

**Assessment of the Effects of Acid-Adaptation on the Thermal Tolerance of
Listeria monocytogenes and *Salmonella* Heidelberg**

by

Jacob Mitchel Smith

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Approved by

Manpreet Singh, Chair, Associate Professor Department of Poultry Science
Sacit Bilgili, Co-chair, Professor and Extension Scientist, Department of Poultry
Science

Christy Bratcher, Associate Professor, Department of Animal Sciences
Luxin Wang, Assistant Professor, Department of Animal Sciences

ABSTRACT

Foodborne illnesses attributed to major pathogens cause more than 9.4 million episodes, 55,000 hospitalizations and 1,351 deaths each year. Two of the most predominant pathogens contributing to these statistics are *Listeria monocytogenes* and *Salmonella* Heidelberg, largely attributed to Ready-To-Eat foods and raw chicken, respectively. In an attempt to eliminate these illnesses-causing pathogens from their respective foodborne vectors, processors may use combinations of acidic and thermal treatments. While such treatments are effective at reducing the microbial load on foods they may cause these pathogens to develop adaptations, both acid and thermal, and survive subsequent treatments. In this study various experiments were conducted and results demonstrated that while *Salmonella* Heidelberg and *Listeria monocytogenes* survive and grow under acid-adapted conditions their growth patterns are not different from their non-adapted counterparts ($P > 0.05$). Additionally, the reduction of non-adapted and acid-adapted cultures of *Salmonella* Heidelberg and *Listeria monocytogenes*, under both *in vitro* and *in vivo* conditions, are not significantly different ($P > 0.05$). These studies demonstrate that while these pathogens can become acid-adapted they are not able to develop cross-protection to additional stressors that could help them survive better than strains that are not acid-adapted.

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CHAPTER 1: INTRODUCTION

Safety of foods is a concern not only in the United States but also throughout the world. In some areas of the world it is less important because food security, the access to sufficient calories, is a preeminent concern. However, as the economic landscape becomes globalized, the issue of food safety will continue to be one of the issues that will prevail in developed as well as undeveloped societies. Oftentimes the food safety is a concern because of the risk of intentional contamination of foods by those intending harm on others. Events of this nature could have a devastating impact on large and small populations, however, these events are difficult to predict and require strategies that are very theoretical in nature. Contrarily, food safety issues that are more systematically explored in research settings are those that are inherent and unintentional. Interventions such as Hazard Analysis and Critical Control Points (HACCP) seek to systematically reduce, eliminate and destroy inherent foodborne pathogens so that these are minimized and not passed on to humans through the food supply. The potential for developing resistance or tolerance to some sub-inhibitory interventions in the processing plants such as heat and pH can lead to adaptation of pathogens to stressors. This is important because it serves as a means that bacteria could use to survive in the human body and cause illness that could be more severe than if the pathogen were not adapted.

The Centers for Disease Control and Prevention (CDC) ranks both nontyphoidal *Salmonella* and *Listeria monocytogenes* as significant contributors to the

estimated 9.4 million annual episodes of foodborne illnesses in the United States. Although the majority of these illnesses exhibit symptoms that are mild and self-limiting for a few days, some can lead to severe complications when certain sectors of the population acquire them. The elderly, neonates and other immune-compromised individuals are at a much higher risk for developing more severe symptoms that can eventually lead to death. Poultry is the prominent source of foodborne salmonellosis and Ready-To-Eat (RTE) products are the prevailing vehicles for listeriosis. *Salmonella* is an enteric pathogen that resides in the poultry gastrointestinal tract but it can persist in processing environments to further contribute to contaminated food products. Additional foodborne sources of *Listeria* are unpasteurized dairy products with delicatessen meats being the leading sources.

In an attempt to eliminate contamination of *Salmonella* and *Listeria*, processing methods that include thermal and acidic treatments in combination or individually are frequently employed. However exposure to these treatments can lead to the development of adaptation to these stressors, which could potentially lead to the survival of these adapted bacteria against treatments that are part of regulatory guidelines. Acidic treatments are used as processing aids during the immersion chilling step of commercial poultry processing and this is effective because of the extended contact time that can be upwards of 2 hours and it contributes to the overall washing effect on the carcasses which helps reduce the bacterial load. Thermal treatments can be employed throughout processing and are not always completely bactericidal. Using acidic treatments to reduce the bacteria that have survived thermal treatments and vice versa is an excellent practice because it does not rely on one

single treatment to contribute to the overall bacterial reduction. However the concern is that bacteria may survive the one treatment they are exposed to and subsequently be more resistant to subsequent interventions, referred to as bacterial cross-protection. Currently there is no evidence that cross-protection occurs *in vivo* but its documentation *in vitro* prompts its examination with the consideration that it could occur and negatively impact public health.

CHAPTER 2: LITERATURE REVIEW

2.1.1. SALMONELLA AS A FOODBORNE PATHOGEN

As the leading cause of domestically acquired bacterial foodborne illness (Scallan et al., 2011) the importance of *Salmonella* spp. cannot be overlooked as it relates to the food-processing environment. *Salmonella* causes over one million of the laboratory confirmed 3.6 million bacterial foodborne illnesses in the U.S. alone (Scallan et al., 2011). Following *Salmonella* spp. the next leading cause of foodborne illness is *Campylobacter* spp. (Scallan et al., 2011). Both of these pathogens are primarily implicated in poultry-related foodborne outbreaks (Batz et al., 2011) and combined they cause more than half of the laboratory confirmed foodborne illnesses in the U.S. (Scallan et al., 2011). Therefore, all attempts to identify methods to decrease the overall contamination of poultry and poultry related products are warranted from a public health perspective.

Salmonellae belong to the *Enterobacteriaceae* family and are characterized as Gram negative, facultative anaerobes and achieve motility using flagella. These rod-shaped organisms grow well at 37°C and are able to utilize D-glucose as a carbohydrate source which will generate organic acids and gas as a growth byproduct (Li et al., 2013). Droffner and Yamamoto (1992) showed that some strains have the ability to reproduce at temperatures higher than 54°C but others can grow at refrigeration temperatures from 2-4°C (D'Aoust, 1991b). Presently there are over 2,400 serovars recognized as members of the *Salmonella* family (Popoff et al., 2000).

While not all of these serovars are pathogenic there are some that can cause serious illness in humans, mainly predisposed populations such as infants and the elderly. These populations are more susceptible because of their decreased immune function. *Salmonella* spp. can be recurrently isolated from meat and poultry products due to their commensal relationship with these livestock animals.

2.1.2. SOURCES AND TRANSMISSION OF *SALMONELLA* SPP.

While there are many *Salmonella* serovars, the top ten isolates from meat and poultry products are Kentucky, Enteritidis, Montevideo, Typhimurium, Infantis, Dublin, Heidelberg, Anatum, Muenster, and Hadar (USDA-FSIS, 2012a). Similarly, the top isolates of laboratory confirmed human *Salmonella* infections were Enteritidis, Typhimurium, Newport, Javiana, 4,5,12:i:–, Montevideo, Heidelberg, Muernchen, Infatis, and Branderup (CDC, 2013).

In commercial broilers, which are produced for meat yield, salmonellae are commensal residents of the intestinal tract. *Salmonella* spp. contamination of broiler carcasses results from antemortem colonization with no apparent clinical symptoms in livestock animals, which amplifies the problem of antemortem identification and intervention of *Salmonella* (Hargis et al., 2001). Salmonellae can be transferred vertically from the reproductive tract of contaminated parent to their progeny. Furthermore, salmonellae tend to favor the colonization of young chickens when there is less competition from additional microflora (Mead, 2001). This can result in further infection of internal organs, including those that may be intended for human consumption. The colonization of *Salmonella* spp. leads to fecal shedding that can be

a source for further contamination. Although not all poultry are colonized by *Salmonella* spp. they have the potential to become colonized while they are in the grow-out facility (Heyndrick et al., 2002). Horizontal transmission may occur through many different vehicles during live production such as rodents, wild animals and contaminated feed before the slaughterhouse.

Transmission of pathogens readily occurs at grow-out facilities but does not stop there. Although they have been withdrawn from feed before transportation to the slaughterhouse to reduce fecal shedding, poultry are still excreting during the transportation period. During processing and procurement of fresh poultry, steps such as scalding, defeathering, evisceration, removal of inedible parts and chilling are all probable sources of cross-contamination. As *Salmonella* spp. contaminated carcasses come into contact with equipment and other possibly non-contaminated carcasses the transmission has occurred and can keep recurring unless an intervention is implemented.

Salmonellae are an unexpected and unwanted part of the food chain, however, it is oftentimes their cross-contamination that can lead to outbreaks from atypical sources. Such outbreaks typically require a great deal of investigative work that can be frustrating for consumers and investigators alike. Recent outbreaks of *Salmonella* have been attributed to uncharacteristic sources such as infant formula (Rodriguez-Urrego et al., 2010), tomatoes (Gupta et al., 2007), tahini sesame paste (Sivapalasingam et al., 2004), and pasteurized milk (Lecos, 1986). These sources are of a preeminent concern when potentially contaminated with *Salmonella* because

these products are considered ready-to-eat (RTE) foods; so the end consumer may unexpectedly fall victim to salmonellosis.

2.1.3. SYMPTOMS AND CHARACTERISTICS OF SALMONELLOSIS

There are a varying degree of reports regarding the quantitative estimation of *Salmonella* spp. contamination on chilled poultry carcasses. Even though the population is typically as low as 1-30 cells per carcass but there may be as many as 10^4 CFU per 100 g of skin (Tokumaru et al., 1990). These estimations can vary due to sample collection differences along with laboratory sample preparation and isolation methods. Irrespective of the overall contamination of poultry products, the infective dose has been reported to be as low as 0.04 to 0.45 cells per gram from an outbreak of dried paprika (Lehmacher et al., 1995) but as high as 10^5 CFU per mL for studies in which human subjects were fed *Salmonella* spp. contaminated foods (McCullough and Elsele, 1951). The degree of clinical manifestation does depend on the fat content of the food consumed, with lower infectious doses associated with higher fat content foods (D'Aoust, 1989).

The susceptibility of healthy adults to foodborne illnesses is typically much lower than that of the younger and older proportions of the population. A lower degree of gastric acid production in infants and the elderly is one way the colonization and spread of *Salmonella* spp. is facilitated (D'Aoust, 1991a). Other factors such as overall immune functionality and the type of contaminated food consumed also affect the colonization of *salmonellae*.

Human infection by *Salmonella* can lead to typhoid fever, enterocolitis and systemic infections by non-typhoidal *salmonellae* (D'Aoust, 1991a). When

Salmonella contaminated foods are consumed the symptoms can be induced in as few as 8 hours but may take over 72 hours (McCullough and Elsele, 1951). Symptoms such as diarrhea, elevated body temperature, abdominal pain, headache, and an overall feeling of exhaustion are common clinical signs of salmonellosis (D'Aoust, 1991a). Most cases of salmonellosis will be self-limiting and symptoms will fade after several days. Diagnosis is carried out through isolation from a stool or blood sample.

The treatment of salmonellosis is predominantly accomplished through therapeutic antibiotics such as ciprofloxacin, ceftriaxone, and cefotaxime in adults (Li et al., 2013). This can be problematic as there are strains of *Salmonella* spp. that have developed resistance to these antibiotics. If left untreated salmonellosis, can lead to development of reactive arthritis, also known as Reiter's syndrome or inflammatory arthritis (Li et al., 2013).

2.1.4. THERMAL TOLERANCE OF *SALMONELLA* SPP.

As with most other foodborne pathogens there can be a wide range of growth temperatures within the salmonellae. During processing higher temperatures are used to assist in removal of poultry feathers, but the overall goal is to reduce the temperature of carcasses below 4°C within 4 h of slaughter. Elevated temperatures are not typically encountered in the supply chain of fresh meat products, however, refrigeration temperatures are very common. Studies regarding the elevated and reduced growth temperatures and proliferation of salmonellae are important because these pathogens do not exhibit any negative quality effects on contaminated meat products, and can be undetectable to the consumer.

Bacterial stress is often not an obvious evaluation, especially with regard to the many different food matrices that exist. Thermal treatments are commonly utilized in many food-processing situations for reasons that are not limited to microbial food control. However, the use of such treatments can have negative implications with regard to foodborne pathogens. As treatments such as heat are applied, foodborne pathogens can become stressed and injured but may recover to a healthy state while not dying. (Yousef and Courtney, 2002). As microorganisms are exposed to one stress they can develop resistance to another stressor. Mild heat shock of *Salmonella* Typhimurium has shown to induce thermal tolerance (Bunning et al., 1990), while an acid adaptation to pH 5.8 induced thermal resistance at 50°C (Leyer and Johnson, 1993). Acidic treatments are commonly employed as processing aids in meat and poultry processing, therefore introducing the potential to induce bacterial cross protection to other stressors that are employed.

A common method to evaluate thermal stress on bacterial populations is known as the D value, or decimal reduction time. This value correlates to the amount of time at a given temperature to reduce a bacterial population by 90%, or 1-log cycle. The D_{52} values for *Salmonella* Enteritidis in culture broth were higher after heat shock (42°C, 60 minutes) when compared to control cultures, however, D_{58} values were not significantly different among controls and thermally adapted cultures (Xavier and Ingham, 1997). The D_{62} values of acid adapted (pH 4.6) *Salmonella* in ground beef increased significantly from 1.93 to 2.98 after 28 days of refrigerated storage (Singh et al., 2006).

2.1.5. ACID TOLERANCE OF *SALMONELLA* SPP.

There are numerous organic and inorganic acids that are used for bacterial control in the food industry. The salmonellae can also survive and propagate throughout a wide pH range. A neutral pH (approximately 7.0) is most favorable, however, Asplund & Nurmi (1999) and Holley & Proulx (1986) demonstrated that salmonellae are able to grow at pH values as acidic as 3.99 or as alkaline as pH 9.5, respectively. Once the ability to survive in low pH environments is gained *Salmonella* spp. display the acid tolerance response (ATR). This involves changes in protein synthesis (Foster, 1991) and aids in their survival and protection in low pH food products. In eliciting human infection, salmonellae are ingested and encounter a lower pH environment in traveling through the stomach. The potential for their survival in such an extreme environment, even in lower volumes, is greater for ATR bacteria since they have gained mechanisms that allow adaptation to low pH environments.

The acid tolerance at pasteurization temperatures in various fruit juices is greater in acid adapted compared to non-adapted *Salmonella* spp. (Sharma et al., 2005). This is a liquid food and may not translate directly to meat products where the overall food matrix is different. Using inoculated ground chicken patties, Jung et al., (2009) demonstrated that acid-stressed *Salmonella* Typhimurium was not resistant to antimicrobials and showed a loss of cellular integrity and membrane partiality.

2.1.6. FOOD SAFETY GOALS FOR *SALMONELLA* SPP. IN POULTRY MEAT

The Hazard Analysis and Critical Control Point (HACCP) approach is an integral part of managing a successful food safety program in the food processing environment. The HACCP system was implemented in meat and poultry processing establishments beginning in 1996 by the USDA-FSIS. By evaluating processing steps microbiological, chemical and physical hazards that can be reduced or eliminated help establish Critical Control Points (CCP) that are monitored and documented. In poultry processing a multi-hurdle approach is an important factor in HACCP systems and helps to control harmful microorganisms in the final products. These practices can have substantial effects on helping processors successfully achieve USDA-FSIS performance standards for *Salmonella*.

Along with the required implementation of HACCP in 1996, the USDA-FSIS also established a *Salmonella* Performance Standard that established there should be no more than 20% positive recovery rate among chilled broiler carcasses (USDA-FSIS, 2000). A new *Salmonella* Performance Standard was issued in 2009 that established a 7.5% positive rate for freshly chilled broiler carcasses. This translates to no more than 5 positive carcasses allowed in a set of 51 carcasses (USDA-FSIS, 2009).

As chicken meat is processed *Salmonella* contamination can become exacerbated. Specifically, the production of ground chicken products is problematic because salmonellae can be distributed throughout the product due to a higher surface area. The USDA-FSIS reported a 15% positive rate for ground turkey samples taken in calendar year 2013 (USDA-FSIS, 2013), while the performance standard for

ground turkey still stands at 49.9% (USDA-FSIS, 2012b). This positive rate was higher than any other product class that the USDA-FSIS monitors, with the next closest product category being ground chicken at 11% positive. (USDA-FSIS, 2012b). Following two large multistate outbreaks of *Salmonella* Heidelberg and Hadar involving ground products, USDA-FSIS required processors to update and reassess their HACCP plans for overall *Salmonella* contamination of not-ready-to-eat (NRTE) ground poultry products (USDA, 2012c).

The USDA-FSIS also has regulations for cooking times for meat and poultry products, referred to as Appendix A (USDA, 1999). This was first published in 1999 and specifically establishes the cooking times that must be adhered to obtain a 7-log reduction in *Salmonella* for ready to eat poultry and beef products. The cooking times are established based on the fat content of the product and multiple sub-lethal temperatures (Juneja et al., 2001). Although these are regulations that are enforced by the USDA-FSIS new product formulations must be tested by individual processors in order to verify the microbial safety of specific products. Additionally, these times were constructed for pathogens that were grown by traditional means, not for pathogens that have been acid adapted or thermally adapted in any manner. Additional research to address these issues is warranted and should be used to evaluate current food safety recommendations.

2.2.1. LISTERIA SPP. AS A FOODBORNE PATHOGEN

The genus *Listeria* is named in honor of Joseph Lister, a British surgeon who was a pioneer in the area of antiseptics. The isolation of *Listeria* occurred only in the

1920s after unusual deaths in laboratory rabbits (Murray et al., 1926) and wild gerbils (Pirie, 1927) in various settings and was first considered a member of the *Bacillus* genus. Further work classified the taxonomy of the genus as containing six species- *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi* (Collins et al., 1991; Graham et al., 1996). The foodborne bacterial pathogens which cause the most illnesses, hospitalizations and deaths-*Escherichia coli*, *Salmonella*, *Campylobacter*- are characterized as Gram-negative, however, the *Listeria* are Gram-positive (Scallan et al, 2011). An additional difference is the psychotropic abilities of *L. monocytogenes* when compared to other foodborne pathogens, specifically growing well at 1-2°C (Walker et al., 1990).

Centers for Disease Control and Prevention (CDC) data shows that *Listeria monocytogenes* causes approximately 1,591 episodes of foodborne illness each year, with the highest hospitalization rate (94%; Scallan et al., 2011). Furthermore, the death rate is an estimated 15.9% with 255 estimated deaths per year (Scallan et al., 2011). *Listeria* is second in number of deaths caused only to *Salmonella* spp. (378) but *Salmonella* spp. accounts for over 1 million episodes of foodborne illness each year, underscoring the severity of *Listeria* related foodborne outbreaks and its 15.9% death rate compared to the 0.5% rate of *Salmonella*.

2.2.2. SOURCES AND TRANSMISSION OF *LISTERIA* SPP.

As a pathogen that can grow and survive at cold temperatures the identification of the initial sources of *Listeria monocytogenes* contamination can be difficult. In the environment it can survive in soil for up to 182 days if not exposed to

sunlight and up to 12 days if exposed to sunlight (al-Ghazali and Al-Azawi, 1990). However, *L. monocytogenes* is found less frequently than *Listeria* spp. in the natural environment (MacGowan, 1994). Botzler et al. (1974) showed an increase in *L. monocytogenes* over a 154-day study in autoclaved soil at winter temperatures (-15 to 18°C). Additionally, recovery of *L. monocytogenes* did not change when compared to initial inoculation over an 8-week period in soil applied with sewage sludge as fertilization (Watkins and Sleath, 1981). While *Listeria* spp. can survive and grow in soil under various conditions substantial evidence of this as a reservoir of *L. monocytogenes* has not been found (Sauders and Wiedmann, 2007).

The contamination of sewage by *L. monocytogenes* and the subsequent use of such sewage as fertilizer has only been linked to one foodborne outbreak (Schlech et al. 1983). Therefore it is not considered a prominent source of transmission of *L. monocytogenes* to humans. Additionally, *L. monocytogenes* have been reportedly found at varying levels in fresh (Fenlon et al., 1996; Arvanitidou et al., 1997) and salt-water (Colburn et al., 1990) environments, however, these have not been linked to any outbreaks of human-acquired listeriosis. Further studies have isolated *L. monocytogenes* from shrimp in the U.S. (Motes, 1991) and a separate study demonstrated that *Listeria* has the ability to survive commercial shrimp processing (Destro et al., 1996).

Although listeriosis was initially thought to be a sporadic disease the majority of outbreaks are directly related to ready-to-eat (RTE) food products (Farber and Peterkin, 1991). *L. monocytogenes* has been isolated from poultry processing environments at multiple stages. In Northern Ireland, Lawrence and Gilmour (1995)

reported raw poultry, cooked poultry and the processing environment all contained *L. monocytogenes* and some strains persisted for up to 1 year. In the U.S., Berrang et al. (2002) reported that the same strain of *L. monocytogenes* was recovered from the raw product at some point during a 1-year study, which suggests consistent reintroduction of the pathogen from raw product into the plant environment. Other studies have suggested that environmental sources are the cause of post-processing *L. monocytogenes* contamination of foods (Ojeniyi et al., 2000).

While *L. monocytogenes* is not consistently isolated from raw poultry it is oftentimes recovered from non-food contact surfaces in poultry processing facilities (Senczek et al., 2000) and is able to survive and reproduce under the conditions which fresh poultry is stored (Walker et al., 1990). This further emphasizes the ability of the organism to be introduced by cross contamination of raw products. Although, isolation of *L. monocytogenes* from raw poultry products at retail is uncommon (Buchanan et al., 1989), some studies have reported that it is recoverable at low levels (Soultois et al., 2003). Furthermore, raw chicken is expected to become noticeably spoiled before *L. monocytogenes* would have the chance to be detectable (Wimpfheimer et al., 1990). However, the implications of mishandling food products are still possible and must be considered as precautions must be taken in order to prevent cross contamination.

2.2.3. SYMPTOMS AND CHARACTERISTICS OF LISTERIOSIS

The clinical manifestation of *Listeria* spp. infection is acknowledged as listeriosis in humans. Demographically, listeriosis is predominantly reported in

newborns, women of childbearing age (approximately 20-35) and the elderly (CDC, 2013). These subsets of the population have the most difficulty in overcoming infection, however, it is not limited strictly to these demographics as healthy adults may also fall victim to infection (CDC, 2013).

As the demographic that presents the highest risk to *Listeria* spp. infection, pregnant women can experience neonatal infection, premature delivery, stillbirth or even fetal loss (CDC, 2013). Bacterial cultures are not always obtained during these complicated events therefore, accurate estimations of the overall impact of listeriosis during pregnancy can be tedious (Painter and Slutsker, 2007). Women that are implicated in listeriosis during pregnancy are otherwise healthy and rarely have any pre-existing conditions (Mylonakis et al., 2002), however, pregnant Hispanic women are 24 times more likely than the general population to have listeriosis (CDC, 2013). Sometimes listeriosis will not result in transplacental transmission and may only lead to mild symptoms such as fever, myalgia and diarrhea (Painter and Slutsker, 2007). Mylonakis et al. (2002) reported the predominant use of ampicillin, amoxicillin and aminoglycoside for treatment in pregnant women when data was available.

Among neonates the onset of *L. monocytogenes* infection can be rapid and debilitating. Neonates can acquire listeriosis before or after birth, referred to as early or late onset. In early onset the infant has acquired the infection *in utero* opposed to acquiring listeriosis in which the mode of transmission is not clearly defined (Painter and Slutsker, 2007). Differentiating the two types of onset usually arises from the infant developing pneumonia or meningitis from early and late onset, respectively (CDC, 2013). Late onset infections have been linked to some sources such as a

contaminated mineral oil used to bathe newborns (Schuchat et al., 1993) and some nosocomial transmission (Nelson et al., 1985), but most listeriosis is foodborne so high risk foods are still considered to be a prevailing vehicle.

Among healthy adults listeriosis usually presents as a noninvasive gastrointestinal event with symptoms that usually show up approximately 24 hours after consumption of the contaminated food product (Frye et al., 2002). Such symptoms may last for as long as 3 weeks but usually subside after about 7 days and healthy adults typically do not have to visit a physician for antibiotic treatment (Frye et al., 2002). Additionally, the infective dose to elicit symptoms of listeriosis is reportedly as high as 10^7 to 10^9 for healthy persons and 10^5 to 10^7 for persons considered high risk (Farber et al., 1996).

2.2.4. THERMAL RESISTANCE OF *LISTERIA* SPP.

Listeria monocytogenes grows over a wide range of temperatures, ranging from as low as 0°C to upwards of 45°C (Ryser and Buchanan, 2013). *Listeria* spp. are also very resistant to freezing injury, unlike most other foodborne pathogens (Ryser and Buchanan, 2013), further emphasizing that *Listeria* spp. contamination of food products is a very large problem for the food industry overall. After heat shock for different timings at 45°C and 48°C Lin and Chou (2004) tested the thermal tolerance of various strains of *L. monocytogenes*. It was observed that increased thermal tolerance (55°C for 60 minutes) was achieved after pre-shocking to 45°C for 1 hour, however pre-shocking to 48°C for 10 minutes showed no significant difference over non pre-shocked controls (Lin and Chou, 2004). Skandamis et al. (2008) further

explored thermal tolerance of *L. monocytogenes* and concluded that exposure of *L. monocytogenes* to 46°C for 1 hour provided a protective ability for the organism at 57°C but not at 52°C or 63°C.

Oftentimes there are processing methods that employ heat as a post-packaging anti-*Listeria* treatment for its ease of use and practicality. However, processors have not always relied on heat alone, but have explored the use of GRAS (Generally Recognized As Safe) antimicrobials during this process. Using summer sausages Roering et al. (1998) achieved D-values of 0.28 to 2.08 minutes at 98°C and 165°F, respectively. In a similar study, Bedie et al. (2000) showed that a water bath of 75°C was effective at achieving a greater than 3-log CFU/cm² reduction of *L. monocytogenes* on individually packaged frankfurters. However, when adding another frankfurter to the package only a 1-log CFU/cm² reduction was achieved. A post-packaging pasteurization treatment of 96°C along with the antimicrobial pediocin was used to effectively reduce the survival populations of *L. monocytogenes* while having very little effect on product quality (Chen et al., 2004).

Similarly, steam can be used instead of completely submerging packages in hot water. Referred to as a flash-heating process, one study combined it with vacuum pressure, to demonstrate the ability to obtain a 4-log CFU/cm² reduction of *L. innocua* (Cygnarowicz-Provost et al., 1994). Furthermore, this same study demonstrated that *L. innocua* that did survive the thermal treatment grew at a slower rate than the untreated frankfurters (Cygnarowicz-Provost et al., 1994). Although Farber and Brown (1990) were not able to induce increased thermal tolerance in a sausage mixture with cells pre-shocked for 30 or 60 minutes (48°C) they did observe

increased thermo-tolerance when cells were pre-shocked for 120 minutes. Further exploring this type of treatment using multiple cycles of steam with vacuum pressure between each treatment showed up to a 5-log reduction of *L. innocua* (Kozempel et al., 2000).

2.2.5. ACID TOLERANCE OF *LISTERIA* SPP.

There are numerous acids that have shown to be effective at controlling *L. monocytogenes* on RTE and other foods that it commonly contaminates. While acids are effective at reducing the overall population of *L. monocytogenes* subsequent refrigeration of *L. monocytogenes* can favor the survival of those that have survived in food products. Incubation in citric acid at pH 5.4 of *L. monocytogenes* Scott A showed resistance to pH 3.35 but no resistance at pH 4.35, indicating that the cross protective behavior was not consistently induced and the organism was still susceptible to the preservative nisin (Okereke and Thompson, 1996). Van Schaik et al. (1999) showed contradictory results after adapting *L. monocytogenes* to a pH 5.5 in lactic acid, indicating that there was increased resistance to lactic acid at pH 3.5 at 37°C. Furthermore, Konstantinos et al. (2003) showed that after being exposed to a mildly acidic (pH 5.0 to 6.0) environment *L. monocytogenes* could survive subsequent acid treatments while those that were not exposed to the acid did not. However this was not a firm rule as the adaptive response elicited depended on the temperature and duration of treatment (Konstantinos et al., 2003).

Aside from the pure culture studies that have shown mixed results, *L. monocytogenes* survived well in cottage cheese for over one month at pH 5.0 when

stored at 5°C (Piccinin and Shelef, 1995). After testing the survival of acid-adapted and non-adapted *L. monocytogenes*, Gahan et al. (1996) showed that the acid-adapted cells survived more favorably in refrigerated (4°C) cottage cheese (pH 4.7), cheddar cheese (pH 5.2), yogurt (pH 3.9), salad dressing (pH 3.0), and orange juice (pH 3.8). However, the same experiment showed that the acid-adapted *L. monocytogenes* cells were only recoverable from cottage cheese until 15 days and cheddar cheese until 70 days post inoculation. The other food products studied showed survival for only 7 hours after inoculation, indicating that the survival is highly dependent on the nutrients and unique characteristics of each food product (Gahan et al., 1996).

Irrespective of the environment that bacteria encounter, their survival depends on their ability to maintain their internal homeostatic pH. While *L. monocytogenes* can regulate its internal pH, the addition of excess H⁺ ions from lactic and more significantly acetic acid disrupts enzymatic activity such that proteins become denatured (Young and Foegeding, 1993), suggesting that conditions of reduced pH can be bacteriostatic and bactericidal to *L. monocytogenes*. Furthermore, survival of *L. monocytogenes* at conditions with elevated free glutamate has been reported to be favorable in simulated gastric conditions (Cotter et al., 2001a). Exploring the issue further, Cotter et al. (2001b) found that foods with more than 0.22 mM free glutamate such as mayonnaise and apple, orange and tomato juices favored survival of *L. monocytogenes* opposed to those with lower glutamate contents. Cotter et al. (2001b) suggested that the glutamate was preventing acidification of the cytoplasm by acting as a source of protons within the cell.

2.2.6 FOOD SAFETY GOALS FOR *LISTERIA* SPP.

As the number of foodborne illness outbreaks is more closely monitored and quantified the direction to food industry guidance regarding contamination of foods with pathogenic bacteria becomes more definitive. As more outbreaks point to RTE foods (FDA, 2003) as the culprit of listeriosis the regulatory tolerance for this *Listeria* spp. must be clarified. Defining the specific attributes of products that are considered RTE is sometimes vague but the Food and Drug Administration (FDA) and United States Department of Agriculture (USDA) refer to them as products that will not be heated or reheated before consumption (FDA, 2003). The Code of Federal Regulations (CFR) Title 9 Part 430.1 defines the products that are considered RTE, mainly deli and hotdog products (CFR, 2010). There are various ways that food processors of these products can meet the zero tolerance regulation, mainly by heat treatments, antimicrobials as part of their HACCP program and by having strict monitoring of the plant sanitation program (CFR, 2010). Since *L. monocytogenes* is an organism most commonly associated with RTE products, the USDA-FSIS has no regulations regarding this on raw products since they will be cooked before consumption. The main focus of USDA-FSIS with regard to *L. monocytogenes* contamination is from processed meat products and it does not focus on the contamination at the retail and foodservice levels. Gombas et al. (2003) reported that delicatessen-sliced RTE meats were contaminated with *L. monocytogenes* at a higher rate than prepackaged RTE meats. Furthermore, Vorst et al. (2008) demonstrated that slicer blades are a mode of transmission when slicing RTE delicatessen meats and control measures need to be implemented to alleviate this type of transmission.

The United States Department of Health and Human Services has target goals for all foodborne illnesses, referred to as the Healthy People Initiative. The current target for 2020 is 0.2 cases of listeriosis for every 100,000 people and the current data suggests that there are approximately 0.3 cases per 100,000 people in the U.S. (HHS, 2014). This reduction will be approximately 30% from the current number of cases but can only be accomplished if scientists, consumers and the food industry remain attentive to the implications of foodborne pathogenic bacteria.

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CHAPTER 3: ACID-ADAPTATION OF *SALMONELLA* HEIDELBERG REDUCES THERMAL TOLERANCE IN NOT-READY-TO-EAT GROUND TURKEY MEAT DURING REFRIGERATED STORAGE

J.M. Smith¹, C. L. Bratcher², L. Wang², S.F. Bilgili¹ and M. Singh³

¹Department of Poultry Science, Auburn University, Auburn, AL 36849

²Department of Animal Science, Auburn University, Auburn, AL 36849

³Department of Food Science, Purdue University, West Lafayette, IN 47907

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3.1 ABSTRACT

Salmonella enterica serovar Heidelberg is the leading cause of human Salmonellosis, and ground poultry products are increasingly implicated in such outbreaks. In this study, the thermal tolerance of acid-adapted and non-adapted *Salmonella* Heidelberg was evaluated in ground turkey meat stored under refrigeration (4°C). *S.* Heidelberg was adapted to acidic conditions by growing in tryptic soy broth supplemented with 1% glucose at 37°C to a final pH of 4.70, while the non-adapted culture was grown in tryptic soy broth with a final pH of approximately 6.10. Irradiated ground turkey was inoculated with either acid-adapted or non-adapted *S.* Heidelberg, and 2 g portions were packaged in plastic pouches and heat sealed for storage at 4°C. Samples were subjected to heat treatment of 62°C and 65°C in a circulating water bath for 230 s after storage for 0, 3, 6, 9 and 12 days and subsequently plated onto Plate Count Agar to determine survival populations. No significant differences ($P > 0.05$) were observed between non-adapted and acid-

adapted *S. Heidelberg* at to both 62 and 65°C, even though lower D values were obtained over the course of the experiments. Recovery of *S. Heidelberg* from inoculated meat samples was constant [ca. 6.50 log₁₀ cfu/g] over the storage period. This suggests that acid-adapted *S. Heidelberg* has the ability to survive in ground meat. However, it is more susceptible to thermal treatments than its non-adapted equivalent in ground turkey over time.

3.2 INTRODUCTION

As the leading foodborne pathogen in the United States *Salmonella* is responsible for over 1 million domestically acquired foodborne illnesses, 19,000 hospitalizations and 378 deaths in the annually (Scallan et al., 2011). This presents an economic burden totaling an estimated \$3.3 Billion each year, representing more than 20% of the cost of all foodborne illnesses acquired in the United States annually (Hoffman et al., 2012). Recent pathogen-food pairings rank salmonellosis acquired from poultry as one of the most probable sources of foodborne illness and also confirms that poultry alone contributes to \$2.4 Billion in foodborne illnesses, more than any other category of food (Batz et al., 2012). With a relatively low infective dose as compared to other foodborne pathogens (Lehmacher et al., 1995) acquiring foodborne salmonellosis can elicit mild self-limiting gastritis for approximately 7 days or be as severe as typhoid fever, systemic infection and possibly death (D'Aoust, 1991a).

In order to combat the issue of *Salmonella* in poultry products the USDA-FSIS has proposed the modernization of poultry slaughter inspection that is estimated to reduce 4,286 of the *Salmonella* related illnesses each year (USDA, 2013). Further

updates to USDA-FSIS *Salmonella* Performance Standards for fresh turkey carcasses have reduced the positive rate to 1.7% post-chill (USDA, 2011). Additionally, regulations for ground turkey, referred to as a Not-Ready-To-Eat (NRTE) products, mandate that the *Salmonella* positive rate shall not exceed 49.9% (USDA, 2011). While these products are NRTE and cooking is the main method of eliminating *Salmonella* products like NRTE ground poultry are still a prevailing vehicle for *Salmonella* related foodborne illnesses (USDA, 2012).

An outbreak of *Salmonella* Heidelberg in ground chicken throughout 29 states with a higher than average hospitalization rate prompted the USDA-FSIS to require processors to update and reassess HACCP plans for overall *Salmonella* contamination of NRTE ground poultry products (USDA, 2012). This outbreak has involved a serotype of *Salmonella* Heidelberg that has exhibited multidrug-resistance and is subsequently challenging to treat clinically (CDC, 2014). Further regulations, known as Appendix A, regulate the cooking time and temperature requirements required to obtain a 7.0 log₁₀ reduction of *Salmonella* in Ready-to-Eat (RTE) for meat and poultry products (USDA, 1999). These regulations are based on the fat content of these products and timings do not factor in any adaptations that pathogens may have undergone which could potentially make them more resistant to heat (Leyer and Johnson, 1993).

During primary processing the multi-hurdle approach is typically employed in order to combat *Salmonella* contamination of final products, however, the production of NRTE products has the tendency to exacerbate this issue as *Salmonella* can be disseminated more widely during the grinding process. While regulations have

mandated more stringent microbiological standards processors have added additional process controls such as post-chill dip tanks immediately following the immersion chilling process to ensure their compliance (Russell, 2010). This technology involves the use of different available acidic antimicrobials as a short contact application to reduce microbiological contamination of fresh poultry carcasses (Nagel et al., 2013). Additional acidic treatments are often applied in the form of spraying at other sites during processing as part of this multi-hurdle approach (Purnell et al., 2014). The use of such treatments is approved by USDA-FSIS, however, there is a concern that the organisms which survive these treatments may become habituated to the provided acidic environment and can therefore exhibit cross protection against additional environmental factors, such as sub-lethal heat, as shown by Leyer and Johnson (1993).

Even though a neutral pH is most favorable to the growth of *salmonellae* this organism has shown the ability to grow in an acidic environment of pH 3.99 (Asplund and Nurmi, 1999) and in an alkaline environment of pH 9.5 (Holley and Proulx, 1986). While *Salmonellae* grow optimally at 37°C they have also shown the ability to survive and proliferate as low as refrigeration temperatures of 4°C and up to 54°C (D'Aoust, 1991b). Foster (1991) showed that when *Salmonella* was pre-shocked for 1 h at pH 6.9 it displayed a more favorable survival when compared to non-adapted cells. Increased thermal tolerance of acid-adapted *Salmonella* has been shown in various fruit juices (Sharma et al., 2005) but was not established on beef carcasses (Dickson and Kunduru, 1995). On inoculated ground chicken patties acid-stressed *Salmonella* Typhimurium did not demonstrate resistance to antimicrobials and

showed a loss of cellular integrity and membrane partiality (Jung et al., 2009). The present study was undertaken to evaluate the thermal tolerance of acid-adapted and non-adapted *Salmonella* Heidelberg. The objective was to compare the D values obtained in inoculated ground turkey during the course of normal, refrigerated storage.

3.3 MATERIALS AND METHODS

Bacterial Culture

For both the *in vitro* and *in vivo* assessments *Salmonella* Heidelberg (USDA/ARS, Athens, GA) was cultured at 37°C for 18 h in Tryptic Soy Broth (TSB; Accumedia, MI) for non-adapted cells and TSB+1% glucose for the adapted cells to reach the stationary growth phase (ca. 9.0 log₁₀ CFU/mL). Buchanan and Edelson (1999) demonstrated that the addition of 1% glucose facilitates the production of organic acids that lower pH of the growth medium. Following this, 350 mL of the cultures were separately dispensed into 50 mL centrifuge tubes and centrifuged at 1069 g for 20 min at 37°C (Sorvall Legend RT+ Centrifuge, Thermo, CT; F13-14x50c rotor, Piramoon Technologies, CA). The resulting supernatant was decanted and the product was then suspended in 175 mL of Phosphate Buffered Saline (PBS; Hardy Diagnostics, CA).

Growth patterns were constructed by plating (100 µL; PCA) non-adapted and acid-adapted cultures grown at 37°C at 1 h intervals for 20 h.

Sample Preparation for in vitro and in vivo Assessments

Non-adapted and acid-adapted *Salmonella* Heidelberg were dispensed into sterile 2 x 2 in pouches (5 mL) and heat sealed for the *in vitro* assessments. For the *in*

in vivo study frozen ground turkey (ca. 8% Fat) was purchased at a retail establishment in Auburn, AL. Ground turkey meat was subjected to gamma radiation (Co^{60}) at a dose of 3.0 kGy (Leach Science Center, Auburn University, AL). Following Co^{60} treatment the meat was divided into two portions weighing approximately 500 g and inoculated with 80 mL of non-adapted and acid-adapted *Salmonella* Heidelberg, respectively. After the inoculum was mixed using sterile forceps to obtain homogeneity it was given a 30 min bacterial attachment period in a biological safety cabinet. The meat was then packaged into individual 2 x 2 in plastic pouches, compressed to approximately 2 mm thick and heat sealed. Pouches were stored at 4°C for 12 days and heat lethality experiments were carried out at 3-day intervals for 12 days and the initial packaging day designated as day 0.

Thermal Assessment

In vitro thermal tolerance experiments for non-adapted and acid-adapted *Salmonella* Heidelberg were carried out in a circulating water bath at either 55°C, 58°C or 62°C. Heat sealed pouches (23) containing *Salmonella* Heidelberg cultures were held in the circulating water bath for heat treatment and removed in 10 s intervals for 230 s total. Following heat treatment samples were placed on an ice-water bath.

The *in vivo* assessment was carried out in a circulating water bath was maintained at either 62°C or 65°C and the decimal reduction time (D values) for non-adapted and acid-adapted *Salmonella* Heidelberg were determined. Twenty-three pouches of inoculated turkey meat were submerged in the water bath (62°C or 65°C) and withdrawn at 10 s intervals for up to 230 s. Time 0 was designated as the time in

which all samples had reached the respective water bath temperature and was measured using a thermocouple probe in a non-inoculated pouch of ground turkey (50 s for 62°C and 90 s for 65°C). Following heat treatment pouches were placed in an ice-water mixture for 10 min.

Microbiological Sampling

In vitro samples were aseptically opened and serially diluted in 0.1% Peptone Water (PW; Accumedia, WI). Samples were then plated (100 µL) on Standard Methods Agar (PCA; Accumedia, WI) and agar plates were incubated at 37°C for 18-24 h and enumerated and results were reported as CFU/g.

For the *in vivo* study pouches were aseptically opened and diluted with 2 mL 0.1% PW. Samples were subsequently serially diluted and plated (100 µL) in duplicate on PCA. Agar plates were then incubated at 37°C for 18-24 h, enumerated and colony forming units reported as CFU/g. Negative (non-inoculated) and positive (acid-adapted and non-adapted) control samples of ground turkey meat were aseptically removed and plated on XLT-4 (Xylose-Lysine-Tergitol 4; Accumedia, WI) agar in addition to PCA.

Statistical Analysis

Survivor curves were constructed following recovery on PCA and D values were calculated as the negative inverse of the slope of the resulting curves obtained following both *in vitro* and *in vivo* studies. $D_{(\text{Temperature})}$ values were reported in minutes for acid-adapted and non-adapted cultures. All experiments were carried out in triplicate and the D values were analyzed using the PROC GLM procedure in SAS 9.2 (SAS Institute, Cary, NC).

3.4 RESULTS

The present study has addressed concerns that acid-adapted *Salmonella* Heidelberg can present in an expected matrix. *In vitro* growth curves (Fig. 1) revealed that there were no significant differences ($P > 0.05$) between the growth rates (Table 1) of *S. Heidelberg* when broth composition (TSB and TSB+1% Glucose) and growth temperature (37°C and 42°C). This revealed that *S. Heidelberg* is able to grow and become habituated to lower pH and elevated temperature conditions but does not exhibit a significantly different ($P > 0.05$) growth rates in these conditions. Subsequent *in vitro* experiments at 55°C, 58°C and 62°C showed (Table 2) that these temperatures produced significantly different D values ($P < 0.05$). D_{55} and D_{62} values of non-adapted and acid-adapted *S. Heidelberg* also showed (Table 2) that there were no differences ($P > 0.05$) achieved between these cultures. However, D_{58} values showed that when comparing non-adapted and acid-adapted *S. Heidelberg* D values of 2.60 and 3.32 were obtained, respectively. This showed that at 58°C the Decimal reduction time was longer for acid-adapted culture opposed to the non-adapted culture.

Following *in vitro* experiments the ensuing *in vivo* experiments revealed that the recovery of *S. Heidelberg* from non-adapted and acid-adapted ground turkey (Fig. 2) was consistent over the 12 d in which the experiment was carried out. Figure 2 also shows that the Negative Control samples that were irradiated but not inoculated with *S. Heidelberg* were below the detection limit of the plating technique utilized in these experiments. *In vivo* decimal reduction values obtained at 62°C and 65°C (Table 3) showed that there were no significant differences ($P > 0.05$) among these values for

non-adapted and acid-adapted *S. Heidelberg* at both temperatures throughout the 12 d of storage. D_{62} values (Fig. 3) showed a decreasing trend over the duration of the experiment for both the non-adapted and acid-adapted samples, although not significantly different. D_{65} values (Fig. 4) did not show a decreasing trend during the course of storage for the non-adapted *S. Heidelberg* samples, as they were consistent throughout the experiment. However D_{65} values for the acid-adapted samples were lower at days 6, 9 and 12 even though the difference was not significant ($P > 0.05$).

3.5 DISCUSSION

Analysis of the growth curves revealed that altering the incubation temperature did not have an impact on bacterial proliferation during the logarithmic phase. This phase is crucial because it is when bacterial populations are logarithmically increasing. *In vitro* experiments were the basis of the *in vivo* experiments that were carried out, with findings that suggested bacterial cross-protection could occur at one of the exposure temperatures (58°C). Previous studies have found mostly similar results to this with different *Salmonella* cultures (Leyer and Johnson, 1993; Singh et al., 2006), but the methods for growing these cultures have differed which has led to some variation in these findings. Considering the stationary phase populations may have a larger application since the overall supply chain would allow for populations to reach this stage once consumers encounter the product.

Regulations set forth by the USDA-FSIS concerning cooking times for meat and poultry products, referred to as Appendix A (USDA, 1999) state that in order to obtain a 7 log₁₀ reduction of *Salmonella* Turkey with approximately 8% fat shall be

cooked for a minimum 17 and 5 min at 62°C and 65°C respectively. Appendix A does not account for the ability of bacteria, such as *S. Heidelberg*, to exhibit cross-protection to processing related stressors. The present study reported non-adapted D_{62} values of 1.76 on sampling day 0, which was similar to those reported by Juneja et al. (2001), although they did decrease over time. Since acid-adapted *S. Heidelberg* showed lower D values than non-adapted cultures it can be concluded that these cultures would not exhibit any additional thermal resistance at 62°C. Furthermore the present study found non-adapted D_{65} values that were higher than those achieved by Juneja et al. (2001), 1.61 min compared to 0.55 min, respectively. However the D_{65} values reported by Singh et al. (2006) were similar to those found in the present study, although Singh et al. (2006) used ground beef instead of ground turkey. The present study also confirms further studies showing non-adapted and acid-adapted *Salmonella* exhibit no differences in resistance to organic acid rinses on inoculated beef (Dickson and Kunduru, 1995).

The evaluation of the thermal tolerance of foodborne bacteria of animal origin is important for the development of cooking temperatures in the commercial food production environment. Since *Salmonella* are presently an unavoidable part of the production of poultry meat and there have been numerous outbreaks in NRTE poultry meat (CDC, 2014) cooking times must be evaluated and reassessed for all inherent pathogenic microorganisms. More importantly the survival of *Salmonella* in acidic environments (Asplund and Nurmi, 1999) and elevated temperatures (Bunning et al, 1990) necessitates the investigation under such conditions, most notably because these are common conditions used in commercial poultry processing and especially

because Leyer and Johnson (1993) suggested that acid-adaptation of *Salmonella* induces more resistance to environmental stressors. Moreover, the present study did not confirm *in vitro* findings by Xu et al. (2008) which showed acid-adapted *S. enterica* cells are more thermal tolerant after exposure to pH 4.0 and storing at 4°C. Additionally Sharma et al. (2005) found that acid-adapted *Salmonella* showed increased thermal tolerance in various fruit juice matrices. Numerous studies have been carried out regarding the thermal tolerance of foodborne pathogens, such as *Escherichia coli* (Buchanan and Edelson, 1999), in many different beef product matrices; however, few studies have considered this both in a matrix of poultry meat and acid-adapted bacterial populations. The present study analyzed the impact of an 8% fat content ground turkey product and this cannot be extrapolated to other meat types or fat contents of turkey products, prompting further research into the topic.

As a microbiological hazard in poultry processing environment, *Salmonella* will continue to be increasingly scrutinized as this it is the top pathogen-food paired cause of foodborne illness (Batz et al., 2012). While bacteria can become habituated to acidic environments and acidic treatments are commonly used in poultry processing the present study shows no differences between non-adapted and acid-adapted *Salmonella* Heidelberg in inoculated ground turkey. This suggests that the use of acidic treatments that could lead to acid habituation in the processing environment do not present any additional risk to the survival of these pathogens. Further studies need to be conducted in order to confirm these results in ground chicken, as this is a highly implicated product in many food safety outbreaks. Finally, an analysis of pathogenic mechanisms of non-adapted and acid-adapted *Salmonella*

needs to be undertaken in order to elucidate the further importance of acid-adaptation of foodborne pathogens.

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Figure 1: Growth patterns of Non-Adapted (37, 42) and Acid-Adapted (37+G, 42+G) *Salmonella* Heidelberg grown at 37°C and 42°C

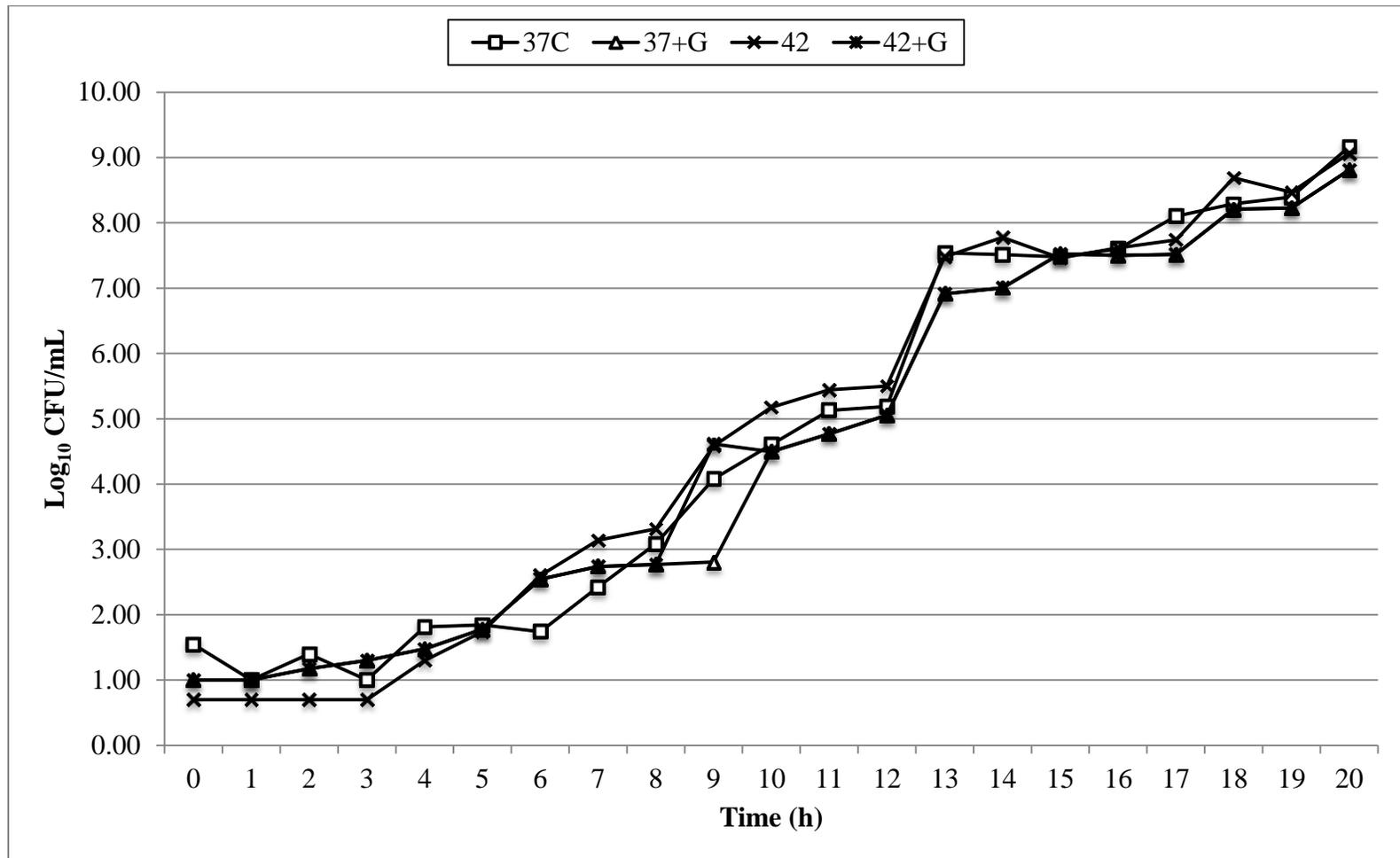


Figure 2: Survival populations recovered from Negative Control, Non-Adapted and Acid-Adapted *Salmonella* Heidelberg inoculated ground turkey samples during the course of stored at 4°C for 12 days

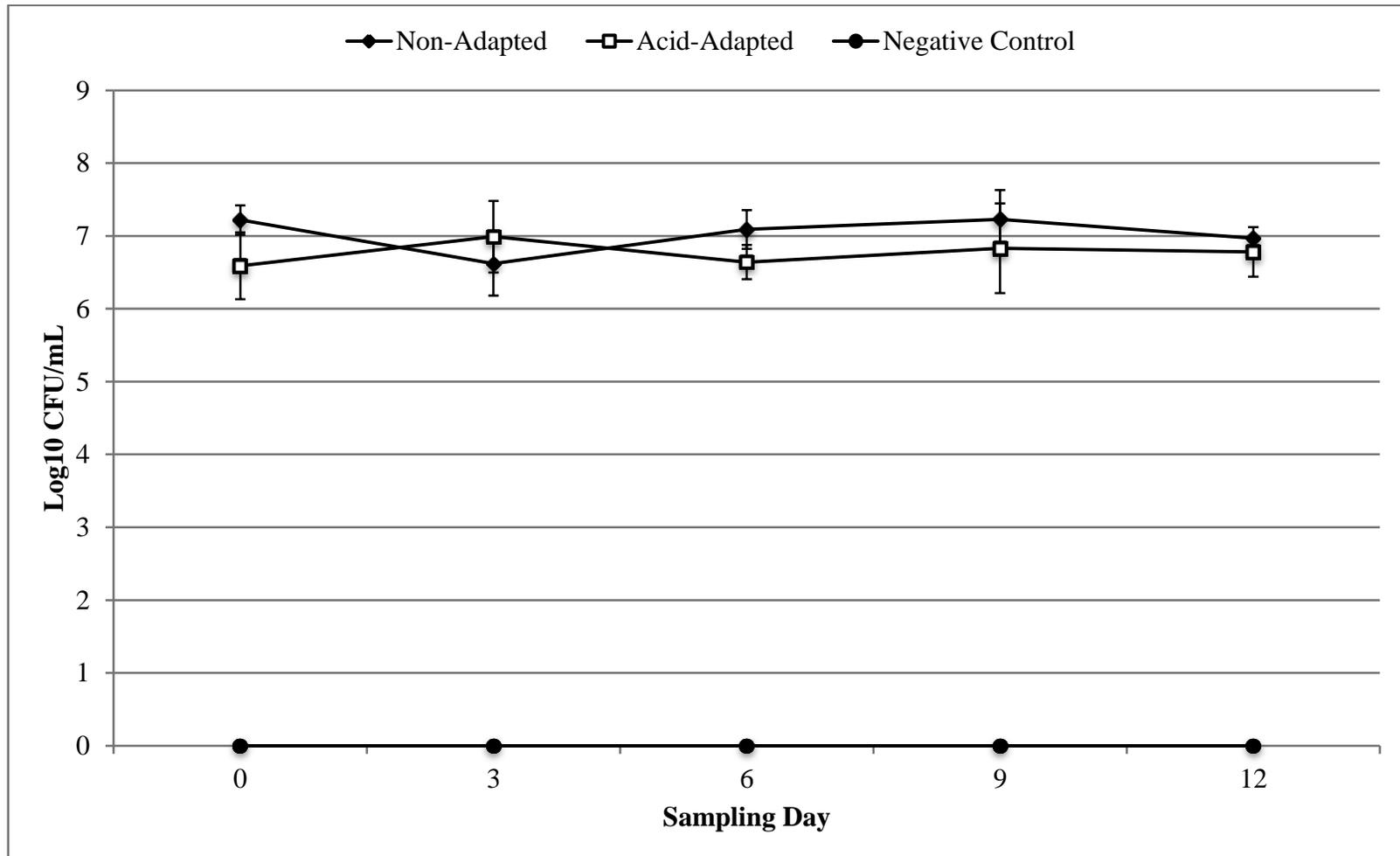


Figure 3: *In vivo* Decimal Reduction Times (min) at 62°C (D62 Values; mean \pm standard deviation) for Non-Adapted and Acid-Adapted *Salmonella* Heidelberg inoculated Ground Turkey Meat stored at 4°C for up to 12 days

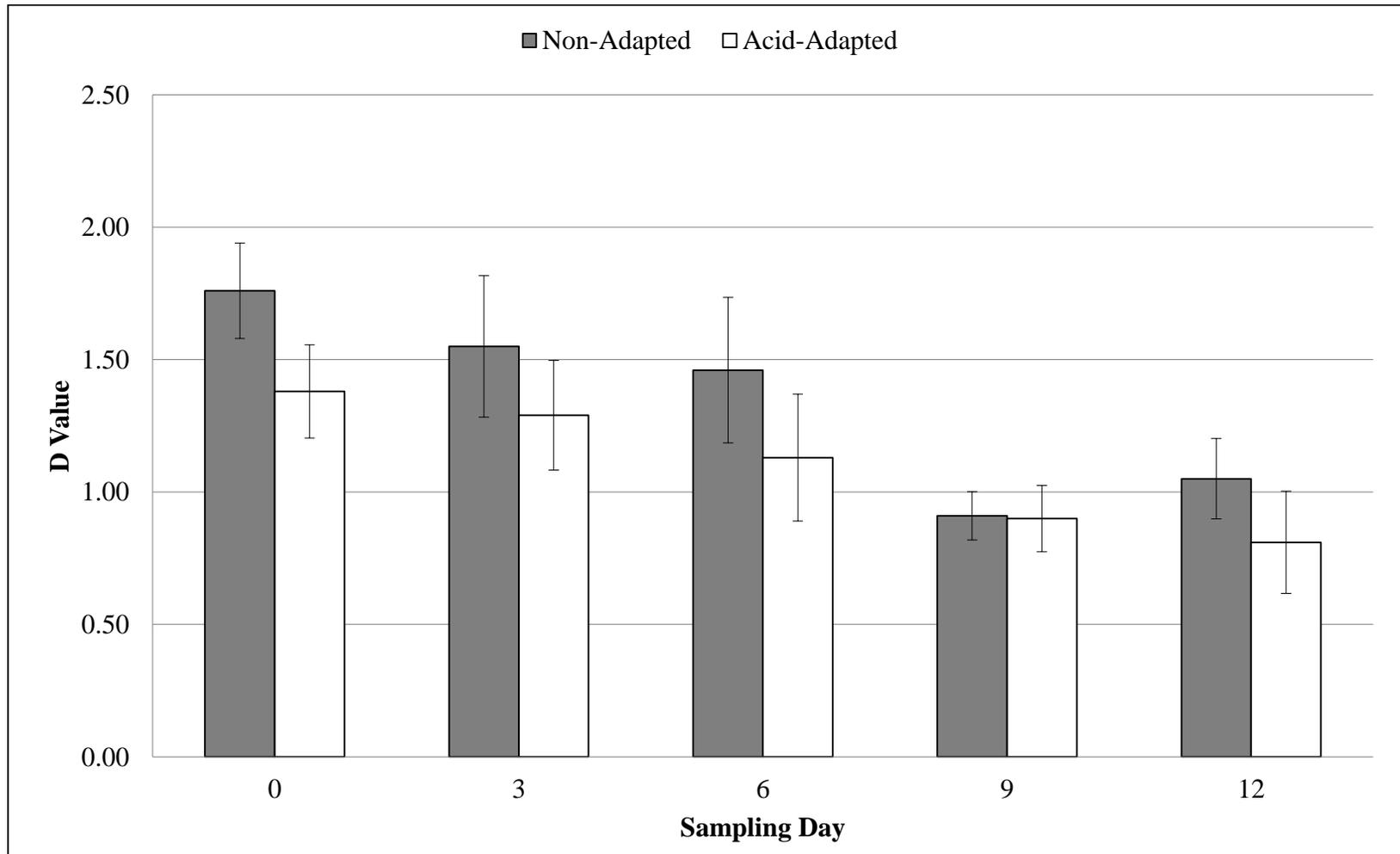


Figure 4: *In vivo* Decimal Reduction Times (min) at 65°C (D_{65} Values; mean \pm standard deviation) for Non-Adapted and Acid-Adapted *Salmonella* Heidelberg inoculated Ground Turkey Meat stored at 4°C for up to 12 days

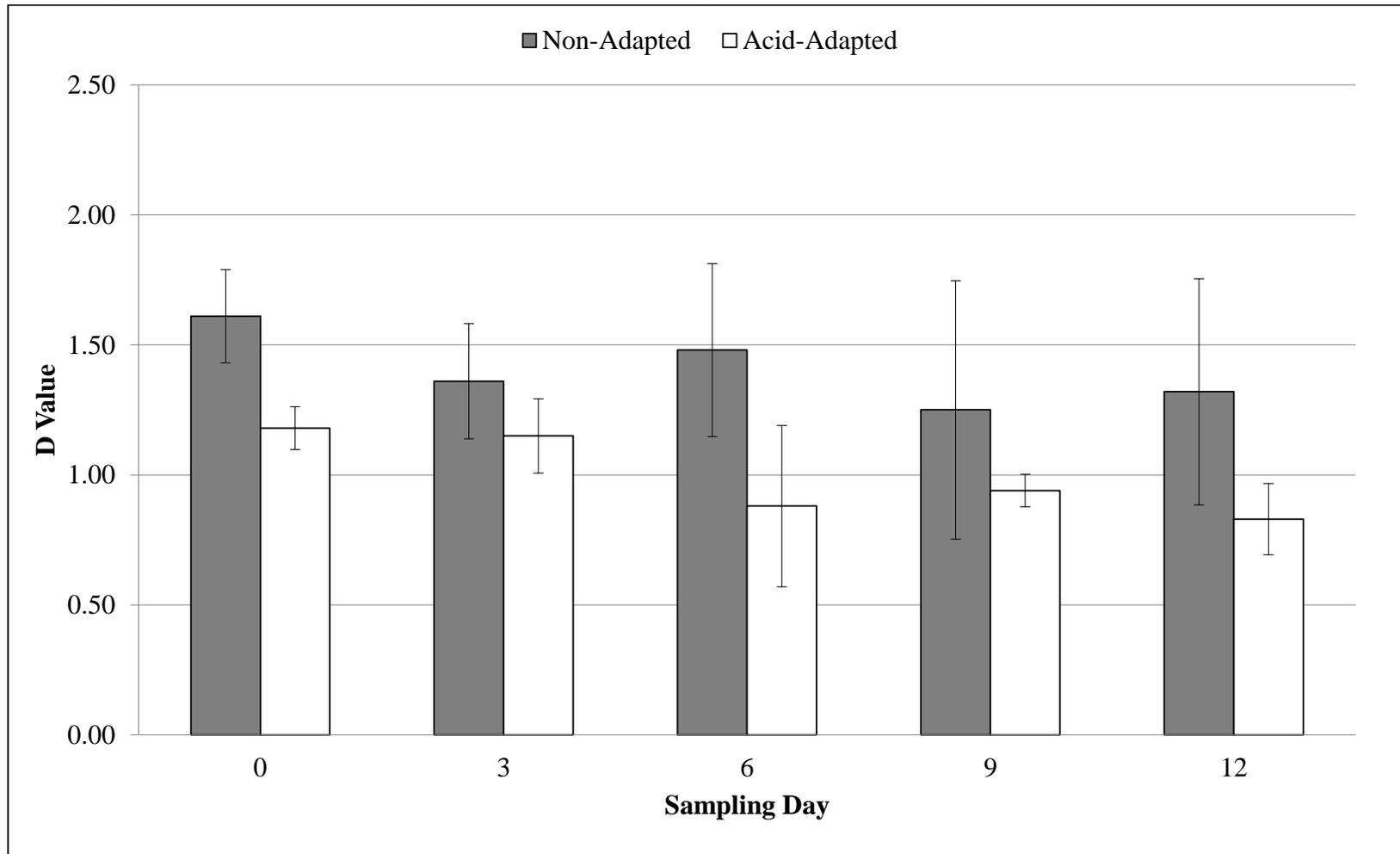


Table 1: Log Phase Growth rates of Non-Adapted and Acid-Adapted *Salmonella* Heidelberg grown in Tryptic Soy Broth (TSB) at 37°C and 42°C

Growth Temperature	Non-Adapted	Acid-Adapted
37°C	0.572	0.539
42°C	0.508	0.509

Table 2: *In vitro* Decimal Reduction Times (D Values; min) for Non-Adapted and Acid-Adapted *Salmonella* Heidelberg grown in Tryptic Soy Broth (TSB) at 37°C

Temperature	Non-Adapted	Acid-Adapted
D ₅₅	5.12±0.72 ^{1,a}	4.39±0.51 ^{1,a}
D ₅₈	2.60±0.23 ^{2,b}	3.32±0.23 ^{1,b}
D ₆₂	0.94±0.14 ^{1,c}	1.13±0.15 ^{1,c}

Numbers indicate significant differences ($P < 0.05$) between rows and letters indicate significant differences among columns ($P < 0.05$)

Table 3: *In vivo* Decimal Reduction Times (min) at 62°C and 65°C (D₆₂ and D₆₅ Values; means) for Non-Adapted and Acid-Adapted *Salmonella* Heidelberg inoculated Ground Turkey Meat stored at 4°C for up to 12 days

Sampling Day	D₆₂		D₆₅	
	Non-Adapted	Acid-Adapted	Non-Adapted	Acid-Adapted
0	1.76	1.38	1.61	1.18
3	1.55	1.29	1.36	1.15
6	1.46	1.13	1.48	0.88
9	0.91	0.91	1.25	0.94
12	1.05	0.81	1.32	0.83

CHAPTER 4: ASSESSMENT OF THERMAL TOLERANCE OF ACID-ADAPTED AND NON-ADAPTED *LISTERIA MONOCYTOGENES* IN READY-TO-EAT TURKEY

J.M. Smith¹, C.L. Bratcher², L. Wang², S.F. Bilgili¹ and M. Singh³

¹Department of Poultry Science, Auburn University, Auburn, AL 36849

²Department of Animal Science, Auburn University, Auburn, AL 36849

³Department of Food Science, Purdue University, West Lafayette, IN 47907

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4.1 ABSTRACT

Listeria monocytogenes is a prominent cause of foodborne illness and is attributed to a high death rate (15.9%). Furthermore, USDA-FSIS regulations mandate a zero-tolerance policy for Ready-To-Eat meat products and acidic treatments in addition to heat are commonly used to adhere to these regulations. This study evaluated the thermal tolerance of non-adapted (pH 5.20) and acid-adapted (pH 4.56) *L. monocytogenes* *in vivo* and *in vitro*. *L. monocytogenes* was cultured at to final populations of 10^9 log₁₀ CFU/mL in tryptic soy broth and tryptic soy broth with 1% glucose to obtain non-adapted and acid-adapted strains, respectively. Individually pre-sliced Ready-To-Eat turkey pieces were separately inoculated with cultures, held at 4°C and this was used to evaluate Decimal reduction values at 62°C and 65°C over the course of 28 days. Results showed that non-adapted and acid-adapted cultures did not show significantly different ($P > 0.05$) Decimal reduction values even though those inoculated with acid-adapted

L. monocytogenes consistently showed lower Decimal reduction values. These results suggest that acid-adapted *L. monocytogenes* have the ability to survive in RTE meat products although they are less resistant 62°C and 65°C heat treatments as compared to non-adapted *L. monocytogenes*.

4.2 INTRODUCTION

In the United States foodborne pathogens cause an estimated 9.4 million episodes of foodborne illnesses each year which result in an estimated 1,351 deaths (Scallan et al., 2011), together costing an estimated \$33 Billion economic burden (Hoffman et al., 2012). One of these pathogens causing such illnesses is *Listeria monocytogenes*, known particularly for its persistence on Ready-To-Eat (RTE) food products (FDA, 2003) and ability to grow at common refrigeration temperatures (Walker et al., 1990). Additionally, *L. monocytogenes* is characterized by its high hospitalization and death rates of 94% and 15.9%, respectively (Scallan et al., 2011).

As one of the more notable Gram-positive foodborne pathogens, *L. monocytogenes* can have severe consequences if acquired during pregnancy, as it is capable of crossing the placental barrier to cause premature delivery, stillbirth or even fetal loss (CDC, 2013). Outside of this particularly vulnerable demographic, neonates and the elderly are also considered high risk for acquiring listeriosis, but healthy adults can also become infected (CDC, 2013). In healthy adults the symptoms of listeriosis include gastrointestinal distress approximately 24 hours after consumption of contaminated food products that may last for up to 3 weeks (Frye et al., 2002).

The USDA-FSIS has a zero-tolerance policy for *L. monocytogenes* in RTE foods (USDA, 2004), specifically meat products that will not be reheated prior to consumption. Code of Federal Regulations (CFR) Title 9 Part 430.4 defines various control measures processors can use to combat *L. monocytogenes* contamination in RTE meats (CFR, 2010). Thermal treatments, antimicrobials and proper sanitation programs are the focus of USDA-FSIS guidance on *Listeria* contamination in processing environments (USDA, 2004). Even though Gombas et al. (2003) reported a higher contamination rate among delicatessen-sliced RTE meats than prepackaged RTE meats it cannot be overlooked that both of these classes of products are potential vectors for *L. monocytogenes* to humans.

L. monocytogenes grows and survives at low temperatures and also shows the ability to grow as high as 45°C (Ryser and Buchanan, 2013). Heat shock at 45°C demonstrated increased thermal tolerance but pre-shocking at 48°C did not show a difference compared to control strains (Lin and Chou, 2004). In *L. monocytogenes* inoculated summer sausages Roering et al. (1998) found D₉₈ and D₆₅ values of 0.28 to 2.08 min, respectively. Preshocking *L. monocytogenes* for 120 minutes at 48°C provided increased thermotolerance but preshocking for 30 or 60 minutes did not provide the same results (Farber and Brown, 1990).

Acidic treatments are also approved additives for the control of *L. monocytogenes* in food processing (USDA, 2004) and could potentially cause cross-protection to other stressors. However, incubation of *L. monocytogenes* Scott A in citric acid (pH 5.4) showed resistance to pH 3.35 treatment but no resistance at pH 4.35, indicating that the cross protective behavior was not

consistently induced and the organism was still susceptible to the preservative nisin (Okereke and Thompson, 1996). Konstantinos et al. (2003) showed that after being exposed to a mildly acidic (pH 5.0 to 6.0) environment *L. monocytogenes* could be afforded the ability to survive some subsequent acid treatments while those that were not exposed to the acid did not survive. Gahan et al. (1996) showed that the acid-adapted cells survived more favorably than their non-adapted counterparts in refrigerated (4°C) yogurt (pH 3.9), salad dressing (pH 3.0), and orange juice (pH 3.8) but *L. monocytogenes* was only recoverable 7 hours after inoculation. In the same study, cheddar cheese (pH 5.2) and cottage cheese (pH 4.7) showed survivability until 15 and 70 days, respectively (Gahan et al., 1996). This indicated that the survivability of non-adapted and acid-adapted *L. monocytogenes* is variable but highly dependent on the nature of the individual food product.

L. monocytogenes has been isolated from poultry processing environments at multiple stages, Lawrence and Gilmour (1995) reported raw poultry, cooked poultry and the processing environment all contained *L. monocytogenes* and some strains were recovered for up to 1 year. Additionally, Berrang et al. (2002) reported that the same strain of *L. monocytogenes* was recovered from raw products at some point during a 1-year study, which suggested reintroduction of the pathogen from raw product into the plant environment was occurring. In commercial processing, meat products are thermally processed after packaging to reduce *L. monocytogenes* contamination in order to comply with USDA-FSIS zero tolerance regulations (Murphy et al., 2003). Therefore the current study was

undertaken to evaluate the response of non-adapted *L. monocytogenes* as compared to its acid-adapted counterpart in RTE, commercially sliced turkey pieces while stored at refrigeration temperatures.

4.3 MATERIALS AND METHODS

Bacterial Culture Preparation

Listeria monocytogenes serotype 4b strain 101M (Dr. H. Thippareddi, University of Nebraska, Lincoln) was cultured in Tryptic Soy Broth (TSB; Accumedia, WI) and Tryptic Soy Broth + 1% Glucose (TSB+G) to obtain non-adapted and acid-adapted cultures, respectively. The addition of glucose (1%) facilitates the production of organic acids as growth byproducts that decrease the pH of the growth medium (Buchanan and Edelson, 1999). Growth was carried out for 18 h at 37°C to a final population of 10^9 log₁₀ CFU/mL in the stationary growth phase. Following this, 350 mL of each culture was centrifuged at 1069 g for 20 min at 37°C (Sorvall Legend RT+ Centrifuge, Thermo, CT; F13-14x50c rotor, Piramoon Technologies, CA). The supernatant was decanted and product suspended in 175 mL of Phosphate buffered Saline (PBS; Hardy Diagnostics, CA).

Growth patterns were constructed by plating (100 µL; PCA) non-adapted and acid-adapted cultures grown in TSB and TSB+G, respectively, at two different temperatures (37°C and 42°C) at 1 h intervals for 20 h.

Sample Preparation and Heat Treatment

***In vitro* experiments.** Five mL aliquots of non-adapted and acid-adapted *L. monocytogenes* were aseptically placed in sterile 5 x 5 cm polyethylene nylon

blend pouches (Oxygen Transmission Rate 90 cc/m²) and heat sealed. Following this 16 of the pouches were placed in a circulating water bath at either 55°C, 58°C or 62°C and removed at 1 min intervals for a total of 15 min. Samples were removed from the heat treatment and placed on an ice-water bath and held until microbiological plating.

***In vivo* experiments.** Packages containing individual, fully cooked, Ready-To-Eat Smoked Turkey Breast pieces were purchased at a retail outlet. The individual pieces were 4.5 cm in diameter, 0.25 cm in thickness and weighed 4 g each. Each piece was individually inoculated with 50 uL of either non-adapted or acid-adapted *L. monocytogenes* and allowed a 30 min attachment period in a biological safety cabinet. Following this period samples were individually placed in sterile 5 x 5 cm polyethylene nylon blend pouches (Oxygen Transmission Rate 90 cc/m²) and vacuum packaged. Samples were held at 4°C until plating which took place on days 0, 7, 14, 21 and 28 after packaging with the initial packaging day representing day 0 of the experiment. Samples (18) were placed in a circulating water bath and removed at 20 s intervals for a total of 330 s. Following heat treatment samples were placed on an ice-water bath for 10 min and held until microbiological sampling.

Microbiological Sampling

***In vitro* experiments.** Samples were aseptically opened and diluted in 0.1% Peptone Water (PW; Accumedica, WI). Samples were then plated in duplicate (100 µL each) on Standard Methods Agar (PCA; Accumedica, WI) and

plates were incubated at 37°C for 18-24 h and enumerated. Results were log transformed and reported as CFU/mL.

***In vivo* experiments.** Individual pouches were removed from the ice-water bath, aseptically opened, and diluted in 2 mL PW and then plated onto PCA (100 µL) in duplicate. Plates were kept at 37°C for 18-24 h, enumerated, results were log transformed and reported as CFU/g. Negative controls (non-inoculated) and positive controls (non-adapted and acid-adapted samples) which were not heat treated were aseptically removed and plated on both PCA and Modified Oxford Agar (MOX; Accumedia, WI).

Data Analysis

Following recovery on PCA for both *in vitro* and *in vivo* studies, survivor curves were constructed and D values were calculated as the negative inverse of the slope of the curves obtained. $D_{(\text{Temperature})}$ values were reported in minutes for acid-adapted and non-adapted cultures. All experiments were carried out in triplicate and the D values were analyzed using the PROC GLM procedure in SAS 9.2 (SAS Institute, Cary, NC) with significance at $P < 0.05$.

4.4 RESULTS

Growth patterns constructed of non-adapted and acid-adapted cultures grown at both 37°C and 42°C in Tryptic Soy Broth (Fig. 1) revealed that growth rates during the logarithmic phase showed no significant differences ($P > 0.05$) between cultures grown at the same temperature (non-adapted or acid-adapted or those grown at different temperatures (37°C or 42°C; Table 1). The final pH

values for the growth curves were 5.20 and 4.56 for non-adapted and acid-adapted *L. monocytogenes*, respectively.

Following this *in vitro* Decimal Reduction times (D values; min) were calculated at 55°C, 58°C and 62°C of non-adapted and acid-adapted cultures grown at 37°C and 42°C in Tryptic Soy Broth. The 55°C exposure temperature revealed significantly higher ($P < 0.05$) D values compared to the 58°C and 62°C exposure temperatures (Table 2). At 55°C the highest D value was for the Non-Adapted culture grown at 42°C and it was significantly higher than the other D values obtained at this exposure temperature ($P < 0.05$; Table 2). D values obtained at 58°C and 62°C revealed no significant differences ($P > 0.05$) among any of the non-adapted or acid-adapted cultures grown at either 37°C or 42°C (Table 2).

Control samples, those not exposed to any heat treatment, of non-adapted and acid-adapted turkey meat showed consistent bacterial recovery throughout the 28 days of study. Additionally, non-inoculated (negative) controls showed no growth of *L. monocytogenes* throughout the course of the study. Further evaluation of inoculated meat samples over the course of 28 days revealed that D_{62} values obtained for non-adapted and acid-adapted cultures did not differ significantly throughout the course of storage (Table 3). However, Fig. 3 shows that the D_{62} values for acid-adapted *L. monocytogenes* were consistently lower than those of the non-adapted *L. monocytogenes*. D_{65} values obtained from inoculated turkey samples revealed that the D values differed significantly ($P < 0.05$) on days 21 and 28 (Table 3). Additionally, the D_{65} value on the initial

sampling day was significantly higher ($P < 0.05$) than subsequent sampling days (Fig. 4). Furthermore, samples inoculated with acid-adapted *L. monocytogenes* displayed a significantly higher D value on day 0 as compared to later sampling days (Table 3). Sampling days 7 and 14 showed no significant difference ($P > 0.05$) but days 21 and 28 were significantly lower than day 14 ($P < 0.05$; Table 3).

4.5 DISCUSSION

Being one of the most fatal pathogens (Scallan et al., 2011) creates mandates studies regarding the overall relationship between *Listeria monocytogenes* and the foods that it is most likely associated with, specifically RTE meats. Even though the USDA-FSIS mandates a zero tolerance policy (USDA, 2004) on RTE meats, prepackaged meats are still a source of listeriosis. While growth patterns (Fig. 1) revealed that incubation temperature (37°C or 42°C) had no significant impact ($P > 0.05$) on the growth rate of non-adapted or acid-adapted *Listeria monocytogenes*. This indicated that the *L. monocytogenes* culture used throughout this study did not exhibit accelerated or decreased growth rate when cultured at an elevated temperature or in an acidic-adapted environment (pH 4.56). Furthermore, *in vitro* D values were also obtained (Table 2) and showed significantly higher Decimal reductions at 55°C compared to 58°C and 62°C, which were not significantly different. At 55°C the 37°C acid-adapted cultures showed a higher D value but this was not true for the 42°C culture ($P > 0.05$). At 58°C and 62°C the acid-adapted cultures displayed lower decimal reduction timings, even though the differences were not significant ($P > 0.05$). Since no significant differences were observed between the log phase growth

rates (Table 1) and Decimal reduction values (Table 2) of 37°C and 42°C cultures this was not pursued in further experiments.

Survival populations recovered from non-adapted and acid-adapted RTE control samples were consistent throughout the experiment (Fig. 2) and non-inoculated samples displayed no growth on selective (MOX) or nonselective agars (PCA). Roering et al. (1998) used summer sausages, considered RTE but not requiring refrigeration, and found a D_{65} value of 2.08, which was lower than the present study at day 0 and at day 12 which were 12.63 and 4.28, respectively. This could be due to the dry nature of the summer sausages, which typically have a lower pH than RTE meats. The lower pH could change the growth dynamics of *L. monocytogenes* in summer sausages as compared to RTE turkey meat.

While the present study did not show cross protection to heat treatments of acid-adapted *L. monocytogenes* as others have shown (Farber and Brown, 1990, Konstantinos et al, 2003). The elicitation of cross-protection may be based on the nutrients within and unique characteristics of individual food products, as suggested by Gahan et al. (1996). Reports by Cotter et al. (2001b) suggested that foods containing glutamate would prevent acidification of the cytoplasm and the RTE turkey product used in the present study did not contain glutamate. Therefore it was expected that sufficient acidification would have occurred and cross-protection would have been successfully induced.

If *L. monocytogenes* remains a major foodborne pathogen it will be necessary to continue to evaluate its survival and death in processing-related situations. As acids continue to be used in food processing, specifically RTE

products, it is necessary to evaluate the different food product matrices that are developed and formulated as it pertains to *L. monocytogenes*. Even though the use of acidic treatments could lead to acid-adaptation of *L. monocytogenes* the present study shows that this will not present any additional risk to the survival of these pathogens in a RTE food product. However, the mechanism of pathogenicity of acid-adapted as compared to non-adapted *L. monocytogenes* needs to be investigated to the knowledge of the severity of these pathogens as it pertains to human health.

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Figure 5: Growth patterns of Non-Adapted (37, 42) and Acid-Adapted (37+G, 42+G) *Listeria monocytogenes* grown at 37°C and 42°C

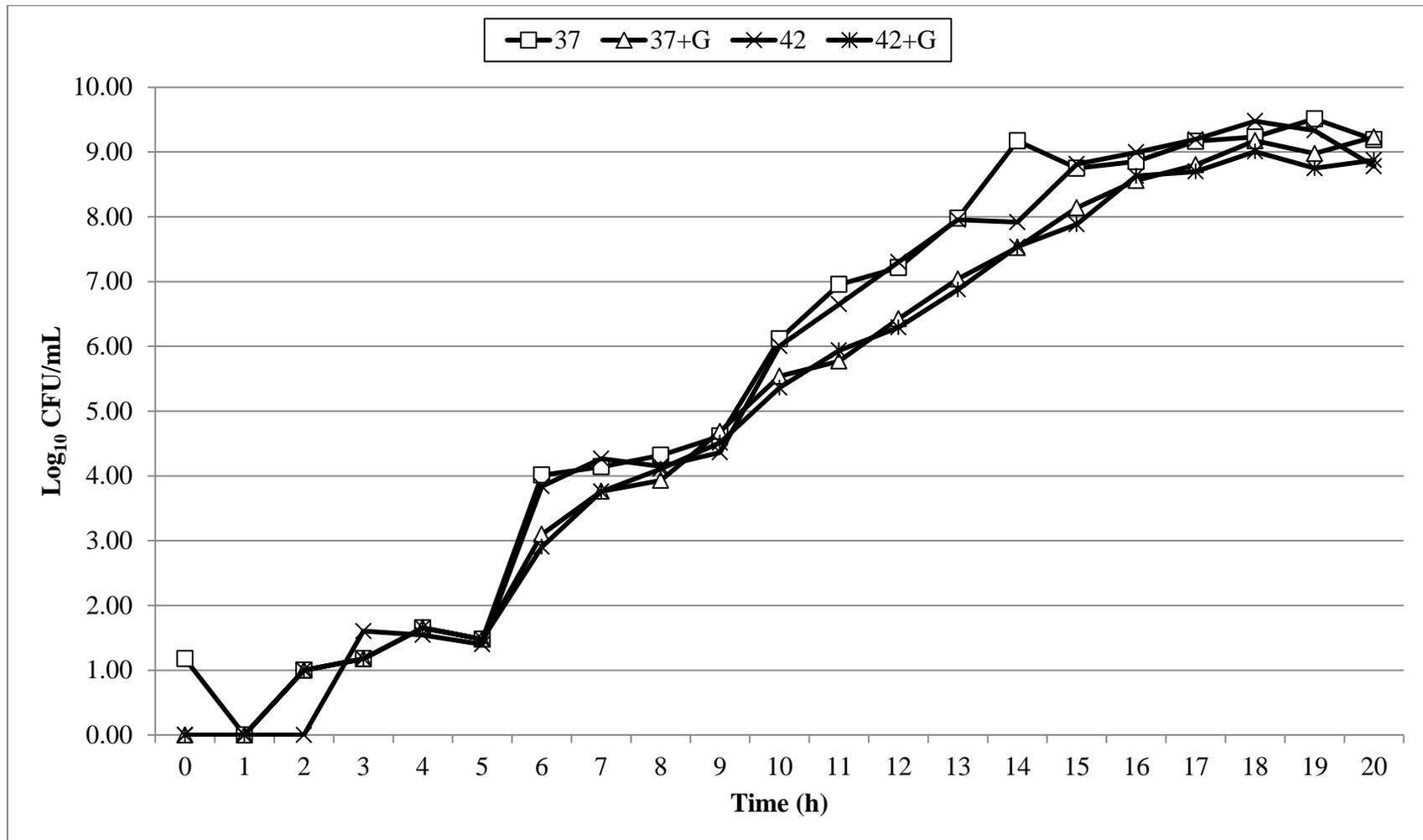


Figure 6: Survival populations recovered from Negative Control, Non-Adapted and Acid-Adapted *Listeria monocytogenes* inoculated RTE Turkey meat samples during the course of stored at 4°C for 28 days

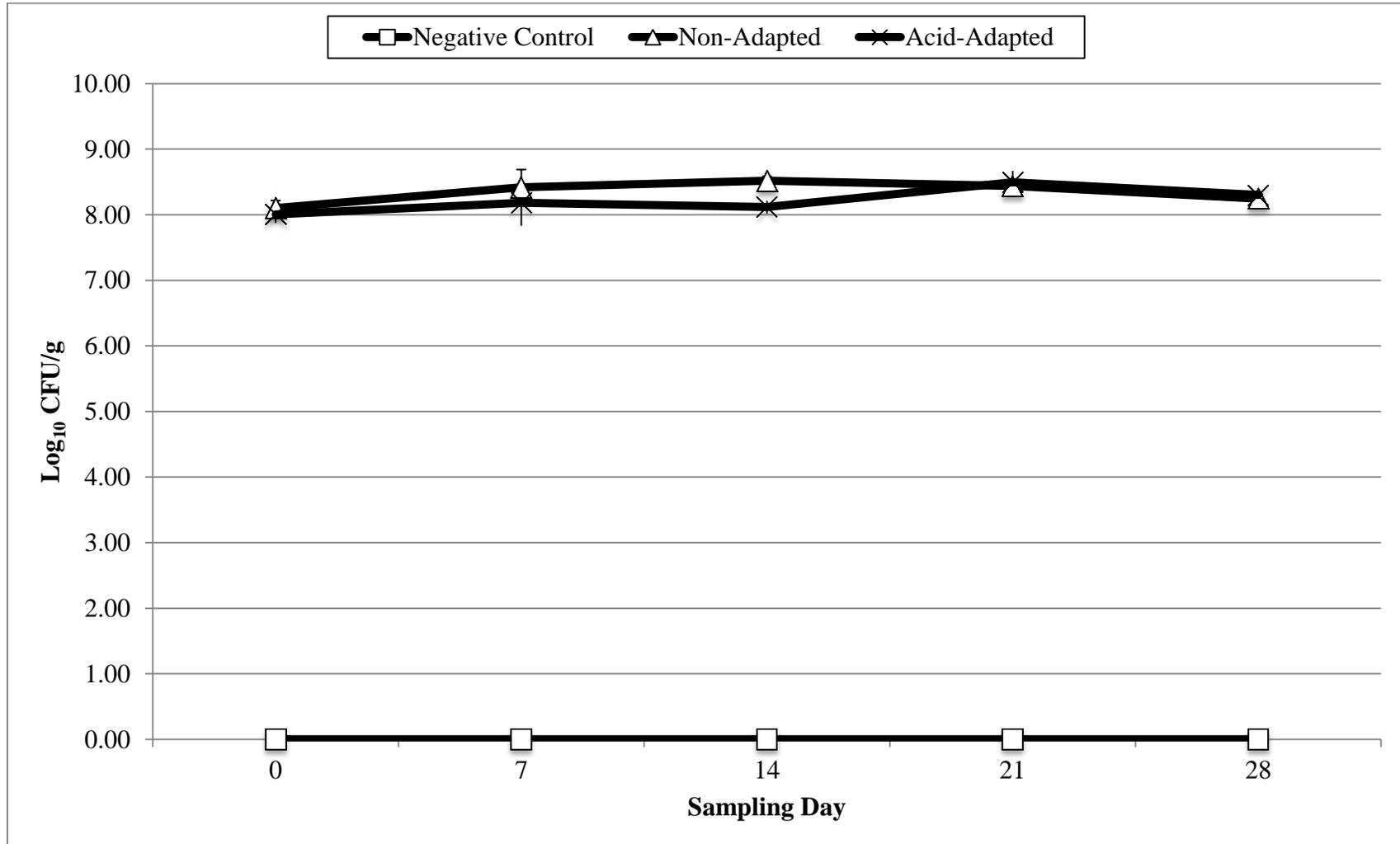


Figure 7: *In vivo* Decimal Reduction Times (min) at 62°C (D_{62} Values; mean \pm standard deviation) for Non-Adapted and Acid-Adapted *Listeria monocytogenes* inoculated RTE Turkey Meat stored at 4°C for up to 12 days

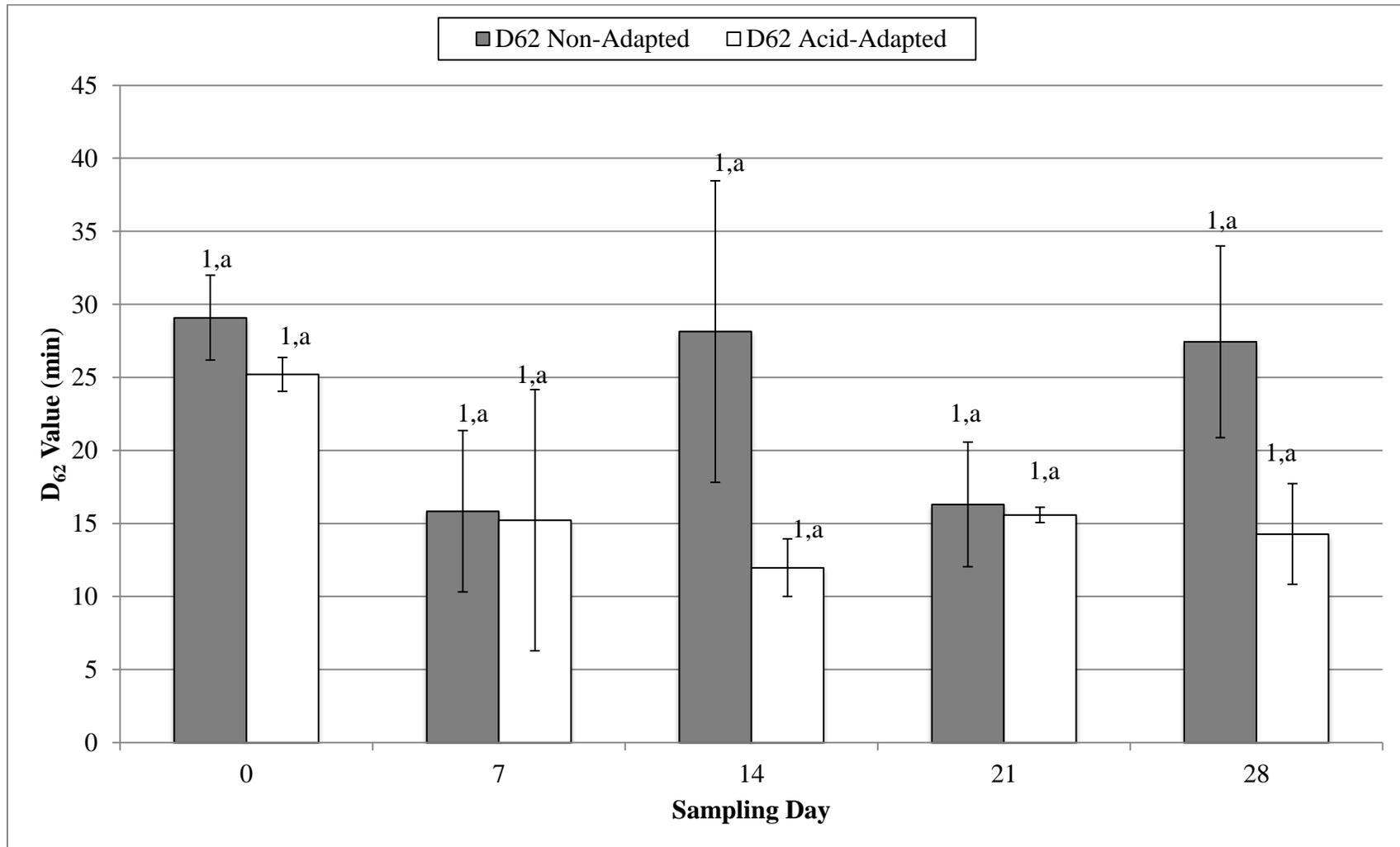


Figure 8: *In vivo* Decimal Reduction Times (min) at 65°C (D_{65} Values; mean \pm standard deviation) for Non-Adapted and Acid-Adapted *Listeria monocytogenes* inoculated RTE Turkey Meat stored at 4°C for up to 28 days

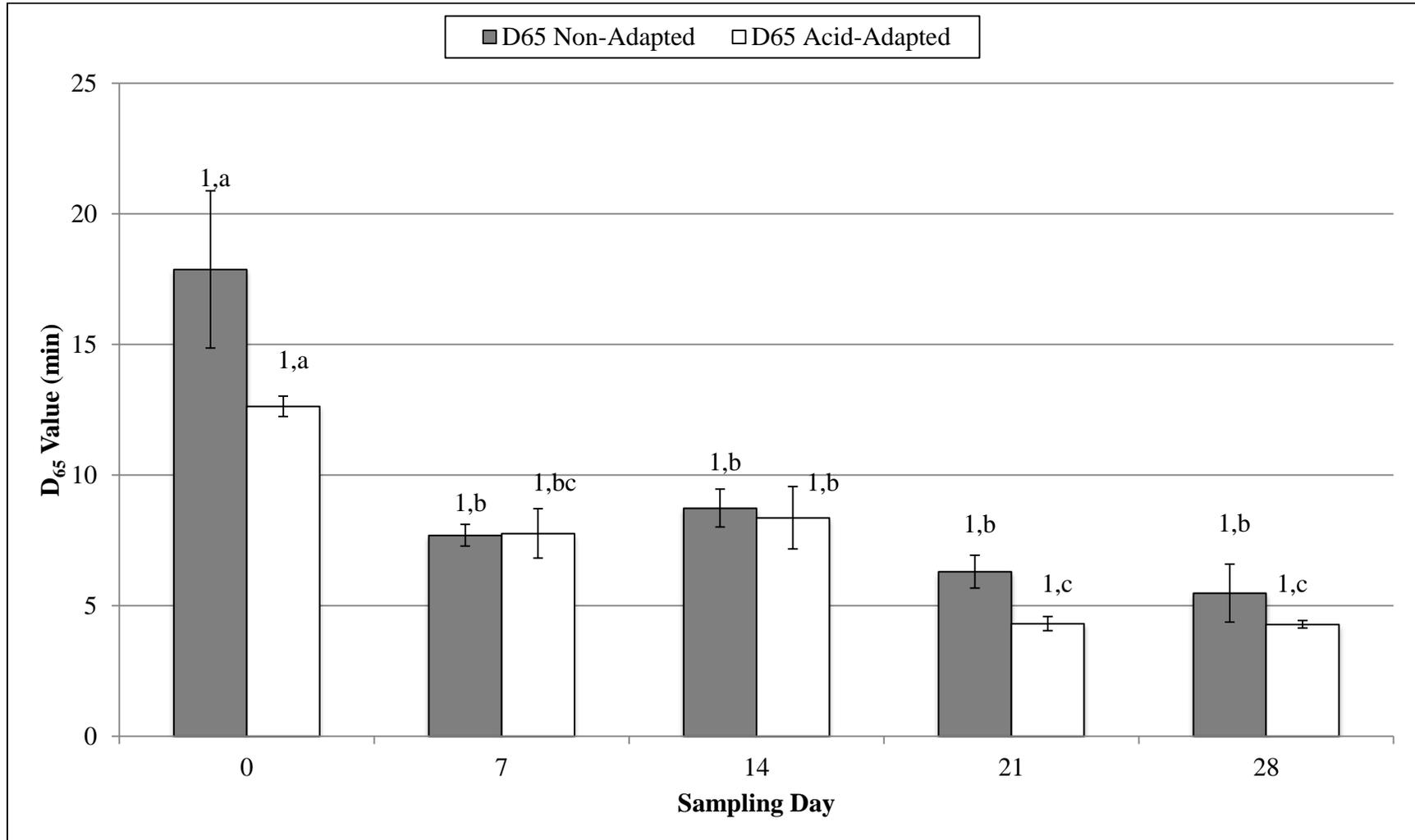


Table 4: Log Phase Growth rates of Non-Adapted and Acid-Adapted *Listeria monocytogenes* grown in TSB or TSB+G at 37°C and 42°C

Growth Temperature	Non-Adapted	Acid-Adapted
37°C	0.52	0.53
42°C	0.53	0.52

Table 5: *In vitro* Decimal Reduction Times (D Values, min) for Non-Adapted and Acid-Adapted *Listeria monocytogenes* grown in TSB or TSB+G at 37°C or 42°C

Exposure Temperature	37°C Non-Adapted	37°C Acid-Adapted	42°C Non-Adapted	42°C Acid-Adapted
55°C	34.97±5.43 ^{2,a}	72.95±12.93 ^{2,a}	121.90±40.72 ^{1,a}	38.16±9.41 ^{2,a}
58°C	20.31±7.90 ^{1,b}	10.93±2.78 ^{1,b}	19.88±0.86 ^{1,b}	4.33±1.44 ^{1,b}
62°C	3.45±0.20 ^{1,b}	1.35±0.22 ^{1,b}	4.57±1.44 ^{1,b}	2.39±0.92 ^{1,b}

Numbers indicate significant differences ($P < 0.05$) between rows and letters indicate significant differences among columns ($P < 0.05$)

Table 6: *In vivo* Decimal Reduction Times (D Values, min) at 62°C and 65°C (D_{62} and D_{65} Values; means) for Non-Adapted and Acid-Adapted *Listeria monocytogenes* stored at 4°C for up to 28 days on inoculated RTE Turkey meat samples

Sampling Day	D_{62}		D_{65}	
	Non-Adapted	Acid-Adapted	Non-Adapted	Acid-Adapted
0	29.09 ^{1,a}	25.21 ^{1,a}	17.87 ^{1,a}	12.63 ^{1,a}
7	15.84 ^{1,a}	15.23 ^{1,a}	7.69 ^{1,b}	7.76 ^{1,bc}
14	28.14 ^{1,a}	11.97 ^{1,a}	8.73 ^{1,b}	8.36 ^{1,b}
21	16.31 ^{1,a}	15.58 ^{1,a}	6.30 ^{1,b}	4.31 ^{1,c}
28	27.44 ^{1,a}	14.28 ^{1,a}	5.48 ^{1,b}	4.28 ^{2,c}

Numbers indicate significant differences ($P < 0.05$) between rows among each Temperature (62°C and 65°C) and letters indicate significant differences among columns ($P < 0.05$)

CHAPTER 5: CONCLUSIONS

The microbiological safety of all foods is an issue that has and will continue to be an issue in the developed world. Studying the mechanisms of survival of all foodborne pathogens in settings that are or could reasonably be encountered in commercial processing settings is an important part of food safety research. As changes in regulatory guidance occur processors will be given more requirements in order for products to be deemed 'safe.' While the regulatory guidance will always have human health in mind it is possible that it could have a contradictory effect if pathogens are able to gain adaptations to certain processing interventions. While each intervention strategy should be evaluated on an individual basis it is important to examine the entire process as it pertains to the survival of pathogens.

Thermal treatments are effective and sometimes unavoidable processes that are able to reduce overall bacterial contamination. However, these treatments are not considered effective if they are the only bactericidal treatment and are frequently accompanied by additional acidic treatments. When modeling the behavior of pathogens that are adapted to processing conditions it is important to consider that there are regulations that have to be adhered to by agencies such as United States Department of Agriculture-Food Safety Inspection Service (USDA-FSIS) and the Food and Drug Administration (FDA).

Just as previous work has shown that cross-protection readily occurs in liquid food products, such as fruit and vegetable juices, cross-protection of bacterial populations should be examined further to completely rule out its contribution to survival in meat and poultry products. There are many other foodborne pathogens that readily survive in both fresh and RTE meat and poultry products and these pathogens should be evaluated for their overall response as it pertains to cross-protection. When research conducted in this area shows that adapted pathogens do not exhibit any additional survivability compared to non-adapted pathogens, as the present studies have shown for *Salmonella* Heidelberg and *Listeria monocytogenes*, there is evidence that cross-protection does not favor the enhanced survivability of these foodborne pathogens. Even though cross-protection can and very well may occur under current food processing conditions it should not be considered to have an impact on the increased survivability of *Salmonella* Heidelberg and *Listeria monocytogenes* given the conditions that were explored in the present study. However, different food matrices will contribute to varying survivability of different foodborne pathogens.