.8656	0.92	y = -6.9899x + 9.2921	0.94
H0622)	Acid	y = -1.1263x + 9.0258	0.96	y = -3.1410x + 9.3009	0.97	y = -6.7254x + 9.4836	0.94
Muenchen	Non	y = -1.2567x + 8.7988	0.87	y = -2.7575x + 8.6942	0.94	y = -5.8555x + 8.8314	0.92
(MDD30)	Acid	y = -1.1233x + 9.0139	0.92	y = -2.8116x + 8.8308	0.93	y = -5.0934x + 8.9485	0.93

Table 4-3. Linear regression equations and R^2 of *L. monocytogenes* strains used in *D*-value calculations at 56, 58, 60°C. Non adapted pathogens were grown in BHI and acid adapted pathogens were grown in BHI supplemented with 1% glucose (BHIG) (n = 6).

		Linear regression equations of strains and R^2 at temperatures					
L. monocytogenes		56°C		58°C		60°C	
Strain	Adaptation	Equations	R^2	Equations	R^2	Equations	R^2
LCDC 81-861	Non	y = -0.6276x + 9.3324	0.96	y = -1.0985x + 9.4175	0.94	y = -1.8173x + 9.2424	0.96
	Acid	y = -0.7472x + 9.2413	0.93	y = -1.0922x + 9.0574	0.93	y = -2.0636x + 8.8346	0.82
Scott A	Non	y = -0.5500x + 9.5018	0.98	y = -0.9529x + 9.4533	0.96	y = -1.5380x + 9.4503	0.96
	Acid	y = -0.5790x + 9.3232	0.96	y = -1.0112x + 9.3661	0.96	y = -1.6027x + 9.3616	0.96
v7	Non	y = -0.6061x + 9.4928	0.96	y = -1.1067x + 9.5121	0.92	y = -1.9095x + 9.4006	0.97
	Acid	y = -0.6794x + 9.4639	0.92	y = -1.1735x + 9.5026	0.92	y = -2.0601x + 9.5129	0.94

Table 4-4. Linear regression equations and R^2 of strains used in *z*-value calculations. Non adapted STEC strains and *Salmonella* serotypes were grown in TSB and acid adapted STEC strains and *Salmonella* were grown in TSB supplemented with 1% glucose (TSBG). Non adapted *L. monocytogenes* strains were grown in BHI and acid adapted *L. monocytogenes* strains were grown in BHI supplemented with 1% glucose (BHIG).

Strain or serotype	Adaptation	Linear regression equations of strains to estimate *z*-value	R^2
E. coli O157:H7	Non	y = -0.1796x + 10.505	0.99
(F4546)	Acid	y = -0.1658x + 9.7404	0.99
E. coli O157:H7	Non	y = -0.1424x + 8.2647	0.99
(MDD338)	Acid	y = -0.1803x + 10.593	0.99
E. coli O111	Non	y = -0.1880x + 11.023	0.99
(MDD339)	Acid	y = -0.1869x + 11.012	0.99
S. Typhimurium	Non	y = -0.1537x + 8.4280	0.99
(ATCC 14028)	Acid	y = -0.1590x + 8.7331	0.98
S. Gaminara	Non	y = -0.1400x + 7.6411	0.99
(CDC H0622)	Acid	y = -0.1332x + 7.2671	0.99
S. Muenchen	Non	y = -0.1486x + 8.1025	0.97
(MDD30)	Acid	y = -0.1528x + 8.3717	0.99
L. monocytogenes	Non	y = -0.1153x + 6.6519	0.99
LCDC 81-861	Acid	y = -0.1115x + 6.3891	0.98
L. monocytogenes	Non	y = -0.1118x + 6.5138	0.99
Scott A	Acid	y = -0.1114x + 6.4707	0.99
L. monocytogenes	Non	y = -0.1254x + 7.2340	0.99
V7	Acid	y = -0.1193x + 6.8472	1.00

Table 4-5. *D* and *z*-values of STEC strains from linear regression equations. Non adapted pathogens were grown in TSB and acid adapted pathogens were grown in TSB supplemented with 1% glucose (TSBG).

	D-value ± SD (min) at temperatures*							
	56°C		58°C		60°C		*z*-value (°C)	
STEC	Adaptation							
Strain	Non	Acid	Non	Acid	Non	Acid	Non	Acid
O157:H7 (F4546)	2.72±0.07Aa	2.81±0.06Aa	1.28±0.07Aa	1.35±0.05Aa	0.52±0.05Aa	0.61±0.06Ba	5.6±0.10	6.0±0.05
O157:H7 (MDD338)	1.93±0.07Ab	3.21±0.08Bb	1.04±0.12Ab	1.31±0.04Bb	0.52±0.13Aa	0.61±0.08Ba	7.0±0.07	5.5±0.09
O111 (MDD339)	3.05±0.06Ac	3.41±0.06Bc	1.39±0.04Ac	1.60±0.05Bc	0.54±0.05Aa	0.61±0.05Ba	5.3±0.11	5.4±0.14

*Capital letters in rows indicate significant difference in *D*-values between acid adapted and non-adapted cells within each temperature and strain. Lower case letters, within columns, indicate significant difference in *D*-values ($P < 0.05$)

Table 4-6. *D* and *z*-values of *Salmonella* serotypes from linear regression equations. Non adapted pathogens were grown in TSB and acid adapted pathogens were grown in TSB supplemented with 1% glucose (TSBG).

	D-value ± SD (min) at temperatures*							
	55°C		58°C		60°C		*z*-value (°C)	
	Adaptation							
Salmonella Serotype	Non	Acid	Non	Acid	Non	Acid	Non	Acid
Typhimirium (ATCC 14028)	0.98±0.09Aa	1.03±0.08Aa	0.30±0.10Aa	0.28±0.10Aa	0.17±0.09Aa	0.17±0.09Aa	6.5±0.17	6.3±0.28
Gaminara (CDC H0622)	0.80±0.10Ab	0.89±0.06Ab	0.33±0.09Ab	0.32±0.05Ab	0.14±0.08Ab	0.15±0.08Ab	7.1±0.10	7.5±0.10
Muenchen (MDD30)	0.80±0.12Ab	0.89±0.09Ab	0.36±0.07Ab	0.36±0.09Ab	0.17±0.09Aa	0.20±0.08Ba	6.7±0.33	6.5±0.21

*Capital letters in rows indicate significant difference in *D*-values between acid adapted and non-adapted cells within each temperature and serotype. Lower case letters, within columns, indicate significant difference in *D*-values ($P < 0.05$)

Table 4-7. *D* and *z*-values of *L. monocytogenes* strains from linear regression equations. Non adapted pathogens were grown in BHI and acid adapted pathogens were grown in BHI supplemented with 1% glucose (BHIG).

Listeria Strain	*D*-value ± SD (min) at temperatures*						*z*-value (°C)	
	56°C		58°C		60°C			
	Adaptation							
	Non	Acid	Non	Acid	Non	Acid	Non	Acid
LCDC 81-861	1.59±0.07Aa	1.34±0.08Ba	0.91±0.08Aa	0.92±0.09Aa	0.55±0.06Aa	0.48±0.14Aa	8.7±0.06	9.0±0.31
Scott A	1.82±0.05Ab	1.73±0.06Ab	1.05±0.07Ab	0.99±0.06Aa	0.65±0.06Ab	0.62±0.07Ab	8.9±0.10	9.0±0.10
V7	1.65±0.07Ac	1.47±0.10Ac	0.90±0.09Aa	0.85±0.10Aa	0.52±0.05Aa	0.49±0.08Ac	8.0±0.06	8.4±0.01

*Capital letters in rows indicate significant difference in *D*-values between acid adapted and non-adapted cells within each temperature and strain. Lower case letters, within columns, indicate significant difference in *D*-values ($P < 0.05$)

Table 4-8. Average *z*-values of STEC and *L. monocytogenes* strains and *Salmonella* serotypes. . Non adapted STEC strains and *Salmonella* serotypes were grown in TSB and acid adapted STEC strains and *Salmonella* were grown in TSB supplemented with 1% glucose (TSBG). Non adapted *L. monocytogenes* strains were grown in BHI and acid adapted *L. monocytogenes* strains were grown in BHI supplemented with 1% glucose (BHIG)

Species	Adaptation	Average *z*-values (°C)
STEC	Non	6.0a
	Acid	5.6a
Salmonella	Non	6.8a
	Acid	6.8a
L. monocytogenes	Non	8.5b
	Acid	8.8b

aWithin the same type of adaptation, *z*-values with different letter in the same column are significantly different ($P < 0.05$)

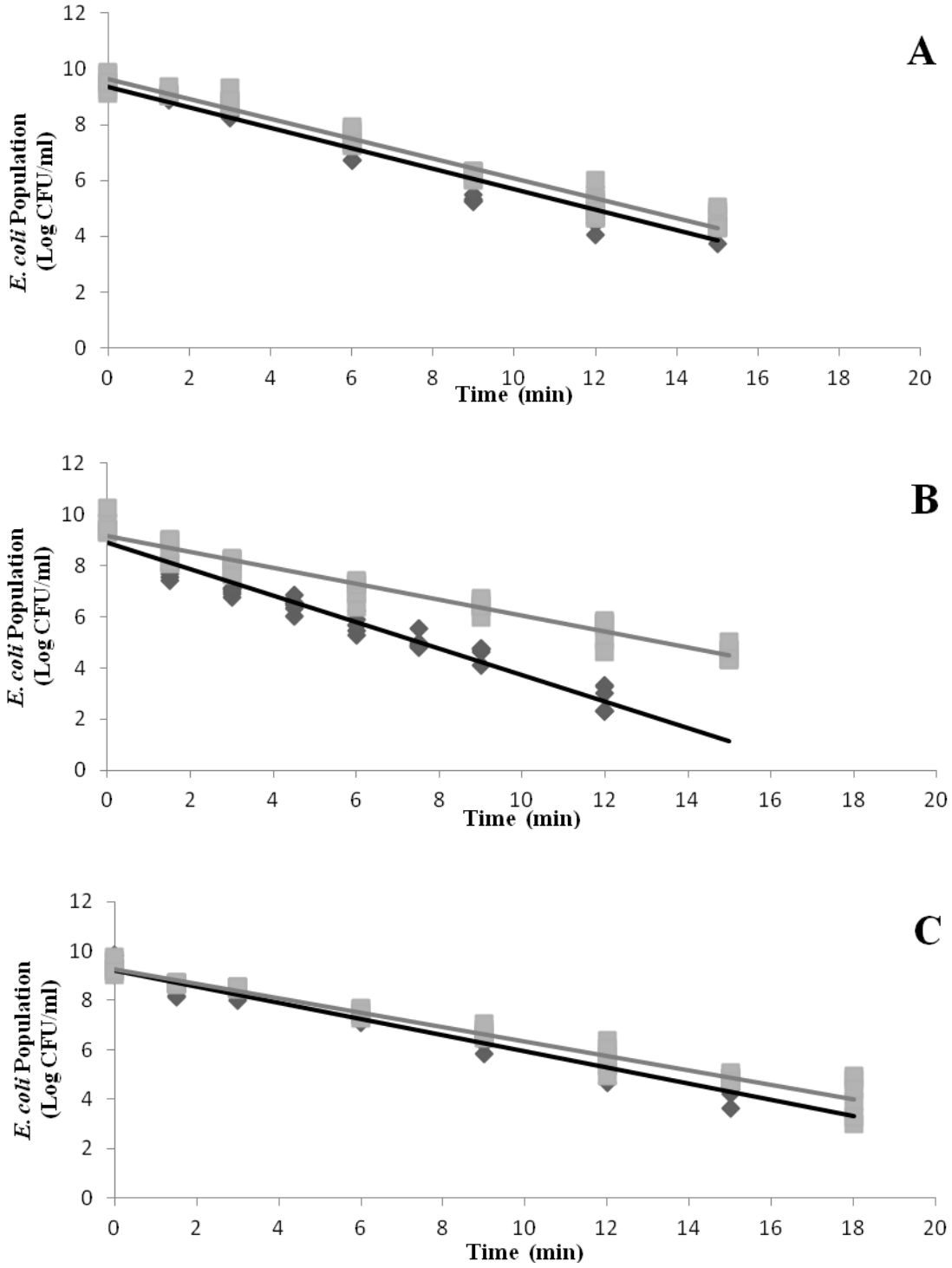

Figure 4-1. Linear regression lines of STEC strain at 56°C to estimate *D*-value. A) *E. coli* O157:H7 from human feces-sprout outbreak (F4546), B) *E. coli* O157:H7 from apple juice outbreak Massachusetts (MDD338), C) *E. coli* from apple cider outbreak New York O111 (MDD339). (■) Acid adapted, (♦) Non-adapted (n=6).

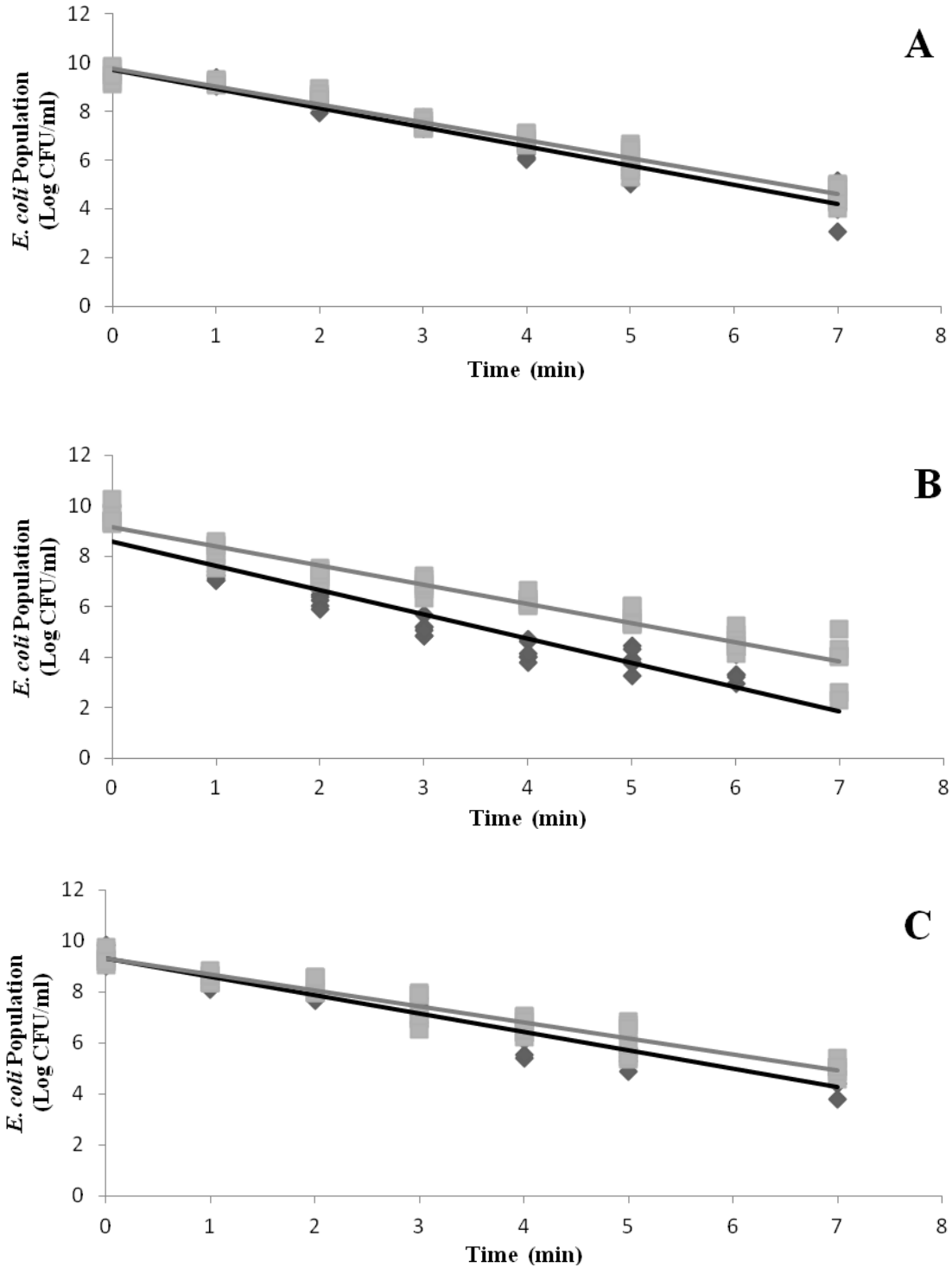

Figure 4-2. Linear regression lines of STEC strains at 58°C to estimate *D*-value. A) *E. coli* O157:H7 from human feces-sprout outbreak (F4546), B) *E. coli* O157:H7 from apple juice outbreak Massachusetts (MDD338), C) *E. coli* from apple cider outbreak New York O111 (MDD339). (■) Acid adapted, (♦) Non-adapted (n=6).

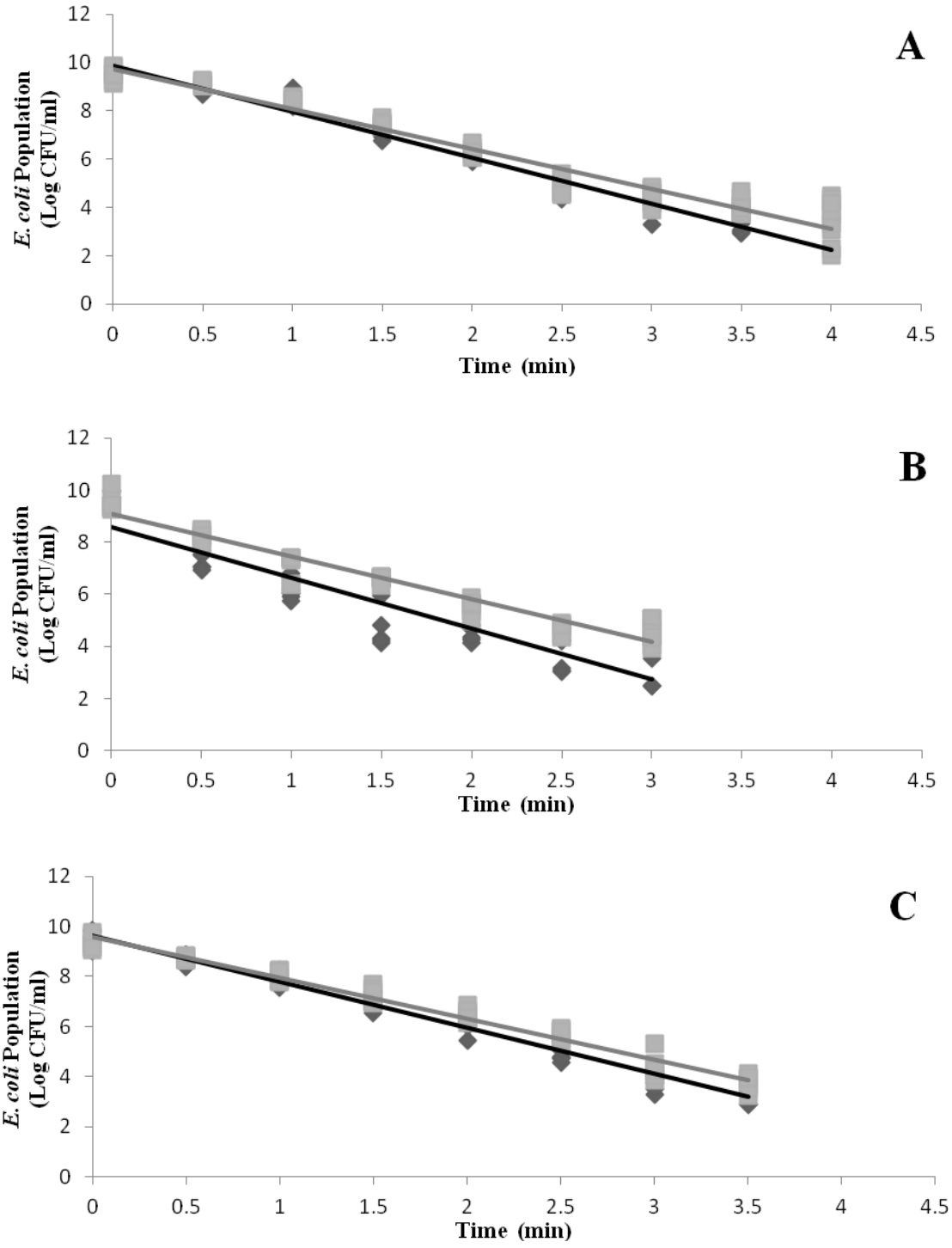

Figure 4-3. Linear regression lines of STEC strains at 60°C to estimate *D*-value. A) *E. coli* O157:H7 from human feces-sprout outbreak (F4546), B) *E. coli* O157:H7 from apple juice outbreak Massachusetts (MDD338), C) *E. coli* from apple cider outbreak New York O111 (MDD339). (■) Acid adapted, (♦) Non-adapted (n=6).

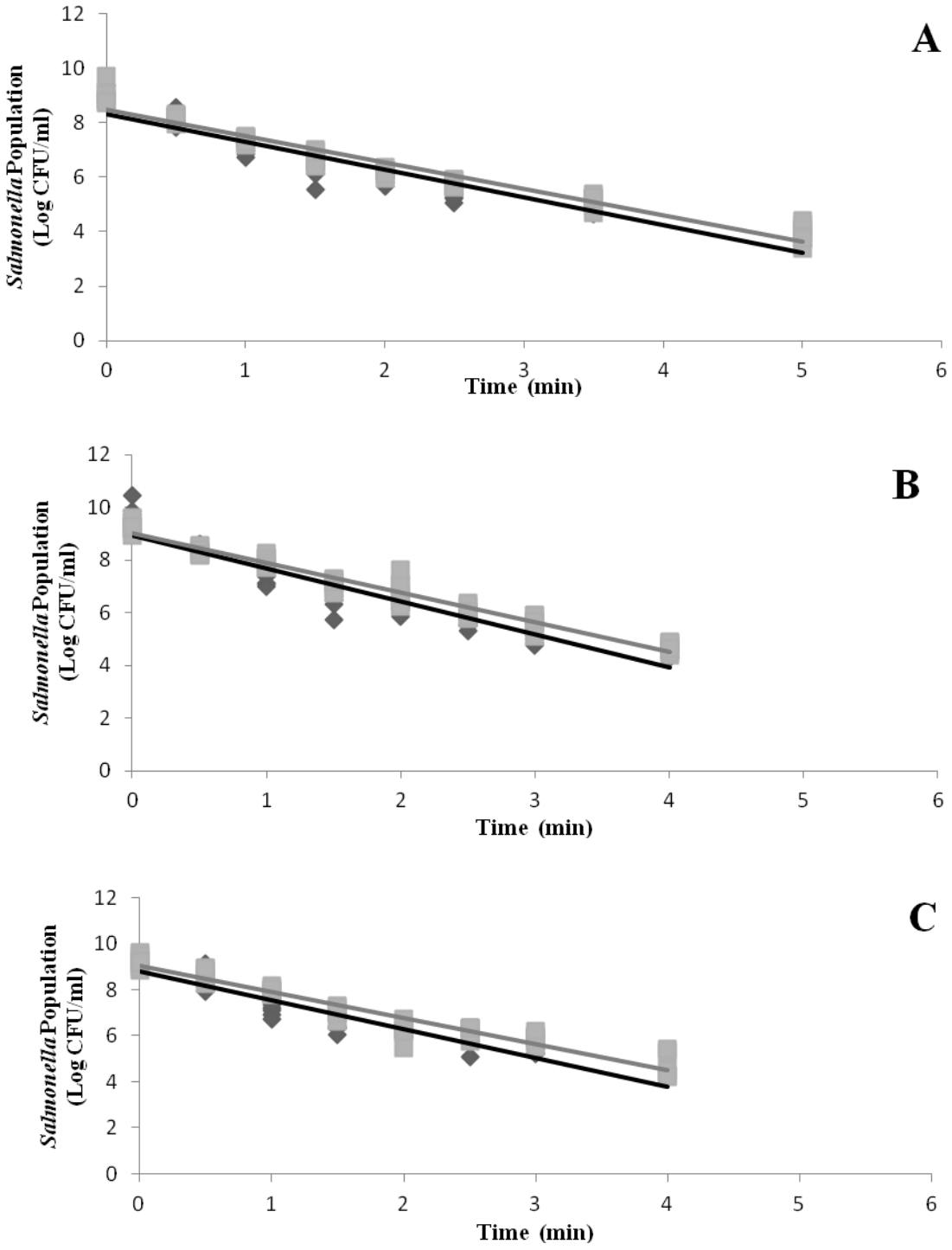

Figure 4-4. Linear regression lines of *Salmonella* serotypes at 55°C to estimate *D*-value. A) *S.* Typhimurium from orange juice outbreak (ATCC 14028), B) *S.* Gaminara from orange juice outbreak (CDC H0662), C) *S.* Muenchen from orange juice outbreak (MDD30). (■) Acid adapted, (♦) Non-adapted (n=6).

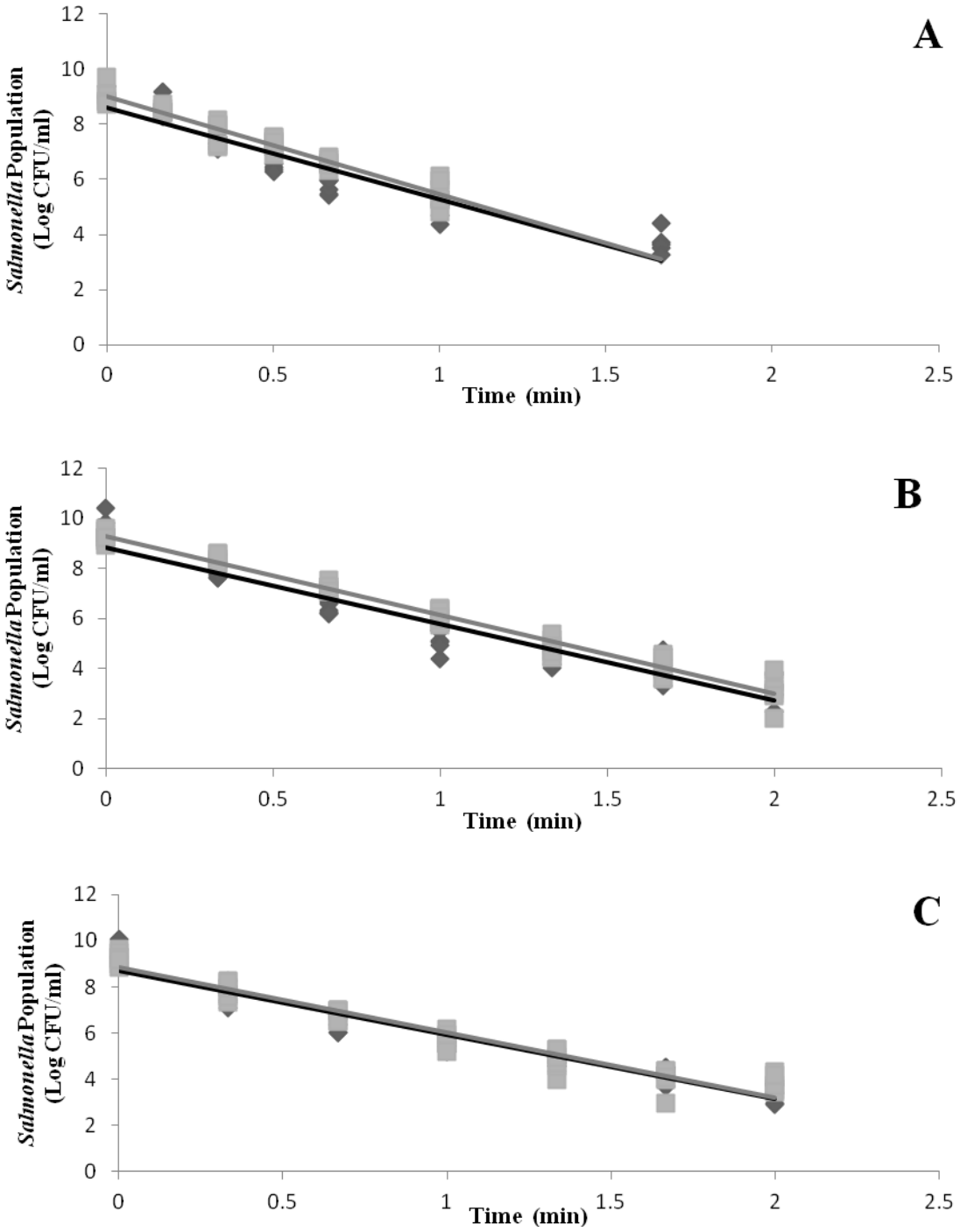

Figure 4-5. Linear regression lines of *Salmonella* serotypes at 58°C to estimate *D*-value. A) *S.* Typhimurium from orange juice outbreak (ATCC 14028), B) *S.* Gaminara from orange juice outbreak (CDC H0662), C) *S.* Muenchen from orange juice outbreak (MDD30). (■) Acid adapted, (♦) Non-adapted (n=6).

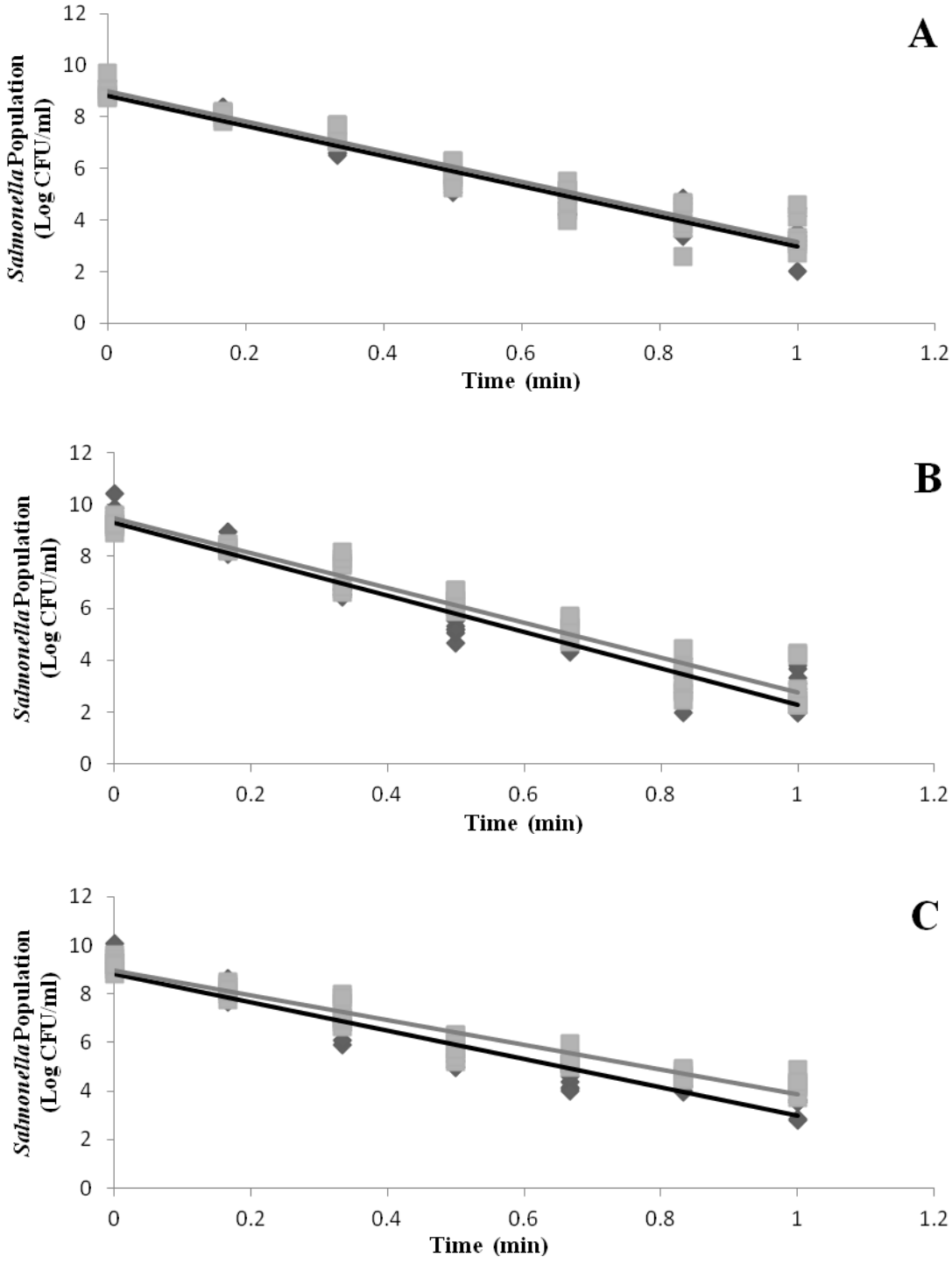

Figure 4-6. Linear regression lines of *Salmonella* serotypes at 60°C to estimate *D*-value. A) *S.* Typhimurium from orange juice outbreak (ATCC 14028), B) *S.* Gaminara from orange juice outbreak (CDC H0662), C) *S.* Muenchen from orange juice outbreak (MDD30). (■) Acid adapted, (♦) Non-adapted (n=6).

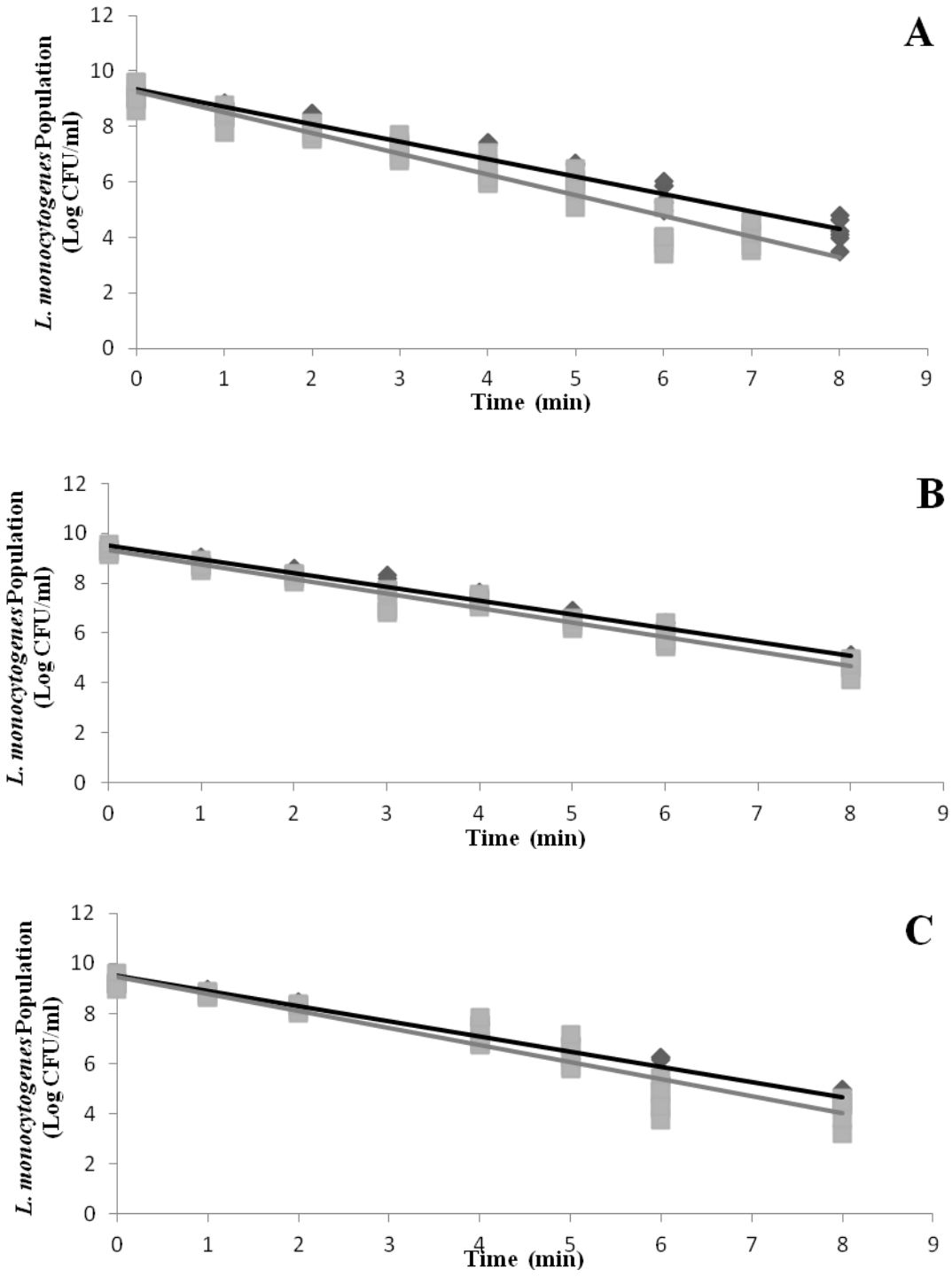

Figure 4-7. Linear regression lines of *L. monocytogenes* strains at 56°C to estimate *D*-value. A) *L. monocytogenes* from raw cabbage outbreak (LCDC 81-861), B) *L. monocytogenes* from human milk outbreak (Scott A), C) *L. monocytogenes* from milk associated outbreak (v7). (■) Acid adapted, (♦) Non-adapted (n=6).

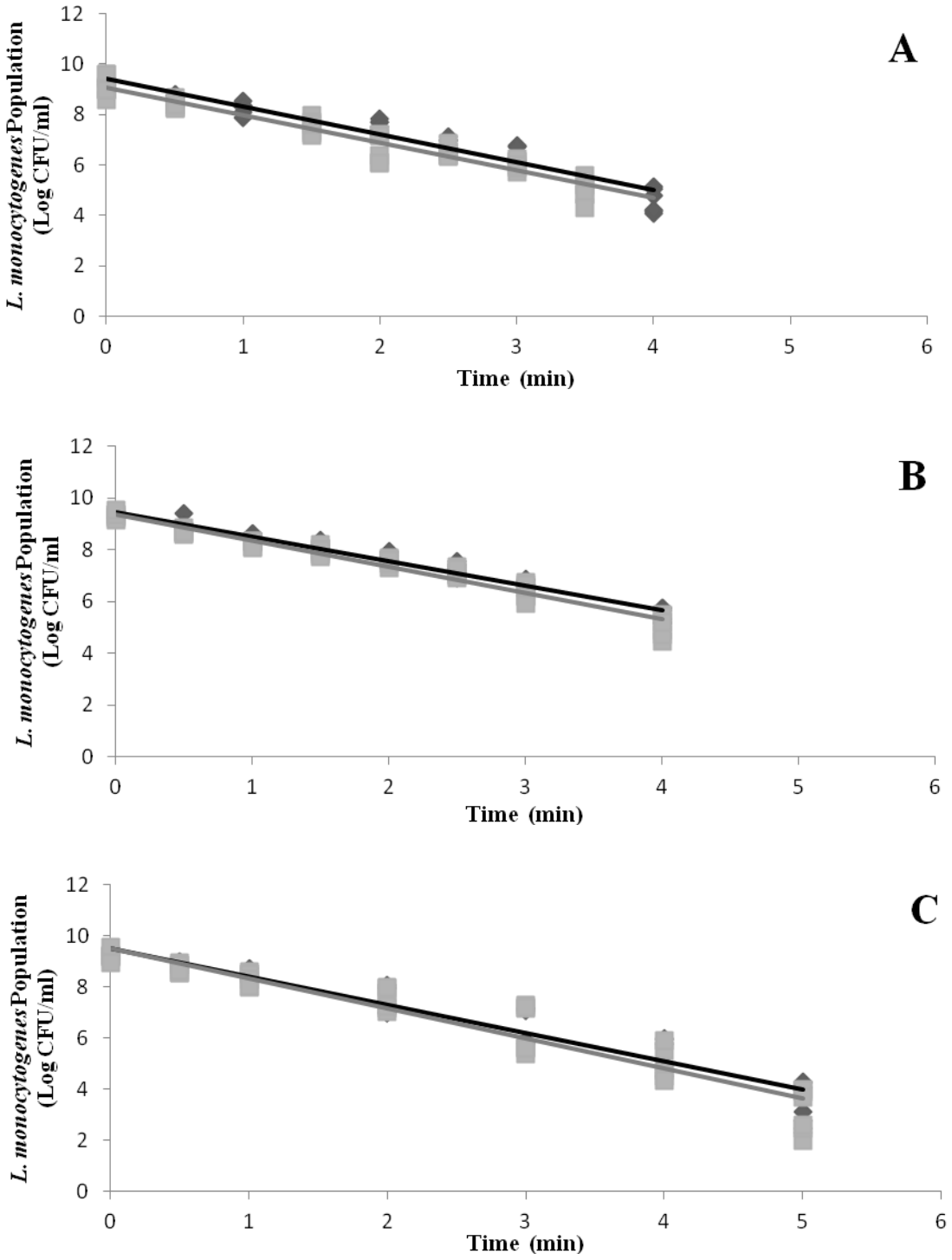

Figure 4-8. Linear regression lines of *L. monocytogenes* strains at 58°C to estimate *D*-value. A) *L. monocytogenes* from raw cabbage outbreak (LCDC 81-861), B) *L. monocytogenes* from human milk outbreak (Scott A), C) *L. monocytogenes* from milk associated outbreak (v7). (■) Acid adapted, (♦) Non-adapted (n=6).

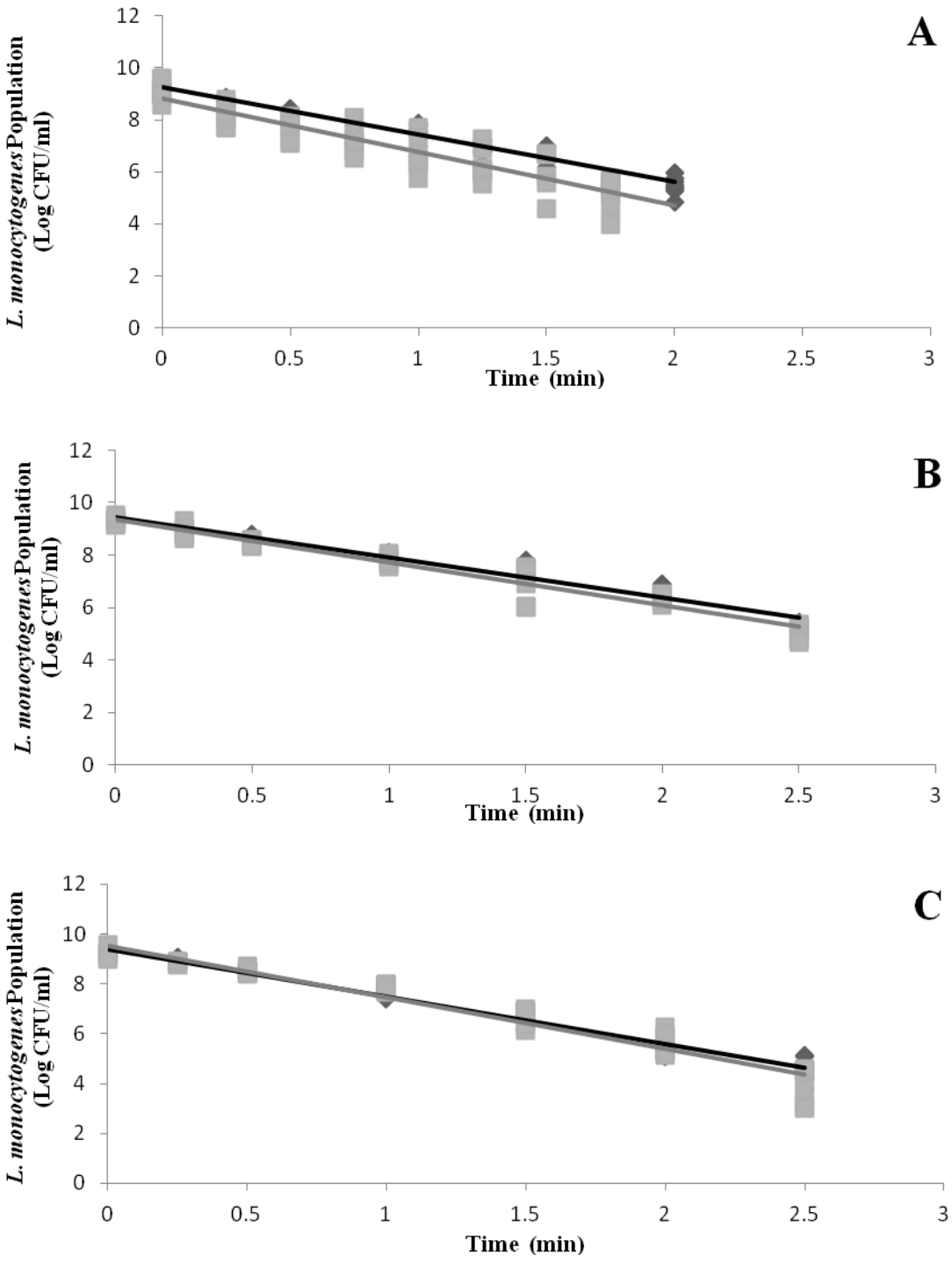

Figure 4-9. Linear regression lines of *L. monocytogenes* strains at 60°C to estimate *D*-value. A) *L. monocytogenes* from raw cabbage outbreak (LCDC 81-861), B) *L. monocytogenes* from human milk outbreak (Scott A), C) *L. monocytogenes* from milk associated outbreak (v7). (■) Acid adapted, (♦) Non-adapted (n=6).

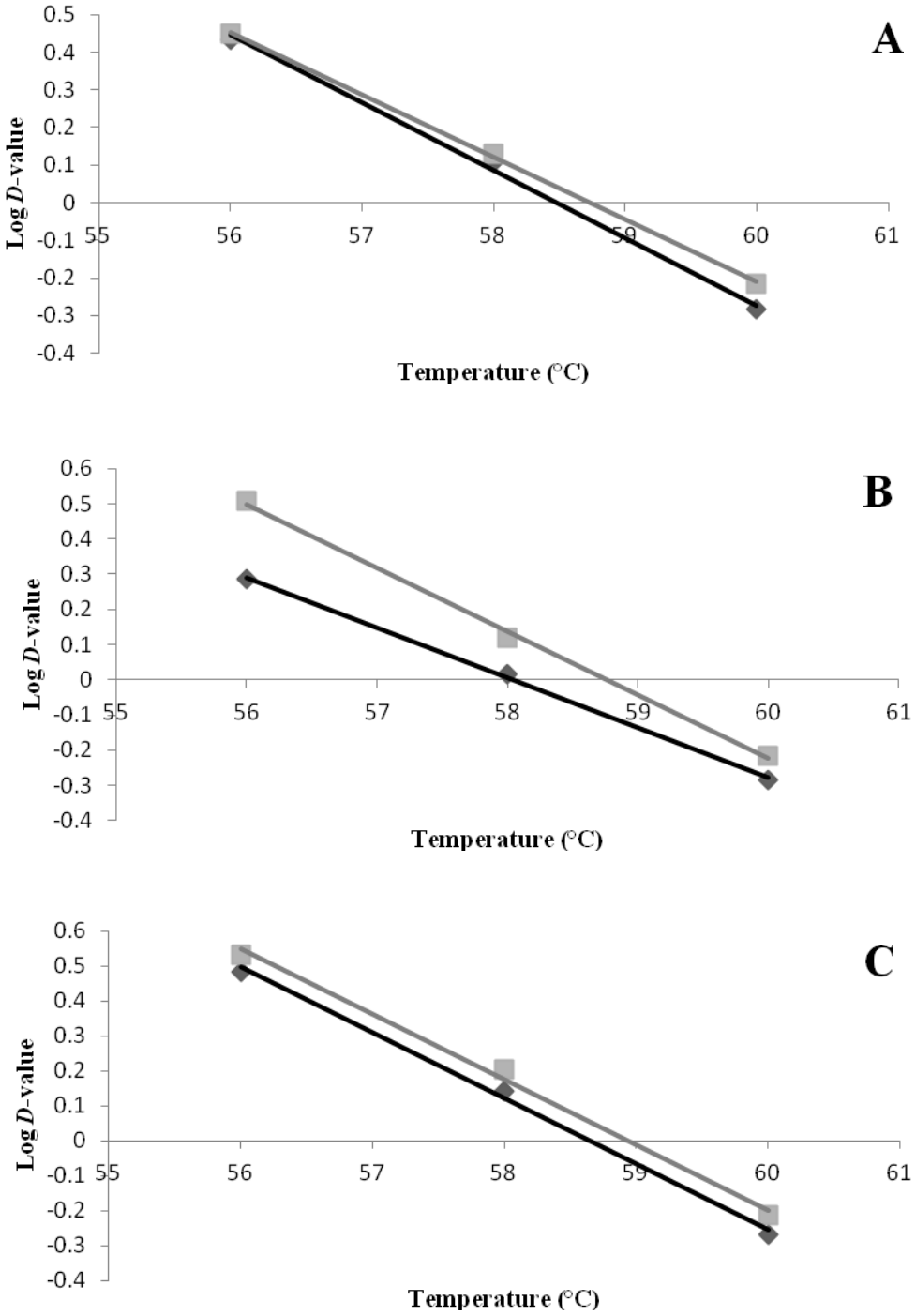

Figure 4-10. Linear regression lines of STEC strains to estimate *z*-value. A) *E. coli* O157:H7 from human feces-sprout outbreak (F4546), B) *E. coli* O157:H7 from apple juice outbreak Massachusetts (MDD338), C) *E. coli* from apple cider outbreak New York O111 (MDD339). (■) Acid adapted, (♦) Non-adapted.

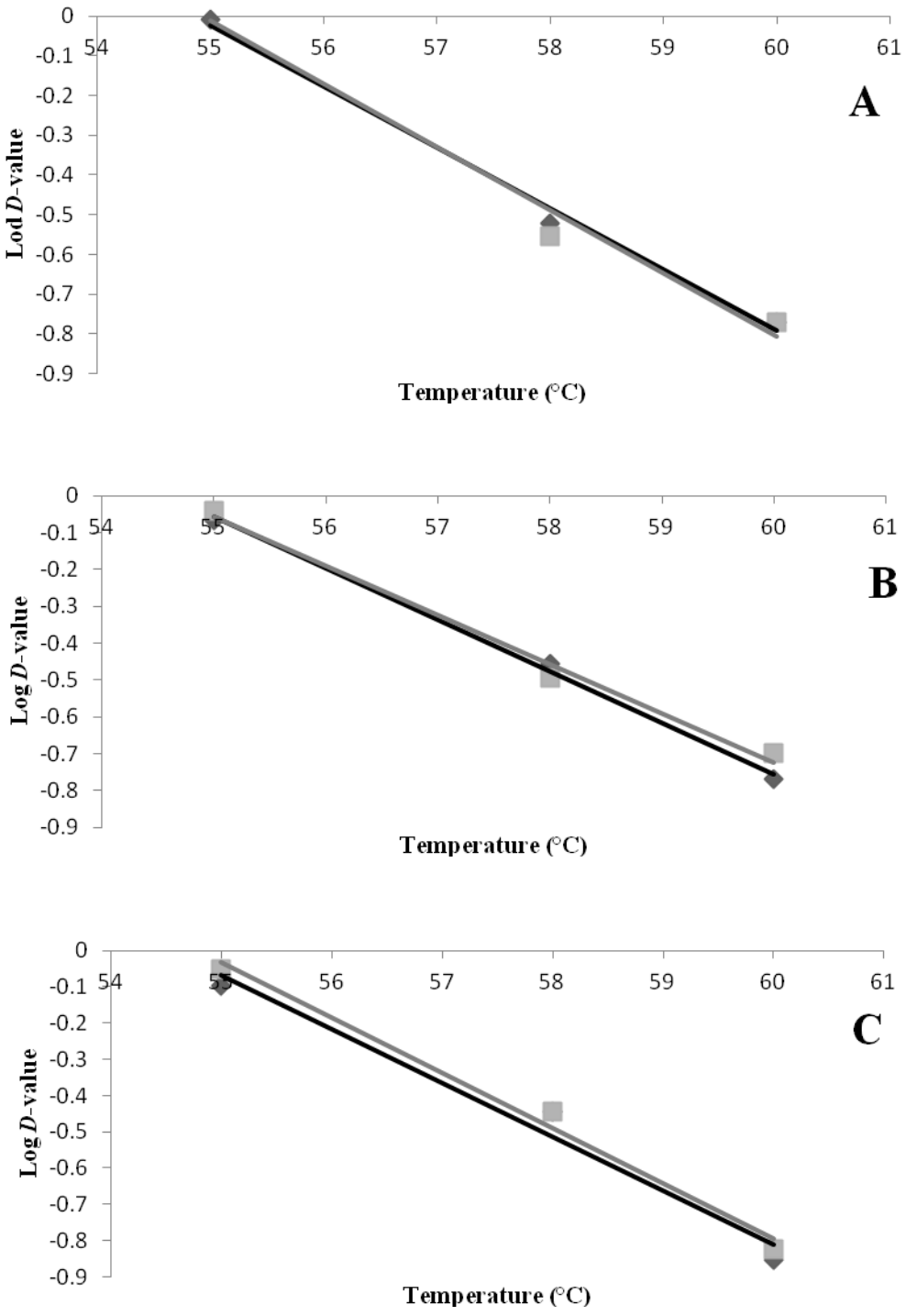

Figure 4-11. Linear regression lines of *Salmonella* serotypes to estimate *z*-value. A) *S.* Typhimurium from orange juice outbreak (ATCC 14028), B) *S.* Gaminara from orange juice outbreak (CDC H0662), C) *S.* Muenchen from orange juice outbreak (MDD30). (■) Acid adapted, (♦) Non-adapted.

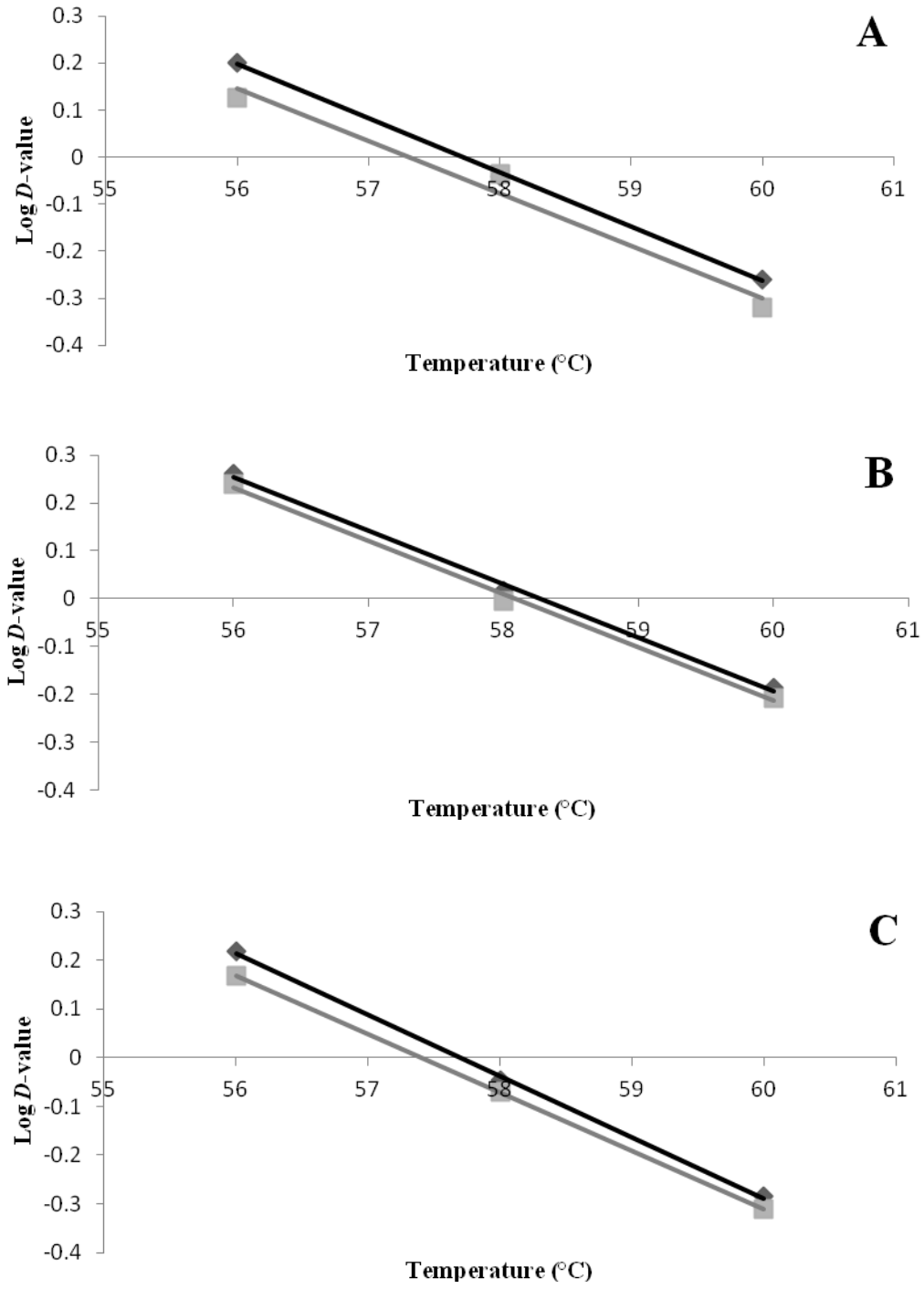

Figure 4-12. Linear regression lines of *L. monocytogenes* strains estimate *z*-value. A) *L. monocytogenes* from raw cabbage outbreak (LCDC 81-861), B) *L. monocytogenes* from human milk outbreak (Scott A), C) *L. monocytogenes* from milk associated outbreak (v7). (■) Acid adapted, (♦) Non-adapted.

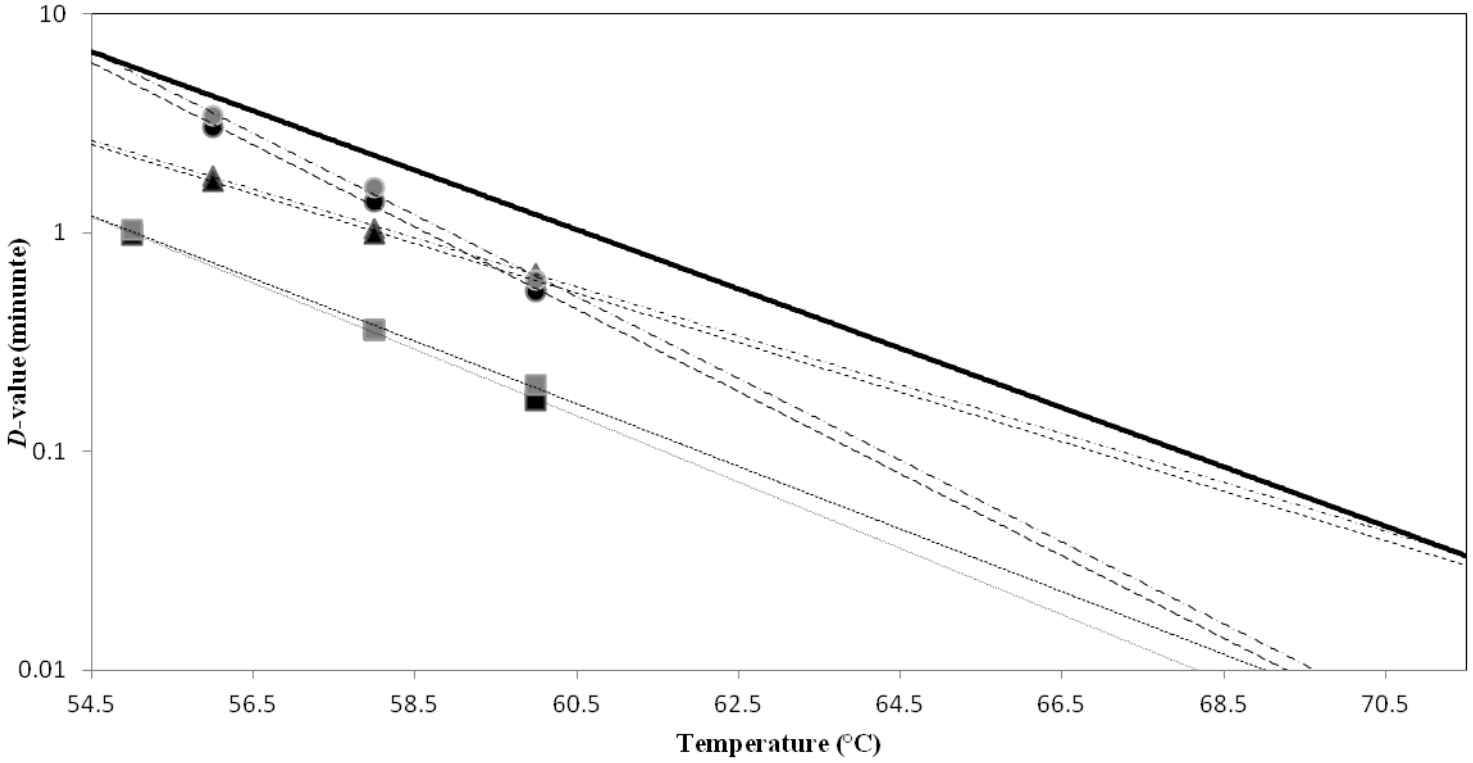

Figure 4-13. Semilogaritmic plot of the highest *D*-values by organism versus temperature of non-acid adapted (−●−) and acid adapted (-●-) STEC strains;non-acid adapted (--▲--) and adapted (··▲··) *L. monocytogenes* strains; and non-acid adapted (···■···) and adapted (···■···) *Salmonella* serotypes. The highest *D*-values obtained for each pathogen were plotted to a linear curve to calculate a minimum process at 71.1°C. A thick solid line was drawn above all the pathogen regression lines to calculate an overall process for all strains. The equation of thick line y = 164,697,794.0085e-0.3122x (R^2 = 1.00).

CHAPTER 5
DISCUSSION

The microbial safety of fruit juices can be ensured by pasteurization treatment with proper time and temperature parameters to destroy sufficient levels of pathogens. Current juice pasteurization parameters describe "pertinent microorganisms" or the most resistant microorganism of public health significance that is likely to occur in the juice (FDA, 2001). Fruit juice processors target 5-log reduction of pertinent microorganisms during pasteurization, as described in the Juice HACCP rule (FDA, 2001). In identification of the pertinent microorganism, outbreaks of pathogen associated with different types of juice have been considered. FDA (2001) states that *Salmonella* species are implicated with outbreaks related to orange juice, and are considered the "pertinent microorganism" in orange juice, while both *Escherichia coli* O157:H7 and *Cryptosporidium* are considered "pertinent microorganisms" in apple juice. Recently, when orange juice moves between facilities, and is required under juice HACCP to be repasteurized, *Listeria monocytogenes* has been suggested as a reasonable pertinent microorganism due to its ubiquitous nature. Both *E. coli* and *L. monocytogenes* survive for extended periods of time in orange juice to be able to cause outbreaks (Piotrowski, 2003; Parish and Higgins, 1989; Oyarzabal et al., 2006). Although not present natural flora of fresh juice, it is believed that *E. coli* can be introduced to juice as a consequence of fecal contamination from reservoirs including cattle, wild birds, deer rodents, goat, sheep, cats, dogs. (Nielsen et al., 2004). The ubiquitous nature of *L. monocytogenes* makes it a potential risk for all juice products. To set conservative pasteurization parameters, thermal death of *Salmonella*, *E. coli* and *L. monocytogenes* should be considered.

Acid adaptation is generally believed to enhance the thermal tolerance of pathogens in fruit juices, and has been demonstrated for *E. coli* O157:H7, *Salmonella*, and *L. monocytogenes* in

apple, orange and white grape juices (Mazotta, 2001), and *E. coli* O157:H7 at 52°C in orange juice and apple cider (Ryu and Beuchat, 1998). Similar to previous studies on strain cocktails (Mazotta, 2001; Ryu and Beuchat, 1998), acid adaptation significantly increased the heat resistance of STEC strains MDD338 and MDD339 at all temperatures tested. However, the thermal resistance of STEC strain F4546 was not significantly increased by acid adaptation at 56 and 58ºC, indicating that there is strain to strain variability in the protective effects of acid adaptation and that acid adaptation may not increase thermal tolerance of all STEC strains. In this study, only small, insignificant ($P > 0.05$), increases in thermal tolerance of *Salmonella* resulted from acid adaptation, unlike those previously reported in the literature with the exception of *S.* Typhimurium at 55ºC (Mazotta, 2001). In a recent study where the authors used individual *Salmonella* serotypes, non-adapted *S.* Newport and *S.* Saintpaul had higher thermal tolerance than acid adapted cells at 56°C in mango and pineapple juice (Yang et al., 2012). Methodology differences, serotype variability and use of cocktails are likely reasons for different thermal tolerance results among the studies. *L. monocytogenes* strains had very different heat resistance trend following acid adaptation when compared to STEC strains and *Salmonella* serotypes. Acid adaptation decreased, but not statistically significantly ($P > 0.05$), the heat resistance of *L. monocytogenes* strains in this study at all temperatures tested, while Mazotta (2001) reports that acid adaptation led to higher *L. monocytogenes* heat resistance in orange juice. Only, acid adapted *L. monocytogenes* strain LCDC 81-861 has higher *D*-values than non-adapted cells at 58ºC. Similar to this study, Sharma et al. (2005) reported that acid adapted *E. coli* strains and *Salmonella* serotypes are more heat resistant, but did not see the same for *L. monocytogenes* in watermelon and cantaloupe juice. The differences between the acid adaptation responses of *L. monocytogenes* among the studies may be explained by the use of different

strains and procedure differences for adaptation. Overall, the most resistant form of bacteria should be considered as reference in determination of pasteurization parameters.

The *D*-values reported here are lower than those reported in Mazotta (2001) at all temperatures, for all species, with the exception of *L. monocytogenes* at 60ºC. *Salmonella* were the most heat sensitive pathogens at all temperatures tested similar to reported in previous studies (Mak et al., 2001, Mazotta, 2001, Sharma et al., 2005). Here, *E. coli* O111 was the most heat resistant pathogen at 56 and 58ºC, while *L. monocytogenes* strain from human milk outbreak had the highest *D*-value at 60ºC. Mazotta (2001) also finds that *L. monocytogenes* is more heat resistant than *E. coli* at higher temperatures, particularly after 65ºC. Enache et al. (2011) report *D*-values varied among different *E. coli* serogroups O26, O45, O103, O111, O121, O145, and O157 in apple juice, and that *E. coli* O157:H7 was the most heat resistant. The *D*-values reported here for *E. coli* O157:H7 were also lower than those reported by Enache et al., (2011), however *D*-values for strain O111 were similar for both non-adapted and acid adapted cells. Unlike apple juice, our results indicate that *E. coli* O111 was more heat resistant than *E. coli* O157:H7 in orange juice at all temperatures tested. We also observed that *D*-values, for all strains in a single species, varied significantly at lower temperatures, while increasing the temperature reduced the *D*-value variability among the strains.

Amongst the three strains/serotypes of STEC, *Salmonella* and *L. monocytogenes* we have tested in orange juice, STEC was the most heat resistant pathogen. However, non-adapted *L. monocytogenes* had a higher *D*-value at 60°C, and the highest *z*-value among all pathogens evaluated, indicating that *L. monocytogenes* have higher thermal tolerance as temperature increases. This result confirms work by Mazotta (2001) where *L. monocytogenes* has higher thermal tolerance than *E. coli* O157:H7 at typical juice-processing temperatures. Lower *D*-value

differences for *L. monocytogenes* strains at tested temperatures resulted in high *z*-values compare to *z*-values reported in Mazotta's (2001) study. Also, higher *z*-values determined in this study indicated that larger changes in temperature were required to reduce the treatment time. As *L. monocytogenes* can survive at 4°C up to 61 days (Piotrowski, 2003), is ubiquitous, and more heat resistance than *E. coli* O157:H7 at higher processing temperatures (Mazotta, 2001), processors should consider *L. monocytogenes* in orange juice pasteurization.

Semi logarithmic plot of the highest *D*-values versus temperature for each pathogen was plotted as described by Mazotta (2001) to determine orange juice pasteurization parameters. The highest *D*-values obtained for both non-adapted and acid adapted cells of each pathogen were plotted to obtain a linear curve to calculate a minimum process at 71.1°C. To determine process parameters that would provide the thermal inactivation of all three pathogens, a regression line covering all regression lines for all three pathogens was drawn above all regression lines for strains and serotypes with the highest *D*-values (Figure 4-13). The equation of this line (log D = 8.2 – 0.14*T* (°C)) was used to calculate a general process for orange juice at 71.1°C, indicating that achievement of 5 log reductions of all three pathogens in orange juice requires 5.29 seconds at 71.1°C with, a *z*-value of 7.1°C. Mazotta (2001) indicates that process of 3 s at 71.1°C is sufficient for fruit juice pasteurization, 2.3 seconds less than our calculated value. In New York, the recommended pasteurization parameters of apple cider are already 6 s at 71.1ºC for most varieties of apples (NYSDM, 1998; Mak et al., 2001). The use of cocktail of strains in thermal destruction experiments might be an explanation for the differences between Mazotta (2001) and results reported here; as is our use of the O111 serogroup of *E. coli*. A validation study of apple cider pasteurization where the authors observed that higher log reductions were observed in two *E. coli* O157:H7 strains compare to the other three strains they used in their cocktail demonstrate

the masking effect strain cocktails may have while determining pasteurization parameters (Mak et al., 2001). In this case the authors note that use of the less heat-tolerant strains would not be appropriate for successful pasteurization of apple cider.

Differences in pasteurization parameters defined here and those produced in previous studies may be due to a number of reasons. Mazotta (2001) only used *E. coli* O157:H7 cocktail, and in this case, acid adapted *E. coli* O157:H7 was the most heat resistant pathogen in calculation of the pasteurization parameter. Here, acid adapted *E. coli* O111 was the most heat resistant pathogen, increasing the time to achieve a 5 log reduction at 71.1°C by 2.3 seconds. As only individual strains were used to determine *D*-values of pathogens in our studies, the possible masking affect among the strains and serotypes was eliminated. Finally, significant methodology differences might have affected the results, which may explain the lower *D*-values we report. Although our individual *D*-values were lower than those reported in literature, the calculated juice pasteurization time at the same temperature is higher. This indicates that proportions of the *D*-values at different temperatures within experiments may play more of a role than calculated *D*-value numbers in determination of pasteurization parameters.

No outbreaks associated with pasteurized orange juice have occurred since FDA required 5 log reduction of "pertinent microorganism" as a mandatory step in fruit juice process. Current fruit juice pasteurization parameters recommended by FDA are likely sufficient to produce safe fruit juices, due to the absence of outbreaks associated with juices being produced under functioning HACCP (Vojdani et al., 2008). However, we should consider other factors that directly affect the success of fruit juice pasteurization. *Salmonella* can survive long enough on orange peel surface to be a contamination source; *Salmonella* population on the orange peel surface declined between 1.5 and 3.0 log CFU/orange after 30 days in any set of post harvest

treatment conditions fruit tested (Danyluk et al. 2010). Pao and Davis (2001) indicate that microbial levels detected in fresh juice are 90–99% lower than microorganism presence on fruit surface indicating that most contamination on fruit can be eliminated by commercial citrus juice extraction. It is unlikely possible that fruit juices will be contaminated with more than 5 logs of foodborne pathogen under good manufacturing practices (GMPs) and standard sanitation operating procedures (SSOPs). However, we cannot ignore the possibility of worst case scenario. Valero et al. (2010) states that high initial microbial population level (10^5 to 10^6 CFU/mL) in citrus juice may occur as a consequence of insufficient sanitation, poor hygiene practices, deteriorated fruits, and poor equipment sanitation to increase the potential risks of level of foodborne pathogen in juice.

CHAPTER 6
CONCLUSIONS AND FUTURE WORKS

FDA (2001) recommends the heat treatment of fruit juices for 3 s at 71.1ºC to achieve 5-log reduction of viable microorganisms. Based on Mazotta's study (2001), these parameters are sufficient to achieve 5-log reductions of pertinent bacterial pathogens in different types of fruit juices. In this study, we determined that these parameters may not be adequate to kill 99.999% of the viable pertinent bacterial pathogens in orange juices. Our findings indicate the achievement of successful 5-log pasteurization under the requirement of FDA can be completed with heat treatment for 5.29 s at 71.1ºC. This implies that if the population of viable pathogens in orange juice somehow reaches over 100,000 CFU/mL, an outbreak associated with orange juice may result under current pasteurization parameters. In practice, orange juice processors pasteurize their products over recommended pasteurization parameters to avoid any violation of critical limits and to inactivate pectin methyl esterase; this study is important in validation of pasteurization parameters for orange juice processors.

D-values reported in this study are lower than those previously reported in literature at all temperatures (Mazotta, 2001; Sharma et al., 2005; Enache et al., 2011). This may be a result of methodology differences and the effect of serotype variability in cocktails used by previous researchers. Although *D*-values reported in here are lower than those previously reported, the calculated overall process parameters to achieve a 5-log reduction are higher. We believe that proportions between the *D*-values at temperatures tested are more important than calculated *D*-values when the purpose of the study is to estimate pasteurization parameters to achieve 5-log reductions.

The use of individual strains and serotypes provides us a better understanding of thermal inactivation response differences amongst strains and serotypes. Different strains and serotypes

responded to heat treatment similarly, but development of acid adaptation varied strain to strain even within the same species. We suggest that more strains of *E. coli* and *L. monocytogenes* strains as well as *Salmonella* serotypes should be studied in orange and other juices types.

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BIOGRAPHICAL SKETCH

Zeynal Topalcengiz is a master graduate research assistant seeking for master degree at Citrus Research and Education Center, off-campus experiment station in University of Florida's Food Science and Human Nutrition department. His main project consists of the estimation of thermal death parameters (*D*-value and *z*-value) of acid adapted and non-adapted stationary phase Shiga toxin-producing *Escherichia coli* (STEC), *Salmonella* spp., and *Listeria monocytogenes* in fruit juices. Zeynal was raised in Mersin, Turkey. After he graduated with a bachelor degree in food engineering from the Department of Food Engineering of Inonu University in Turkey, he worked in two main sectors of food industry; dairy, catering. He has been honored with several scholarships, including his current scholarship for master degree by the Republic of Turkey the Ministry of National Education. In the near future, Zeynal will pursue his PhD. in food microbiology, particularly, in the field of food safety