2006

Thermal tolerance of acid adapted and non-adapted Escherichia coli O157:H7 and Salmonella enterica in ground beef

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Thermal tolerance of acid adapted and non-adapted *Escherichia coli* O157:H7 and *Salmonella enterica* in ground beef

by

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A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Food Science and Technology

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Iowa State University
Ames, Iowa
2006

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ACKNOWLEDGEMENTS

The author wishes to express a sincere gratitude to his major professor Dr. James S. Dickson for his enthusiasm, guidance, and encouragement throughout the course of his study. The author is thankful to Drs. Aubrey F. Mendonca, Joseph G. Sebranek, Joseph C. Cordray, and Philip M. Dixon for their support, advice, and service on the program of study committee.

Special thanks to the entire faculty in food science and graduate/ undergraduate students especially in the Food Safety Research laboratory for their cooperation and friendship during my graduate studies at Iowa State University.

A special note of thanks to Dr. Harshavardhan Thippareddi for his encouragement, suggestions, and discussions during the course of this study; and his friendship.

The author wishes to thank his parents Drs. Avtar Singh and Balvinder Kaur for their understanding, support, encouragement and inspiration.

ABSTRACT

D-values of acid adapted *Escherichia coli* O157:H7 and *Salmonella* were determined in meat serum and ground beef stored at 4 and -20 °C as an indicator of thermal tolerance. Pathogens were acid adapted by growing in Tryptic Soy Broth with 1% glucose (TSB+1%G). Five-strain cocktail of both bacteria were grown and inoculated meat serum was heated to 58, 62, and 65 °C, while inoculated ground beef was heated to 62 and 65 °C on day 1, 7, 14, 21, and 28 (4 °C), and on day 1, 30, 60, 90, and 120 (-20 °C). Higher ($P<0.05$) D-values were observed for acid adapted *E. coli* O157:H7 in meat serum at 58, 62, and 65 °C; and for acid adapted *Salmonella* at 58 and 62 °C, but no difference ($P>0.05$) was observed at 65 °C. In ground beef D_{62}-values of non-adapted *E. coli* O157:H7 were higher ($P<0.05$) on day 21 and 28 (4 °C) and on day 90 and 120 (-20 °C). Higher ($P<0.05$) D_{62}-values were observed on day 21 and 28 at 4 °C amongst non-adapted strains and on day 28 for acid adapted strains of *Salmonella*. Higher ($P<0.05$) D_{62}-values of acid adapted *Salmonella* were observed on day 30, 60, and 90 when stored at -20 °C while no differences ($P>0.05$) were observed in D_{65}-values of acid adapted and non-adapted strains throughout storage (4 and -20 °C).

Toxicity of acid adapted *E. coli* O157:H7 to African Green Monkey kidney cells (Vero cells) was also determined. Vero cells were grown separately and subjected to toxin produced by acid adapted and non-adapted *E. coli* O157:H7 grown in laboratory media and meat serum. Comparison of toxicity was made after storing samples for 1 and 7 days at 4 and -20 °C. No differences ($P>0.05$) were observed between the toxicity of acid adapted and non-adapted cells in laboratory media and meat serum at both temperature. Higher ($P<0.05$)

toxicity was observed on day 1 as compared to day 7 at both storage temperatures. Higher ($P<0.05$) verotoxin production was observed in meat serum inoculated with non-adapted cells on day 1 as compared to acid adapted cells.

GENERAL INTRODUCTION

Organic and inorganic acids have been widely used in the food industry by deliberate addition to control foodborne pathogens and through controlled fermentations. Addition of organic acids and their salts during the manufacture of meat products is well documented. Although these applications in a variety of processes has been effective in controlling certain pathogenic bacteria, new hazards to the safety of the food supply are being posed due to the emergence of microorganisms resistant to the physical and chemical processes of traditional preservation (Bower and Daeschel, 1999). Acids have a lethal or inhibitory effect on microorganisms when they are used in high enough concentrations, but when used in moderate concentrations they often encourage the bacteria to have increased acid tolerance (Marshall, 2003). This increased tolerance to acidic conditions can lead to long term survival in acidic foods and increased probability of survival in the gastric environment.

Leyer and Johnson (1993) suggested that acid adaptation of pathogenic bacteria can induce cross protection of many of these acid adapted pathogens to other environmental stresses such as increased tolerance to thermal stress, osmotic stress, and some surface active agents. Studies have been done to demonstrate the increased resistance of acid adapted *Escherichia coli* O157:H7 in laboratory media and liquid food systems (Buchanan and Edelson, 1999a), but variable results are available in regards to resistance responses of acid adapted *Salmonella* to environmental stressors. While Leyer and Johnson (1993) reported increased resistance of *Salmonella* to environmental stresses, Dickson and Kunduru (1995) suggested no difference in the resistance of acid adapted strains to environmental stressors as compared to the non-adapted strains of *Salmonella*. Although trends in the food industry

have been towards convenient, refrigerated foods with extended shelf life, these acid adapted pathogens with increased resistance to environmental stressors may persist in food products during processing and pose a threat to human illness as a result of undercooking or raw consumption. The efficiency of thermal processing protocols to eliminate/ reduce the risk of foodborne pathogens in processed meats is generally targeted towards non-adapted strains whereas the increased thermal tolerance of acid tolerant/ adapted strains could be a reason for concern.

Despite the widely published literature on the thermal tolerance of acid adapted pathogens such as *Salmonella* and *E. coli* O157:H7 there are some limitations to the existing research. These limitations range from research being done in laboratory media and liquid foods to foods being held under conditions that do not reflect the current practices of storage and distribution in the industry. Another weakness of this widely researched area is that the data obtained from conducting studies on laboratory media and liquids foods may or may not be extrapolated to actual food systems including meat matrices such as ground beef and conditions that are closely related to the processing, storage and distribution of these food systems. Although, the major meat processors operate under a Hazard Analysis Critical Control Point (HACCP) program to reduce, control and/ or eliminate foodborne pathogens in raw meat and processed meat products, it is essential to provide new and revised guidelines for thermal and non-thermal measures to control stressed bacteria (including acid adapted pathogens) that exhibit increased tolerance to environmental stressors (physical and chemical).

LITERATURE REVIEW

Importance of *Escherichia coli* O157:H7 in meat products

The microflora of food consists of microorganisms associated with the raw material, those acquired during handling and processing, and those surviving any preservation treatment and storage conditions (Jay, 2000). A bacteriological survey of ground beef reported that *Escherichia coli*, *Enterobacter* spp., and *Clostridium perfringens* were the most commonly isolated organisms and were introduced by improper processing and poor sanitary conditions of the meat plant (Foster et. al, 1977).

The presence of coliforms in foods is usually associated with fecal contamination (Tompkin, 1983; Jay, 2000). These groups of organisms, particularly *E. coli*, has been regarded as indicator organisms of food and waterborne pathogens and are still used to assess the microbiological quality of foods (Jay 2000). All coliforms are aerobic and facultative anaerobic, gram negative, non-sporeforming bacteria capable of fermenting lactose with the production of acid and gas at 32-35 °C with 24-48 h on solid or liquid media. Most strains of *E. coli* O157:H7 possess several characteristics that are not common in other *E. coli* such as inability to grow well at temperatures > 44.5 °C, inability to ferment Sorbitol within 24 h and the possession of an attaching and effacing gene (Meng et. al., 2001). *E. coli* O157: H7 can grow over a pH range of 4.0-9.0 which is different than other *E. coli* that grow from 4.4-9.0. Genetically, *E. coli* is closely related to the genus *Shigella*. Although it is biochemically more active, the inert strains are difficult to distinguish from *Shigella*.

E. coli O157:H7 has been recognized as the most important etiological agent of hemorrhagic colitis which is characterized by severe abdominal pain and bloody diarrhea

(Bopp et. al., 1987). Doyle and Schoeni (1987) also reported isolation of the organism from specimens of foods implicated in outbreaks of hemorrhagic colitis and hemolytic uremic syndrome (HUS). *E. coli* O157:H7 was formally associated primarily with dairy cattle and their products, milk, and beef, but it has also caused outbreaks associated with water and apple cider (Mermelstein, 1993).

The bacteria constituting the species *E. coli* are commonly found in the intestinal flora of humans and warm blooded animals (Meng et. al., 2001), and were recognized as non-pathogenic normal co-inhabitants. However, certain strains have been implicated in outbreaks of foodborne disease and should be considered as potential pathogenic organisms. Many types of diseases are caused by *E. coli* depending upon the virulence factors expressed. Some virulence factors identified in pathogenic *E. coli* include possession of adhesions or colonization factors, ability to invade epithelial cells or the small intestine, hemolysin production and toxin production (heat stable, ST; heat labile LT; Vero cytotoxin 1, VT1; and Vero cytotoxin 2, VT2). VT1 and VT2 are also referred to as shiga-like toxins (SLT) 1 and 2 (Bell and Kyriakides, 1998). *E. coli* strains that cause diarrheal illness are categorized into specific groups based on the virulence properties, mechanisms of pathogenicity, and clinical syndromes (Meng et. al., 2001; Jay, 2000). The various strains of *E. coli* can attach and multiply in the small intestine, producing illness by invading epithelial cells, producing one or more enterotoxins, or vero or cytotoxins, or by adhering to and destruction of microvilli without invasion. Four different types of *E. coli* known to produce human gastroenteritis include: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), and enterohemorrhagic *E. coli* (EHEC) or Verotoxin producing *E. coli* (VTEC; Marth and Kornacki, 1982).

The original definition of EPEC is "diarrheagenic *E. coli* belonging to serogroups epidemiologically incriminated as pathogens but whose pathogenic mechanisms have not been proven to be related to either heat-labile enterotoxin, heat-stable enterotoxin, or Shigella-like invasiveness" (Meng et. al., 2001). EPEC was first characterized in 1955, and causes diarrhea in children generally under 1 year of age (Jay, 2000). EPECs do not produce Shiga like toxins but may produce one or more of the cytotoxins (Olsvik et. al., 1991) and have been determined to induce adherence and invasion of the epithelial cells (Donnenberg et. al., 1989). Cleary et. al. (1985) suggested the Shiga-like cytotoxins may be involved in the pathogenesis of these organisms.

ETEC is a major cause of infantile diarrhea in developing countries and is the most common cause of traveler's diarrhea (Meng et. al., 2001). These strains attach to and colonize in the small intestine producing heat-labile or heat-stable enterotoxins leading to watery diarrhea (Jay, 2000). In a study on the prevalence of enterotoxigenic *E. coli* in some processed raw food from animal origin Reis et. al. (1980), suggested that the frequencies of these strains was about 5 % in keebe (Arabian food made from bovine meat and wheat), 7.5 % in hamburgers, and 10 % in sausages. It is estimated that relatively high inoculum (10^6 – 10^{10} cells) are necessary to cause illness in adults (Jay, 2000) although it has been reported that ETECs from some animals may also attach to human intestinal cells and result in illness (Deneke et. al., 1984).

EIEC serotypes have been reported to cause non bloody diarrhea and dysentery like symptoms in humans similar to that caused by *Shigella* spp. and they enter and multiply in the colonic epithelial cells and then spread to adjacent cells (Meng et. al., 2001 and Jay, 2000). EIEC has an affinity for the colon causing bloody or non bloody diarrhea but rarely

causing dysentery. Very young or very old people are the most susceptible members of the population. Humans are a major reservoir for EIEC and the serogroups frequently associated with illness include O112, O124, O136, O144, O152, O164, and O167 (Meng et. al., 2001). The infective dose of EIEC appears to be substantially higher than *Shigella* and this is attributed to greater sensitivity of EIEC to gastric acidity (Adams and Moss, 2000).

EHEC were first identified as human pathogens in 1982 in the United States, when *E. coli* O157:H7 was implicated in two outbreaks of hemorrhagic colitis (Meng et. al., 2001). Most EHEC infections are caused by contaminated water or food; however, they may also be transmitted through human contact. EHECs have been reported to produce one or more toxins that are cytotoxic to Vero (African green monkey kidney) cells, which have been described as verotoxins or Shiga-like toxins. The terms Verotoxin producing *E. coli* (VTEC), Shiga-like toxin producing *E. coli* (SLTEC), and Enterohemorrhagic *E. coli* (EHEC) are synonymous and are used interchangeably. The term VTEC refers to *E. coli* strains that produce verotoxin and EHEC refers to the strains that have the same clinical, epidemiological, and pathogenetic features associated with *E. coli* O157:H7. In contrast to EPEC, EHEC strains affect only the large intestine and produce large amounts of Shiga-like toxins (Jay, 2000). Benjamin and Datta (1995) reported that most EHEC strains isolated from humans with infections carry a large plasmid which has been implicated in the adherence of the bacterium to intestinal mucosal walls.

Table 1: Earliest known foodborne gastroenterititis cases cause by pathogenic *Escherichia coli* (Source: Jay, 2000)

Year	Location	Food/ Source	No. of victims/ No. at Risk	Toxin/ Strain Type	Serotype
1947	England	Salmon (?)	47/ 300	EIEC	O124
1961	Romania	Substitute coffee drink	10/ 50	EPEC	O86:B7; H34
1963	Japan	Ohagi	17/ 31	EIEC	O124
1966	Japan	Vegetables	244/ 435	EIEC	O124
1967	Japan	Sushi	835/ 1,736	?	O11 (?)
1971	United States (14 states)	Imported Cheeses	387/?	EIEC	O124:B17
1980	Wisconsin	Food Handler	500/ > 3,000	ETEC	O6:H16
1981	Texas	Not Identified	282/ 3,000	ETEC (LT)*	O25:H+
1982	Oregon	Ground Beef	26/?	EHEC	O157:H7
* LT = Heat labile enterotoxin					

Life threatening complications such as hemorrhagic colitis and hemolytic uremic syndrome (HUS) make EHEC infection a serious public health concern. Although most episodes of HUS are caused by EHEC strains belonging to serotype O157:H7, other serotypes have also been implicated (Griffin and Tauxe, 1991). Only two VTEC serotypes (O157:H7 and O26:H11) have been classified as EHEC (Levine, 1987). *E. coli* O157:H7 is recognized as the most common cause of VTEC-associated illness in humans and is the only serotype implicated in foodborne illness (Bopp et. al., 1987).

Reservoirs of *E. coli* O157:H7

Person-to-person contact has been identified as a means of spread of *E. coli* O157:H7 in day care settings (Meng et. al., 2001) and has been implicated as a source of secondary transmission by humans at a nursing home where the staff became a second wave of transmission (Bell et. al., 1994). There have been reports on confirmed cases on human illness due to *E. coli* O157:H7 as a result of consumption of undercooked hamburgers and rarely unpasteurized milk that has lead to these being the primary sources implicated in human illnesses. These outbreaks have led to the identification of dairy cattle as a primary reservoir for *E. coli* O157:H7 (Wells et. al., 1983). According to Schoeni and Doyle (1994), preoral administration of small populations (25 cells) of *E. coli* O157:H7 can lead to colonization in chicks that could then possibly lead to eggs being carriers of this organism and chicks being long term shedders.

Outbreaks of *E. coli* O157:H7 infection have been reported from direct contact with cattle (Crump et. al., 2002), environmental contamination with cattle manure (Varma et. al., 2003); recreational (Friedman et. al., 1999) and drinking water (Licence et. al., 2001), and produce such as lettuce and sprouts that were likely contaminated with cattle manure (Ellen-Swanson et. al., 2005). Of the outbreaks and clusters reported in the United States, 83.4 % occurred in the months from May to October as a result of the following three reasons: (1) increased prevalence of the pathogen in cattle or other livestock during summer, (2) greater human exposure to *E. coli* O157:H7-contaminated foods during cook-out months, and (3) greater improper handling including temperature abuse during summer months (Griffin and Tauxe, 1991; Meng et. al., 2001). Hussein and Bollinger (2005) reported prevalence rates of *E. coli* O157:H7 in cattle ranging from 0.3 to 19.7 % in the feedlot, 0.7 to 27.3 % on pastures,

and 0.9 to 6.9 % on the range, which suggests that there is a high potential for infection and reinfection of cattle with *E. coli* O157:H7 during grazing of dense vegetation on pasture.

Meat as a source of *E. coli* O157:H7

Foods of animal origin, dairy cattle in particular have been epidemiologically linked as a source of *E. coli* O157:H7 infections. Doyle and Schoeni reported in 1987 that the isolation of *E. coli* O157:H7 from 3.7 % of beef, 1.5 % of pork, 1.5 % of poultry and 2 % of lamb samples obtained from retail indicating that this bacterium is associated with foods of animal origin and not specifically beef. Samadpour et. al. (2006) analyzed a total of 1,750 samples of ground beef, 100 samples of mushrooms, and 200 samples of sprouts over a period of 12 months. In their study PCR assays and were followed by cultural confirmations to determine the presence or absence of *E. coli* O157:H7. The authors reported 3.5 % positive samples for EHEC and 1.1 % positives for *E. coli* O157:H7 in ground beef, while 6 % positives for EHEC and 1.5 % positives for *E. coli* O157:H7 were found in sprouts. Out of the 100 mushroom samples 4 % were positive for EHEC but none of them were positive for *E. coli* O157:H7.

Even though ground beef has been implicated as the primary vehicle of transmission for *E. coli* O157:H7 (Griffin and Tauxe, 1991), outbreaks involving other foods such as mayonnaise, apple cider and yogurt (Morgan et. al., 1993) might be a concern for the safety of high-acid foods and the acid tolerance properties of this pathogen .

Isolation and Identification of *E. coli* O157:H7 from meat

E. coli O157:H7 can be isolated and identified from raw meats and other food products by two official methods: USDA and FDA method. Both these methods use an

enrichment technique, a rapid screening test, a highly selective medium for isolation and confirmational tests using biochemical and serological techniques.

Isolation and identification of *E. coli* from raw ground beef products according to the USDA method requires sample collection of five 65 g (+/- 2 g) samples that are representative of a batch and modified *E. coli* broth (with novobiocin) is added. A latex agglutination test is performed following incubation at 35 °C for 24 h. The latex agglutination test has a specificity > 90 %, sensitivity > 98 %, and the rate of false negatives if less than 2 %. The samples negative for *E. coli* O157:H7 are discarded while the positive samples are reported as potential positives and isolation procedures are followed (USDA-FSIS, 2005a).

The FDA method for testing for *E. coli* O157:H7 is outlined in the Bacteriological Analytical Manual (BAM). According to this method 25 g of a sample is required and at times 50 g portions can also be used with appropriate scale up of the suspending media (Selective enrichment broth). Enrichment broths are made highly selective by adding certain antibiotics such as cefixime, vanomycin, and cefsulodin that help to inhibit growth of the microflora associated with the food products (BAM, 2002). Unlike typical *E. coli*, isolates of O157:H7 do not ferment Sorbitol and are negative with the MUG assay. Therefore, this criterion is commonly used for selective isolation of *E. coli* O157:H7. Latex agglutination tests using O157 and H7 antiserum are performed for confirmation of presumptive positives. These confirmed tests are then isolated using commercial biochemical identification kits. Polymerase chain reaction (PCR) methods are performed once *E. coli* O157:H7 is identified to determine the type of toxin production. Feng et. al. (2000) suggested that the use of an

O157:H7 specific DNA probe and multiplex PCR assay can be employed to detect *E. coli* O157:H7 and the toxins produced by this pathogen.

Table 2: Microbiological results of raw ground beef products analyzed for *Escherichia coli* O157:H7, summarized by calendar year (Source: USDA-FSIS, 2005b).

	2003		2004		2005[a]	
Source	Analyzed	Positive	Analyzed	Positive	Analyzed	Positive
Federal Plants	5,735	20	7,683	14	10,866	18
Retail Stores	779	0	311	0	95	0
State Plants	39	0	0	0	0	0
Imports	31	0	16	0	15	1
Totals	6,584	20	8,010	14	10,976	19

[a] During October 2005, a new screening method was introduced to reduce the number of screen positives that do not confirm positive.

Outbreaks due to *E. coli* O157:H7

There have been a number of very large outbreaks due to *E. coli* O157:H7 around the world and their public impact has often been dramatic. The Centers for Disease Control and Prevention (CDC) has reported that *E. coli* O157:H7 causes an estimated 73,000 infections resulting in more than 2,000 hospitalizations and 61 deaths annually in the United States (Mead et. al., 1999). The annual cost of illness due to O157:H7 STEC was $405 million (in 2003), including $370 million for premature deaths, $30 million for medical care, and $5 million in lost productivity (Frenzen et. al., 2005). Over the last two decades this organism

has frequently been implicated in causing diarrhea with bloody stools and the primary cause of HUS with a fatality rate of 5 % (Laine et. al., 2005).

Table 3: Major outbreaks of *Escherichia coli* O157:H7 infections.

Year	Location	No. of Cases	Vehicle	Reference
1982	Oregon	26	Ground Beef	Wells et. al. (1983)
1982	Michigan	21	Ground Beef	Wells et. al. (1983)
1982	Ontario	31	Ground Beef (primary) Person-to-person (Secondary)	Bell et. al. (1994)
1984	Nebraska	34	Ground Beef	Ryan et. al. (1986)
1984	North Carolina	36	Person-to-person	Spika et. al. (1986)
1985	Ontario	73	Ham, turkey, cheese sandwiches (Primary) Person-to-person (Secondary)	Carter et. al. (1987)
1986	Ontario	46	Raw Milk	Anonymous (1986)
1986	Alberta	16	Ground Beef	Honish (1986)
1986	Washington	37	Ground Beef	Griffin et. al. (1988)
1987	England	26	Turkey roll sandwiches	Salmon et. al. (1989)
1987	Utah	51	Ground Beef	Pavia et. al. (1990)
1988	Minnesota	30	Ground Beef	Belongia et. al. (1989)
1993	Multi-state	600	Ground Beef	Adams and Moss (2000)
1996	Scotland	500	Cross contamination of cooked meats from raw meats	Adams and Moss (2000)
1996	Japan	11,826	White radish sprouts	Michino et. al. (1998)

Two outbreaks of hemorrhagic colitis in the states of Oregon (26 cases) and Michigan (21 cases) were the first incidences of *E. coli* O157:H7 outbreaks causing human illness in the US in 1982 (Wells et. al., 1983). These outbreaks were epidemiologically linked to eating hamburger sandwiches at outlets of the same fast food chain. Since then several outbreaks of *E. coli* O157:H7 have occurred in the United States and other parts of the world. Ground beef has been implicated or suspected to be the vehicle or infection in most of these outbreaks.

Table 2 provides a summary of major outbreaks of *E. coli* O157:H7. The CDC reported that a total of 38 confirmed outbreaks of *E. coli* O157:H7 infections occurred in 1999 which is fewer than the 45 reported for 1998. But five of the 38 outbreaks in 1999 involved 2 or more states compared to only two of the 45 outbreaks in 1998.

E. coli O157:H7 surveillance systems

The Centers for Disease Control and Prevention currently has six surveillance systems for obtaining information about *E. coli* O157:H7. These six systems provide various features of the organism's epidemiology and serve different purposes. These six systems are as follows (CDC, 2006a):

1. **Public Health Laboratory Information System (PHLIS):** is a passive laboratory based surveillance system that collects data from the state public health laboratories about many infections including *E. coli* O157:H7.
2. **National Electronic Telecommunications System for Surveillance (NETSS):** is a passive physician based surveillance system that gathers both laboratory based and clinically suspected cases of all nationally notifiable diseases including *E. coli* O157:H7.
3. **FoodNet:** this is an active surveillance system for identifying and characterizing confirmed infections that may be foodborne, including *E. coli* O157:H7. In addition to monitoring the number of *E. coli* O157:H7 infections, investigators monitor laboratory techniques for isolation of bacteria
4. **National Molecular Subtyping Network for Foodborne Diseases Surveillance (PulseNet):** is a network of public health laboratories that perform pulsed-field gel electrophoresis (PFGE) on certain foodborne bacteria including *E. coli* O157:H7. This

system helps to determine if individual infections are related or if an outbreak is occurring. PulseNet is not a surveillance system itself but a laboratory subtyping method that aids in surveillance.

5. **National Antimicrobial Resistance Monitoring System (NARMS):** this passive surveillance system monitors the antimicrobial resistance of food borne bacteria including *E. coli* O157:H7.
6. **Foodborne Outbreak Detection Unit:** CDC monitors outbreaks of foodborne disease, including outbreaks caused by Shigella. While outbreaks account for a small percentage of the total number of illnesses that occur each year, these investigations provide valuable information about sources of foodborne infection. It also provides important information in regards to prevention methods to minimize and/ or inhibit number of cases during outbreaks.

Syndromes caused by *E. coli* O157:H7

E. coli O157:H7 infection typically leads to quite severe illness and can be expressed as hemorrhagic colitis, hemolytic uremic syndrome (HUS), or thrombocytopenic purpurea (TPP; Meng et. al., 2001). Hemorrhagic colitis is an illness consisting of crampy abdominal pain and watery diarrhea progressing to a bloody diarrhea and hemorrhagic discharge resembling lower gastrointestinal bleeding. The symptoms generally persist for several days to a few weeks.

Hemolytic uremic syndrome (HUS) is a serious illness that usually affects children and is the leading cause of acute renal failure in children. It starts with a bloody diarrheal illness resembling hemorrhagic colitis, and then is followed by a triad of features that define

HUS: (1) acute renal failure, (2) microangiopathic hemolytic anemia (intravascular coagulation of erythrocytes resulting in mechanical damage of erythrocytes), and (3) thrombocytopenia (low circulating platelets). Patients with HUS generally require dialysis and blood transfusions and may prove to be fatal.

Thrombocytopenia purpurea (TPP) is a typical feature but causes symptoms similar to HUS and is therefore occasionally missed. TPP largely affects adults and has histological resemblance to HUS, but is a rare syndrome of *E. coli* O157:H7 infection. Patients suffering from TPP often develop blood clots in the brain and usually results in death.

Heat resistance and acid tolerance of *E. coli* O157:H7

The primary means of eliminating pathogenic microorganisms in foods is cooking and the degree of microbiological control that can be achieved is dependent on a number of factors such as time, temperature, thermal tolerance of the microorganisms and characteristics of foods (Buchanan and Edelson, 1999a). The success of a cooking step for elimination of pathogenic bacteria depends upon the accurate information about the thermal tolerance of target pathogens. Effective clinical treatments are available for most human pathogens, however when microorganisms develop resistance to commonly used preservation methods, serious complications can occur especially for very young, the elderly and immunocompromised individuals (Bower and Daeschel, 1999).

Bacteria often become acid-adapted in that exposure to moderate acid environments increases their acid tolerance (Marshall, 2003). But the acid resistance of bacteria varies from organism to organism and on the environment they were subjected to before and during acid challenge. Organic acid sprays including acetic, citric, and lactic acid at concentrations of up

to 1.5 % on beef have revealed the *E. coli* O157:H7 populations were not appreciably reduced (Brackett et. al., 1994). Acid tolerance is an important virulence determinant that contributes to the survival and pathogenicity of infectious foodborne pathogens such as EHEC, *Salmonella*, and *Shigella* spp. to cause disease (Buchanan and Edelson, 1999b).

The mechanism of acid tolerance has not yet been fully elucidated but has been associated with proteins that can be induced by preexposing the bacteria to acidic conditions. Acid resistance (habituation) refers to the extended exposure of a microorganism to moderately acidic conditions leading it to being able to withstand pH values of < 2.5, while acid tolerance refers to enhanced survival of a microorganism exposed to pH values between 2.5 and 4.0 after a brief exposure to moderately acidic conditions (Foster, 1995). Bearson et. al. (1997) suggested that acid tolerance and acid resistance/ habituation responses can produce cross-protection against several other stresses including heat.

Doyle and Schoeni (1984) reported that *E. coli* O157:H7 did not have any unusual heat resistance and is more sensitive to heat than salmonellae, however *Salmonella* and *E. coli* O157:H7 can survive in ground beef at -20 °C for several months without a major change in numbers. Line et. al. (1991) conducted studies to compare D-values of *E. coli* O157:H7 in lean (2 % fat) and fatty (30.5 % fat) ground beef and suggested a higher D-value for the fatty ground beef. The D-values were reported to be 78.2 and 115.5 min at 125 °F; 4.1 and 5.3 min at 135 °F; and 0.3 and 0.5 min at 145 °F for the lean and fatty ground beef respectively. According to Leyer et. al. (1995), acid-adapted *E. coli* O157:H7 showed an increased resistance to lactic acid and survived better than non adapted cells during sausage fermentation showing enhanced survival in shredded dry salami (pH 5.0) and apple cider (pH 3.4). Glass et. al. (1992) suggested that *E. coli* O157:H7 can survive during fermentation,

drying, and storage of fermented sausage (pH 4.5) for up to 2 months at 4 °C, with only a 100-fold decrease in the cell population.

Studies conducted by Arnold and Kaspar (1995) indicated that although the degree of acid tolerance of *E. coli* O157:H7 may vary among the strains, survival of most of these strains exceeded that of other related pathogens in a synthetic gastric fluid. Cell density of *E. coli* O157:H7 is an important factor that has an impact on the acid sensitivity. Research has shown that at low cell densities (ca. 2 X 10^7 per ml) approximately 100 % of the stationary phase cells survived in Luria broth (pH 2.5) at 37 °C for at least 7 h, while at higher cell densities (ca. 2.5 X 10^9 per ml) they were a 1000-fold more sensitive under identical conditions (Datta and Benjamin, 1999). Cheng et. al. (2002) reported that the survival of acid adapted *E. coli* O157:H7 varied with strain and the type of subsequent stress. Results from their study indicated that there was an increased thermal tolerance noticed in the acid adapted strains while no difference was observed from their non adapted counterparts when stressed with bile salts. Juneja et. al. (1998) reported that the thermal tolerance of heat shocked *E. coli* O157:H7 in ground beef was lost after 14 h when stored at 4 °C, however the thermal tolerance of these heat shocked *E. coli* O157:H7 was maintained in ground beef for at least 24 h when held at 15 and 28 °C.

Results from a study conducted by Buchanan and Edelson (1996) suggested that prior growth of *E. coli* in a medium with and without a fermentable carbohydrate is a convenient way of studying the induction of acid tolerance, and that pH-dependent or pH-independent stationary phase acid tolerance phenotypes may exist among different strain foe EHEC. Prerigor beef carcass surface tissue inoculated with bovine feces containing either acid-adapted or unadapted *E. coli* O157:H7 and treated with water wash or a 2 % acetic acid wash

lead to larger populations of acid-adapted cells remaining on the carcasses as compared to non-adapted cells and these differences were observed over a period of 14 days at 4 °C storage (Berry and Cutter, 2000).

USDA-FSIS Directive for *E. coli* O157:H7

The purpose of this directive is to provide FSIS inspection program personnel, program investigators, and import inspection personnel instructions for sampling raw beef products as part of verification testing for *E. coli* O157:H7 to ensure the protection of public health. According to this directive all establishments in the United States that produce raw ground beef products including ground beef patties and raw ground beef components will be subject to FSIS sampling and testing for *E. coli* O157:H7 (USDA-FSIS, 2004a). In 1994 the FSIS declared all raw ground beef contaminated with *E. coli* O157:H7 to be adulterated unless it is further processed to destroy the pathogen and on January 19, 1999 FSIS stated that intact cuts of beef that are to be further processed into non-intact cuts prior to distribution for consumption must be treated in the same manner as non-intact cuts of beef because pathogens may be introduced below the surface of these products when they are processed into non-intact (USDA-FSIS, 2004b). The directive also states the "sampling may vary depending upon the prevalence and exposure of *E. coli* O157:H7 such as volume of production of ground beef in an establishment, season of the year, and number of suppliers for an establishment".

Importance of *Salmonella enterica* in foods

Over 2,000 serotypes of *Salmonella* have been identified to date, some of which cause serious illness in humans particularly in vulnerable populations such as elderly. The widespread occurrence of salmonellae in the natural environment and their prevalence in various global food markets are the primary reasons that *Salmonella* spp. is the leading cause of bacterial foodborne illnesses. Salmonellosis is described as a zoonotic infection since the major source of human illness is infected animals. This pathogen is transmitted by the fecal-oral route, whereby a period of temperature abuse allows Salmonellae to grow in the food and an inadequate final heat treatment is a common means of foodborne outbreaks (Adams and Moss, 2000). Meat, milk, poultry, and eggs are primary vehicles that lead to human illness due to undercooking or cross contamination.

Salmonella spp. are facultatively anaerobic, gram negative, rod-shaped bacteria that grow optimally at 37 °C (D'Aoust et. al., 2001). Some Salmonella strains can grow at elevated temperatures (≤ 54 °C), and others can exhibit psychrotrophic properties by growing in foods stored at 2-4 °C (D'Aoust, 1991). Studies have been done to suggest that pre-conditioning of the cells to low temperatures significantly increases the growth and survival of salmonellae under refrigerated temperatures (Airoldi and Zottola, 1988). The psychrotrophic nature of *Salmonella* spp. can be of major concern due to the widespread refrigerated storage of vacuum or modified atmosphere packaged foods. The optimal growth of salmonellae is demonstrated at pH 6.5 to 7.5 but this organism has also been known to grow over a wide range of pH (4.5 to 9.5).

Reservoirs of *Salmonella*

Salmonella spp. is widely spread in the natural environment. The primary habitat of *Salmonella* spp. is the intestinal tract of animals such as birds, reptiles, farm animals, humans and occasionally insects (Jay, 2000; Adams and Moss, 2000). Polluted water and foods that have been contaminated with insects or by other means when consumed by humans and other animals, *Salmonella* is shed through the fecal matter which helps in the continuation of the cycle. This continuation of the cycle through import and export of animal products and feeds is a major factor for the world wide distribution of Salmonellosis (Jay, 2000). Direct person-to-person spread by the fecal oral route is also possible but usually this is restricted to outbreaks that involve institutions such as hospitals, nursing homes, and nurseries.

In the many sectors within the meat industry, poultry and eggs are the predominant reservoir of *Salmonella* spp. in many countries and tend to overshadow the importance in other meats such as pork, beef, and mutton as potential vehicles of infection (D'Aoust et. al., 2001). Food animals may acquire *Salmonella* infection on the farm from rodents, wild birds and primarily from other animals that are carriers of this organism. According to Adams and Moss (2000), transfer of *Salmonella* between animals is particularly linked with situations where animals are stressed and crowded such as during transport, at the market, and at the slaughter house.

Meat as a source of *Salmonella*

A study of meat and poultry products during 1983-1986 reported salmonellae on 17.5 % of 596 pork samples, 69.1 % of 230 turkey samples, 60.9 % of 670 chicken samples, and only 2.6 % of beef samples (Lammerding et. al., 1988). From this study and table 4 it can

be seen that salmonellae is not very highly prevalent in beef as compared to pork and poultry meat product. *Salmonella* serovars are a significant hazard in raw meat and poultry products and processors of raw meat products may have limited control over whether this pathogen is present in the raw meat that they receive for processing (Ingham et. al., 2004). Therefore, meat processors must select a Critical Control Point (CCP) and associated critical limits in order to minimize the risk that will arise from contaminated raw ingredients.

Table 4: Percentage of salmonellae recovered from various raw commodities and animal matter (Source: Jay, 2000).

Products	Country	Years	No. of Samples	%
Broiler carcasses	USA	1994-95	1,297	20
Steer/ heifer carcasses	USA	1992-93	2,089	1.0
Ground beef	USA	1993-94	563	7.5
Pork carcasses	Belgium	1998	49	27
Beef carcasses	Belgium	1998	62	0
Poultry carcasses	Spain	1997	192	60
Poultry livers	Spain	1997	192	80

Outbreaks due to *Salmonella*

Salmonella infections cause an estimated 1.4 million human illnesses and 400 deaths annually in the United States (Voetsch et. al., 2004). However, most cases of salmonellosis do not result in a visit to the doctor and are not reported to the public health agencies leading to a high proportion of unreported cases that makes it difficult to determine the true incidence of *Salmonella* infection. According to the 2005 FoodNet surveillance a total of 16,614

laboratory-confirmed cases of infections were identified out of which 6,471 were due to *Salmonella* (CDC, 2006b). This report also identified that of the 5,869 isolates of *Salmonella* serotyped, six accounted for 61 % of the infections as follows: Typhimurium (19 %), enteritidis (18 %), Newport (10 %), Heidelberg (6 %), and javiana (5 %). Mead et. al. (1999) reported that out of a total of approximately 1.4 million cases of salmonellosis occurring annually, 824 illnesses are caused by *S.* Typhimurium out of which 659 are foodborne that lead to 494 hospitalizations and 3 deaths. The estimated annual costs (in 1998) including medical care and lost productivity due to foodborne *Salmonella* infections were $0.5 billion based on the human capital approach and while using the more conservative labor market approach the annual costs were estimated at around $2.3 billion (Frenzen et. al., 1999).

Outbreaks have historically been linked but not limited to raw foods of animal origin together with cross-contamination to ready-to-eat foods through inadequate processing or personal hygiene as being significant contributory factors to the cause of outbreaks. Consumption of raw and undercooked ground beef was implicated in a multi-state outbreak involving *S.* Typhimurium (CDC, 2006c). Dechet et. al. (2006) identified 58 cases of *S. enterica* serotype Typhimurium definitive type 104 infection in 9 stated by PFGE as a result of consuming store-bought ground beef prepared as hamburgers at home. Product trace back linked all these cases to a single large ground beef manufacturer that had previously been implicated in a multistate outbreak of highly drug resistant *S. enterica* Newport infections in 2002. Three outbreaks of *Salmonella* infection associated with eating Roma tomatoes were reported in the United States and Canada in the summer of 2004 (CDC, 2005). In the three outbreaks 561 outbreak-related illnesses from 18 states and one province in Canada were identified.

According to the CDC (1997) most of the DT104 infections in the United States are sporadic and only five outbreaks have been reported in literature that have been associated with consumption of contaminated dairy products or contact with animals, suggesting a cattle reservoir (Dechet et. al., 2006). Illnesses caused by multi-drug resistant *Salmonella* are more severe than those caused by susceptible *Salmonella* species, resulting in increases rate of hospitalization and death (Helms et. al., 2002).

Symptoms of disease caused by *Salmonella enterica*

The infective dose for causing foodborne salmonellosis in humans was believed to be very high (~ 10^5 to 10^6 cells) for a number of years, but there have been various outbreaks in which the infective dose was found to be as low as < 10-100 cells (Bell and Kyriakides, 2002). Newborns, infants, the elderly, and immunocompromised are more susceptible to *Salmonella* infections as compared to healthy adults. *Salmonella* infection in humans can lead to several clinical conditions including enteric (typhoid) fever, systemic infections by non-typhoid organisms and enterocolitis. According to D'Aoust (1991), enteric fever is a severe disease in humans that is associated with the typhoid and paratyphoid strains that are adapted for invasion and survival within the host.

The incubation period for enteric fever ranges from 7 to 28 days and common symptoms include diarrhea, prolonged and very high fever, abdominal pain, and headaches (D'Aoust et. al., 2001). Human infection with nontyphoid *Salmonella* spp. commonly results in enterocolitis, which appears 8 to 72 h after contact with the invasive pathogen. This is usually self limiting and characteristic nonbloody diarrhea and abdominal pain occur which generally disappear after 5 days of onset of symptoms. Some chronic conditions induced by

Salmonella spp. include aseptic reactive arthritis, Reiter's syndrome, and ankylosing spondylitis which are caused by bacteria that have the ability to infect mucosal surfaces, presence of an outer lipopolysaccharide, and the virulence to invade host cells (Smith, 1994).

Table 5: Human infectious dose of *Salmonella* (Source: D'Aoust, 2001).

Food	*Salmonella* serovar	Infectious dose (Cells)
Eggnog	Anatum	10^5-10^7
Imitation ice cream	Typhimurium	10^4
Hamburger	Newport	10^1-10^2
Cheddar cheese	Heidelberg	10^2
	Typhimurium	10^0-10^1
Chocolate	Typhimurium	$\leq 10^1$
Alfalfa sprouts	Newport	$\leq 4.6 \text{ X } 10^2$
Ice cream	Enteritidis	$\leq 2.8 \text{ X}10^1$

Heat resistance and acid tolerance of *Salmonella enterica*

Goodfellow and Brown (1978) conducted studies to determine the fate of Salmonella in ground beef during cooking. Results from this study suggested that the D-values were 61-62, 3.8-4.2, and 0.6-0.7 min. at 51.6, 57.2, and 62.7 °C respectively. Conditions such as reduced water activity, increased pH, elevated processing temperatures and a variety of spice and cure mixtures have been thought to reduce and eventually eliminate viable salmonellae (Masters et. al., 1981), but there have been studies that suggest the ability of salmonellae to survive such conditions that are encountered during the production of fermented meats (Smith et. al., 1975).

Preshocked cells of *Salmonella* at pH 6.0 for 1 h have demonstrated 100 to 1000-fold better survival to challenge exposure pH 3.3 (acid shock) as compared to non-adapted cells (Foster, 1991). Walsh et. al. (2005) studied the thermal resistance of antibiotic resistant (AR) and antibiotic sensitive (AS) *Salmonella* spp. on chicken meat and reported no significant differences between the D-values of the AR and AS strains of *Salmonella* although D-values of *S.* Typhimurium DT104 (multidrug resistant strain) were significantly higher. In the same study it was also suggested that heat shocked cultures of *S.* Typhimurium DT104 had significantly higher D-values than their non heat shocked counterparts.

According to Foster and Hall (1990), this organism possesses a novel system of acid stress management including an inducible pH homeostasis system. pH homeostasis has been defined by Foster and Hall (1991) as "the process whereby a cell maintains a relatively constant intracellular pH over a broad range of external pH values". *S. Dublin* was able to survive in fermented and unfermented products during the 15-30 days commercial drying period and was present in the products after 43 days of drying (Smith et. al., 1975). The authors also reported that *S.* Typhimurium was not able to survive in fermented sausage (pepperoni) after 42 days of drying, and heating salmonellae-contaminated beef-pork pepperoni to an internal temperature of 60 °C eliminated all foodborne pathogen from the sausage product.

Masters et. al. (1981) conducted studies on the fate of *S. Newport* and *S.* Typhimurium in summer sausages. Their studies suggested that the rate of fermentation during sausage production along with other factors such as processing time/ temperature, level of *Salmonella* contamination, and the serotype of *Salmonella* present as a contaminant affect the survival of this organism in summer sausages. Uhart et. al. (2006) reported that

Salmonella Typhimurium DT104 growth was inhibited or inactivated when spices were in direct contact with a food system but did not inhibit growth when added to a complex food system such as ground beef and stored at 4 °C for 10 days.

Table 6: Minimum pH at which salmonellae would initiate growth under optimum laboratory conditions (Source: Jay, 2000).

Acid	**pH**
Hydrochloric	4.05
Citric	4.05
Tartaric	4.10
Gluconic	4.20
Fumaric	4.30
Malic	4.30
Lactic	4.40
Succinic	4.60
Glutaric	4.70
Adipic	5.10
Pimelic	5.10
Acetic	5.40
Propionic	5.50
Tryptone-yeast extract-glucose broth was inoculated with 10^4 cells/ ml of S. Anatum, S. Tennessee, or S. Seftenberg.	

Broths acidified with hydrochloric acid (pH 3.0), citric acid (pH 3.0), or lactic acid (pH 3.8) and inoculated with acid shocked and unshocked serovars of *Salmonella* have been evaluated for growth and survivability of these cells. Arvizu-Medrano and Escartin (2005) found that the biggest difference in reduction of survival between shocked and unshocked strains (~ 2 CFU/ ml) was observed when the microorganisms were shocked with lactic acid and then challenged with citric acid. Acid adaptation of *Salmonella* Typhimurium induces cross-protection against heat, high osmolarity, and the lactoperoxidase system which could

be reasons for the survival mechanism enabling this organism to persist in fermented dairy products and possibly other acidic foods (Leyer and Johnson, 1992).

Sharma et. al. (2005) suggested that pasteurization conditions necessary to eliminate pathogens such as *Salmonella* and *E. coli* O157:H7 from cantaloupe and watermelon juice would need to be more severe if cells are habituated to acidic environments as a result of increases thermal tolerance in liquids foods but did not find any correlation between the soluble solids content of the two juices and thermal resistance. Studies have also been conducted on effects of prior heat shock on thermal tolerance of *Listeria monocytogenes* and *S.* Typhimurium (Bunning et. al., 1990). Results from this study suggested that prior heat shock induced increased thermotolerance in *S.* Typhimurium but did not affect the thermal tolerance of *L. monocytogenes* in broth cultures. Oscar (1999) reported that exposing *Salmonella* to a previous temperature ranging from 16 to 34 °C does not effect the lag time and specific growth rate of the organism when exposed to temperatures of 16 to 34 °C on cooked ground chicken breasts. Another study conducted by Calicioglu et. al. (2003) reported that acid adaptation of *Salmonella* did not increase the survival of the pathogen and may have reduced its population during storage when marinades (modified with 1.2 % sodium lactate, 9 % acetic acid, and 68 % soy sauce containing 5 % ethanol) were used in jerky processing.

USDA-FSIS Performance standards for *Salmonella*

On July 25, 1996, FSIS published its final rule on Pathogen Reduction and Hazard Analysis Critical Control Point (HACCP) Systems, which established new requirements for all meat and poultry products to improve food safety (USDA-FSIS, 2000). To verify that

HACCP systems are effective in controlling contamination of raw product from harmful bacteria, the rule sets pathogen reduction performance standards for *Salmonella* that slaughter plants and plants that produce raw ground products must meet. FSIS verifies that plants are meeting the standards by taking product samples and analyzing them for *Salmonella* in FSIS laboratories (USDA-FSIS, 2000). *Salmonella* was selected as the target pathogen because it is one of the most common causes of foodborne illness and it can be isolated from a variety of food products.

FSIS requires that beef, swine, and chicken carcasses be sampled for *Salmonella* testing. There are no published performance standards for turkey carcasses. Yet the goal of the *Salmonella* testing program is to protect the consumer from contaminated products, especially from fecal contamination, by verifying that each establishment's performance meets the *Salmonella* standards as per regulatory requirements. Samples are taken in sets and the results of an entire set are used to determine if an establishment is meeting the performance standards. Failure to meet *Salmonella* performance standards is based on whether or not a set passes, not on individual samples. A *Salmonella* test is positive when any *Salmonella* organisms are found (USDA-FSIS, 2004c).

Acid tolerance responses in *Salmonella*

S. Typhimurium has the ability to grow over a wide range of pH values, and during the course of infection it is exposed to potential lethal acidic environments such as those found in the stomach of humans. Acid tolerance response (ATR) is an adaptive system triggered at external pH values of 5.5 to 6.0 that protect cells from severe acid stress (Foster and Hall, 1991). The adaptive ability of Salmonella to survive harsh acidic environments has

been explained by Foster and Hall (1990) and they characterized the ATR phenomenon in which exposure to slight or moderate acid stress results in synthesis of proteins that protect the bacterium from more severe acid challenge. Wilmes-Riesenberg et al. (1996) suggested that this ATR is essential for acid adapted bacterium to survive acidic environments within the host cells. There have been studies conducted recently that provide evidence that at least two distinct pH-dependent ATR systems exist in *S.* Typhimurium (Lee et. al., 1994). The two ATR systems are observed in the logarithmically growing cells (induced after a shift of pH 5.8) and at the stationary phase of growth of the cells (induced after a shift of pH $\leq$ 4.5). The logarithmic ATR is referred to as a pre-acid shock and the proteins induced at pH 5.8 induce pH homeostasis that helps maintain intracellular pH, whereas the ATR at stationary phase is shown to exhibit sustained induction over a course of several hours as compares to the transient induction during the log phase (Lee et. al., 1994).

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Effect of acid adaptation on thermal tolerance of *Escherichia coli* O157:H7 and *Salmonella enterica* in meat serum

A paper to be submitted to the JOURNAL OF FOOD PROTECTION

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Abstract

Escherichia coli O157:H7 and *Salmonella* were adapted to acidic conditions by growing in Tryptic Soy Broth with 1 % glucose (TSB+ 1%G). Ability of acid adaptation of these pathogens to provide resistance against thermal stress was evaluated in meat serum. Five-strain cocktail of both bacteria were grown separately in TSB and TSB+1%G for 24 h at 37 °C to provide cells with or without acid adaptation. Meat serum was prepared from irradiated ground beef and inoculated with either acid adapted or non-adapted *E. coli* O157:H7 and *Salmonella*. Inoculated meat serum was then subjected to heat treatment at 58, 62, and 65 °C to determine D-values of the pathogens. Significantly higher ($P<0.05$) D-values of the acid adapted strains was observed as compared to the non-adapted *E. coli* O157:H7 at 58, 62, and 65 °C. Higher ($P<0.05$) D-values of acid adapted *Salmonella* were observed at 58 and 62 °C, but no statistical difference ($P>0.05$) was seen at 65 °C. D-values were observed to be 22.46 and 10.58 min. at 58 °C; 3.58 and 1.38 min. at 62 °C; 1.02 and 0.75 min. at 65 °C for acid adapted and non-adapted *E. coli* O157:H7 respectively. D-values of the acid adapted *Salmonella* were 9.36 min. at 58 °C, 1.66 min. at 62 °C, and 1.14 min. at 65 °C whereas the non-adapted counterparts had D-values of 6.44, 0.88, and 0.95 min. at 58,

62, and 65 °C respectively. This indicates that acid adaptation of food borne pathogens provides cross-protection against heat treatments.

Introduction

Escherichia coli O157:H7 has become a pathogen of concern in the food industry since its initial implication in various foodborne outbreaks (Mermelstein, 1993). An estimated 73,000 cases of infection and 61 deaths occur in the United States each year as a result of *E. coli* O157:H7 infection (CDC, 2005). Infection often leads to bloody diarrhea and occasionally kidney failure. The primary reason for outbreaks of *E. coli* O157:H7 have been linked to undercooked ground beef (Pandhye and Doyle, 1992), however extensive research has shown that this organism does not possess any unusual heat resistance (Doyle and Schoeni, 1984; Ahmed et. al., 1995). Person-to-person contact in families and child care centers is also an important mode of transmission. Salmonellosis is another important public health problem in the United States with an estimated number of nontyphoidal *Salmonella* infections ranging from 800,000 to 4,000,000 annually (Voetsch et. al., 2004). Although most outbreaks cause mild to moderate self limited illness, serious disease resulting in death does occur particularly in elderly and immunocompromised populations. The Centers for Disease Control and Prevention (CDC) along with the Foodborne Disease Active Surveillance Network (FoodNet) have indicated about 1.4 million cases of salmonellosis annually (Mead et. al., 1999). Accounting for medical costs and lost productivity the estimated costs associated with salmonellosis is approximately $2.3 billion (Frenzen et. al., 1999). *S. typhi* has been shown to have the ability to adapt and increase its acid tolerance during its exponential phase of growth in culture medium (Tiwari et. al., 2004).

Resistance and/ or tolerance to acidic conditions has an important impact on virulence determinants that contribute to the survival and pathogenicity of certain foodborne pathogens such as *E. coli*, *Salmonella*, *Shigella* spp., and *Listeria monocytogenes*. These acid resistant/ tolerant pathogens have a greater likelihood of surviving in acidic foods for extended period (Buchanan et. al., 1994; Leyer and Johnson, 1995; and Miller and Kaspar, 1994) and increased portion of the population that can survive the gastric environment (Gorden and Small, 1993) which enhances the infectivity once these pathogens attach to the intestinal tract (O'Driscoll et. al., 1996). Bearson et. al. (1997) reported that acid tolerance and acid resistance/ habituation produce cross-protection against several other stresses including heat, with *S.* Typhimurium showing increased resistance to heat, osmotic stress, and crystal violet (Leyer and Johnson, 1993). Heat shocked *E. coli* O157:H7 has been reported to have longer survivability than the non-heat-shocked cells. Juneja et. al. (1998) suggested a 1.56-fold increase in the 'time of 4D inactivation' (T_{4D}) values at 60 °C in beef gravy. Oyarzabal et. al. (2003) showed recoverable populations of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* in acidic juices such as apple, orange, pineapple, and white grape juice concentrates for up to 12 weeks when inoculated with levels of $\geq 10^3$ CFU/ g and stored at -23 °C.

Numerous studies have been conducted on the thermal tolerance of acid adapted foodborne pathogens in fruit juices and laboratory media but there is limited literature on the thermal tolerance of acid adaptation of these foodborne pathogens in actual food systems. Studies in the past have been conducted at mild heating temperatures whereas effects of heating to temperatures that are more indicative of cooking temperatures have rarely been

researched. The objective of the present study was to assess the ability of acid adapted *E. coli* O157:H7 and *Salmonella* spp. to survive thermal treatment at 58, 62, or 65 °C in meat serum.

Materials and Methods

Preparation of bacterial cultures.

A five-strain cocktail of *Escherichia coli* O157:H7 [ATCC 35150 (human feces from outbreak of hemorrhagic colitis); ATCC 43894 (human feces from outbreak of hemorrhagic colitis); ATCC 43895 (isolate from raw hamburger implicated in hemorrhagic colitis); WS 3062 (clinical isolate); and WS 3331 (clinical isolate)] and *Salmonella enterica* (*S. Newport*, *S. Uganda*, *S. Heidelberg*, *S. Typhimurium*, and *S. enteritidis*) was used. The five bovine strains of *Salmonella* spp. were obtained from the veterinary diagnostic laboratory at Iowa State University, Ames. IA. The stock cultures were maintained on Tryptic Soy Agar (TSA; Difco, Becton Dickinson, Sparks, MD) slants at 4 °C. Working cultures were maintained by daily transfers in Tryptic Soy Broth (TSB; Difco, Becton Dickinson, Sparks, MD) and TSB + 1% glucose for the non-adapted and acid adapted strains respectively. Growth curves for individual strains of *E. coli* O157:H7 and *Salmonella* spp. were constructed to determine the time at which stationary phase was reached in order to make subsequent transfers (Data not shown). The non-adapted and acid adapted inoculum was prepared by inoculating bacterial cultures into 10 ml TSB and TSB + 1% glucose respectively and then incubating at 37 °C for 24 h. Addition of glucose to the growth medium allows the production of organic acids, which lower the final pH of the culture medium significantly (Buchanan and Edelson, 1999). Cultures (1ml) were then transferred into 25 ml TSB centrifuge tubes and further incubated at 35 °C for 18 h. The cultures were then centrifuged at 5,738 X g for 10 min. at 4 °C

(SORVALL SUPER T21, Newton, CT). The supernatant was decanted and resultant pellet resuspended with 10 ml of 0.1% sterile peptone water (PW; Difco, Becton Dickinson, Sparks, MD). A cocktail was prepared by mixing the five cultures in a sterile bottle to get a final volume of 50 ml of the inoculum.

Preparation and inoculation of meat serum.

Ground beef was obtained from the Iowa State University Meat Laboratory and irradiated at the Iowa State University Linear Accelerator Facility to an average absorbed dose of 8.05 kGy. Ground beef was irradiated to remove any background gram negative microflora. Meat serum was then prepared by making a 1:1 dilution of the irradiated ground beef with 0.1 % PW and homogenizing in a stomacher (Stomacher 400, Tekmar, Cincinnati, OH) for 2 minutes. This was followed by centrifugation at 5,738 X g for 10 min. at 4 °C. The supernatant was decanted into a sterile Wheaton bottle and then filtered through a 0.22 µm Millex-GP® sterilizing filter unit (Millipore Corp., Billerica, MA). Filtered meat serum was then divided into four-120 ml parts in sterile 250 ml wheaton bottles. This 120 ml sterile meat serum was inoculated with acid adapted or non inoculated *E. coli* and *S. enterica*. All the inoculated bottles of meat serum were stored at 4 °C for 30 minutes. 5 ml of the inoculated meat serum was then measured into 3" X 7" WHIRL-PAK® bags (2.25 mil thick; Nasco, Fort Atkinson, WI) prior to determination of D-values. Bags containing inoculated meat serum were kept at 4 °C until the time of thermal treatment.

Thermal resistance in meat serum (D-value determination).

Thermal tolerance of non-adapted and acid adapted strains was determined by calculating D-values at 58 (136.4 °F), 62 (143.6 °F) and 65 °C (149 °F). Thermal tolerance

was conducted for up to 1 h at 58 °C and up to 10 minutes at 62 and 65 °C. At each test temperature the 5 ml WHIRL-PAK® bags were completely immersed in a water bath maintained at 58, 62, or 65 °C. For D-values at 58 °C the bags were removed at 0, 10, 20, 30, 40, 50, and 60 min. while for 62 and 65 °C the bags were removed from the water bath at 0, 1, 2, 3, 4, 5, 7.5, and 10 min. An additional 5 ml meat serum WHIRL-PAK® bag was placed in each water bath at the three temperatures to monitor temperature increase. The timing for each experiment was not started until the 5 ml portions of inoculated meat serum had reached the same temperature as the water bath. This was designated as time "zero" in the study. Typical come up times were 45 s at 58 °C, 50 s at 62 °C, and 60 s at 65 °C. The WHIRL-PAK® bags were removed from the water bath and immediately placed in an ice water bath at each time interval. Each WHIRL-PAK® bag was allowed to cool down in the ice water bath for 10 min. prior to sampling.

Microbial sampling and enumeration.

The samples that had been subjected to 58, 62 or 65 °C were then aseptically opened and serially diluted in 0.1 % sterile PW. These serially diluted samples were then spread plated on to Tryptic Soy Agar (TSA). Since the ground beef was irradiated it was not necessary to use selective media for the recovery of the pathogens. Clavero and Beuchat (1996) suggested that a higher recovery of *E. coli* was achieved on TSA as compared to MacConkey agar and modified eosin methylene blue agar. The plates were then incubated at 37 °C for 48 h and colony forming units were manually counted and reported as log Colony Forming Units per ml ($\log_{10}$ CFU/ ml).

Experimental Design and Statistical Analysis.

Three independent replications of randomized complete block design were used to prepare four 120 ml bottles of the filtered meat serum which was inoculated with 25 ml of either non adapted or acid adapted *E. coli* O157:H7 and *Salmonella* spp respectively. The inoculated meat serum was divided into 5 ml WHIRL-PAK® bags in a completely randomized design prior to D-value testing at each temperature. Survival curves were constructed for organisms recovered on TSA, with Y-axis representing $\log_{10}$ CFU/ ml of each inoculum tested and X-axis representing time in minutes. Decimal reduction time (D-value) was calculated as the negative reciprocal of the slope of the survivor curve for each inoculum type exposed to 58 °C (D_{58}), 62 °C (D_{62}) and 65 °C (D_{65}). Three replications of the experiment were performed and the mean D-value of each inoculum was analyzed using analysis of variance (ANOVA) with the SAS PROC MIXED procedures (2002-03, SAS Institute, Cary, N.C.).

Results

Tolerance of acid adapted and non-adapted *Escherichia coli* O157:H7 to thermal stress.

Table 1 shows decimal reduction times (D-values) of acid adapted and non-adapted *E. coli* O157:H7 at 58 °C (D_{58}), 62 °C (D_{62}) and 65 °C (D_{65}). It can clearly be seen that there are significant differences ($p<0.05$) in D_{58}, D_{62} and D_{65} values of acid adapted and non-adapted strains of *E. coli* O157:H7. D-values of acid adapted *E. coli* O157:H7 were slightly more than twice the D-values of their non adapted counterparts at 58 and 62 °C. D-values at 65 °C

were 1.02 and 0.75 minutes for the acid adapted and non-adapted *E. coli* O157:H7 respectively.

Comparing survival populations of acid adapted and non-adapted *E. coli* O157:H7 at 58 and 62 °C (Fig. 1A and 1B) it was observed that the acid adapted populations had a higher survival rate than non-adapted populations. Populations of acid adapted and non-adapted *E. coli* O157:H7 were reduced by 2.71 and 5.85 $\log_{10}$ CFU/ ml when heated at 58 °C for 1 h. Figure 1A suggests a more linear reduction of the non-adapted *E. coli* O157:H7 as compared to their acid adapted counterparts at 58 °C. At 62 °C non-adapted *E. coli* O157:H7 were reduced from 7.10 $\log_{10}$ CFU/ ml to below detection limits (< 50 CFU/ ml of meat serum) after 10 min. while the population of acid adapted *E. coli* O157:H7 was reduced from 4.96 to 1.91 $\log_{10}$ CFU/ ml in the meat serum. Survival population curve of *E. coli* O157:H7 at 65 °C (Fig. 1C) suggests that both acid adapted and non-adapted strains were reduced to below detection limits (< 50 CFU/ ml) after 4 min. The acid adapted populations were reduced from 4.51 $\log_{10}$ CFU/ ml to below detection limits whereas the non-adapted counterparts were reduced from 6.84 $\log_{10}$ CFU/ ml to below detection limits within 4 min. of heating at 65 °C.

Tolerance of acid adapted and non-adapted *Salmonella* to thermal stress.

Table 2 shows D-values of acid adapted and non-adapted *Salmonella* at 58 °C (D_{58}), 62 °C (D_{62}) and 65 °C (D_{65}). Significant differences ($p<0.05$) in D_{58} and D_{62} values were observed whereas no significant differences ($p>0.05$) were seen for D_{65} values of acid adapted and non-adapted *Salmonella*. D-values of acid adapted *Salmonella* were approximately 1.5 times the D-values of non-adapted *Salmonella* at 58, 62, and 65 °C.

From figure 2A it can be seen that the non-adapted strains of *Salmonella* were reduced to below detection limit (<50 CFU/ ml) after 50 min. of heating at 58 °C while the acid adapted strains survived until 60 min. before they were reduced to below the detection limit. Non-adapted *Salmonella* displayed a fairly linear inactivation curve while the curve suggests less linearity for the acid adapted strains (Fig. 2A) at 58 °C. Acid adapted populations of *Salmonella* were reduced from 6.02 $\log_{10}$ CFU/ ml to below detection limit after 7.5 min. of heating at 62 °C whereas the non-adapted populations were reduced from 7.98 $\log_{10}$ CFU/ ml to below detection limit after 5 min. at the same temperature (Fig. 2B). Populations of acid adapted *Salmonella* were reduced from 4.35 $\log_{10}$ CFU/ ml to below detection limits after 4 min. at 65 °C while the populations of non-adapted *Salmonella* were reduced from 4.32 $\log_{10}$ CFU/ ml to below detection limits after 3 min. at 65 °C (Fig. 2C).

Discussion

It has been reported in numerous studies and well documented that environmental factors such as pH can affect the thermal resistance of microorganisms (Bearson et. al, 1997; Leyer and Johnson, 1993; and Jay 2000). During the process of carcass decontamination up to the stage of processing to produce various meat products for consumers food borne pathogens such as *E. coli* O157:H7 and *Salmonella* are exposed to low pH environments that may trigger protective responses to heat (Mazzotta, 2001). Hence when choosing the target organism in order to calculate lethality of a heat treatment it is necessary to select the most resistant pathogens that are likely to occur in food. This will help in adding an extra safety factor to the minimum regulatory requirements for heating/ cooking meat and poultry products.

Results from our study were consistent with a study conducted by Buchanan and Edelson (1999) that reported a higher D-value for three different strains of acid adapted *E. coli* O157:H7 grown in laboratory media. The D-values reported in their study were lower than those that were observed in our study at 58 °C, which can be attributed to the use of meat serum that could provide more protection to the bacteria as compared to laboratory media. Cheng et. al. (2002) demonstrated that acid adapted *E. coli* O157:H7 were more thermally tolerant than their non-adapted counterparts. As a result of a study conducted to determine $D_{52°C}$ -values of acid adapted cells of *E. coli* O157:H7 in apple cider and orange juice Ryu and Beuchat (1998) suggested that heat tolerance of *E. coli* O157:H7 can be substantially enhanced by acid adaptation as compared to acid shock.

Most of the studies that have been conducted to determine thermal tolerance of acid adapted *E. coli* O157:H7 have been conducted at lower temperatures ranging from 52 to 58 °C in laboratory medium. But in our study we have shown that acid adapted *E. coli* O157:H7 are less susceptible than their non-adapted counterparts at 62 and 65 °C. Although similar results were observed with acid adapted *Salmonella* at 65 °C in this study, there was no statistical difference in the D-values and the acid adapted *Salmonella* were less susceptible than their non-adapted counterparts. Similar results were reported in a study conducted by Leyer and Johnson (1993) that suggested increased thermal tolerance of acid adapted *Salmonella* at 50 °C.

Studies have shown that acid adaptation of food borne pathogens including *E. coli* O157:H7 and *Salmonella* increases thermal tolerance in fruit juices. Sharma et. al. (2005) reported significant differences in the D-values of *Salmonella* and *E. coli* O157:H7 grown in

TSB+1% glucose in comparison to those strains grown in TSB regardless of the type of fruit juice they were heated in, whereas thermal resistance of *Listeria monocytogenes* was not affected by adaptation in acidified broth. In our study, increased thermal tolerance of acid adapted *E. coli* O157:H7 and *Salmonella* is in general agreement with other studies that have reported increased thermal tolerance of food borne pathogens as a result of acid adaptation.

Conclusions

Our study shows that acid adapted strains of *E. coli* O157:H7 and *Salmonella* have increased thermal tolerance as compared to non-adapted counter parts in meat serum which is an example of a liquid food system. Most of the studies have been conducted to determine D-values of acid adapted food borne pathogens at lower temperatures where significant differences have been observed. Our study is not only in agreement with these previously observed results but also indicates that at higher temperatures (62 and 65 °C) these differences are observed between the acid adapted and non-adapted *E. coli* O157:H7 and *Salmonella.* Use of higher temperatures in our study are more indicative of cooking temperatures used for preparation of meat commercially as well as by consumers in the household as compared to some of the other studies which were reported at mild heating temperatures. Adaptation capabilities of food borne pathogens such as *E. coli* O157:H7 and *Salmonella* to stress has been scarcely researched and not been given the same attention as compared to non-stressed pathogens. This study stresses the need for regulatory agencies and large scale meat manufacturers to take into consideration the increased thermal tolerance of acid adapted pathogens, as healthy growing cultures in a laboratory medium may inaccurately represent their survival in natural food environment. A better understanding of the effects of acid adaptation on subsequent stresses is needed in food systems to allow for

more accurate risk assessments to be made on enhancing safety of processed meat products. Further studies need to be conducted on the thermal tolerance of these acid adapted food borne pathogens in actual raw and processed meat systems to determine pathogenicity and answer questions on transient and prolonged affects of acid adaptation on thermal tolerance.

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Figure Legends

Figure 1: Survival populations of acid adapted and non-adapted *Escherichia coli* O157:H7 at (A) 58 °C, (B) 62 °C, and (C) 65 °C. ● = Acid adapted; ■ = Non-adapted. Each data point is an average of three independent heating trials.

Figure 2: Survival populations of acid adapted and non-adapted *Salmonella* (A) 58 °C, (B) 62 °C, and (C) 65 °C. ● = Acid adapted; ■ = Non-adapted. Each data point is an average of three independent heating trials.

Table 1: Decimal reduction time (D-values) of acid adapted and non-adapted *Escherichia coli* O157:H7 at 58, 62, and 65 °C in meat serum.

Temperature	Inoculum	D-value (min.)*
58 °C	Acid adapted	22.46[b]
	Non adapted	10.59[a]
62 °C	Acid adapted	3.58[b]
	Non adapted	1.38[a]
65 °C	Acid adapted	1.02[b]
	Non adapted	0.75[a]

* Different superscripts indicate statistical differences within a temperature

Table 2: Decimal reduction time (D-values) of acid adapted and non-adapted *Salmonella* at 58, 62, and 65 °C in meat serum.

Temperature	Inoculum	D-value (min.)*
58 °C	Acid adapted	9.36[b]
	Non adapted	6.44[a]
62 °C	Acid adapted	1.66[b]
	Non adapted	0.88[a]
65 °C	Acid adapted	1.14[a]
	Non adapted	0.95[a]

* Different superscripts indicate statistical differences within a temperature

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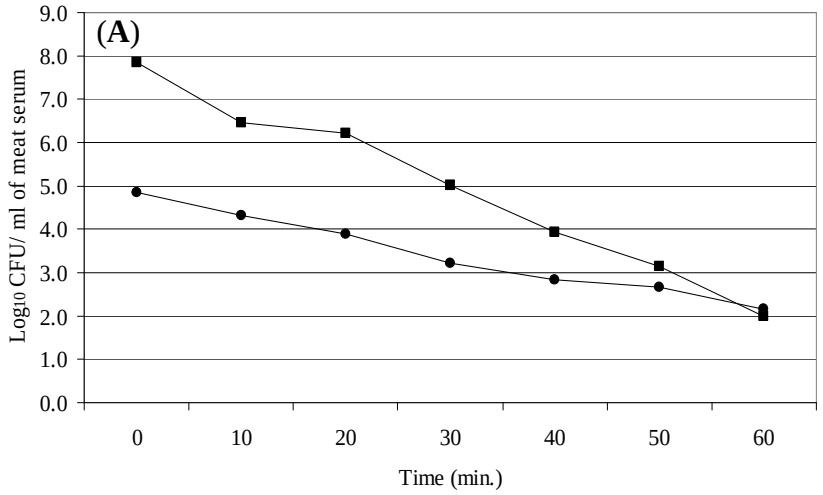

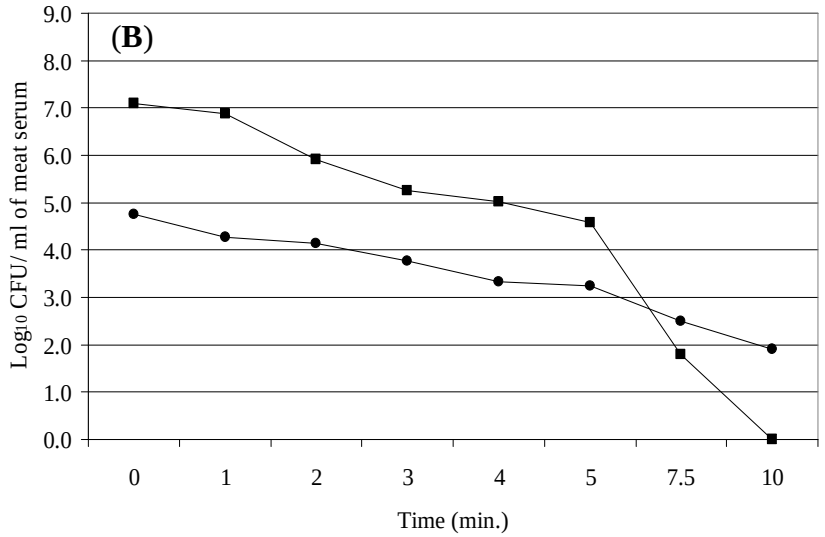

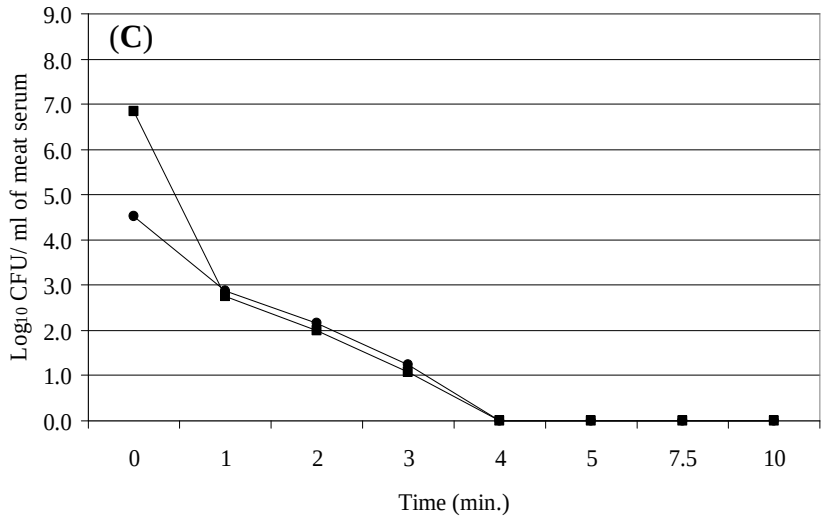

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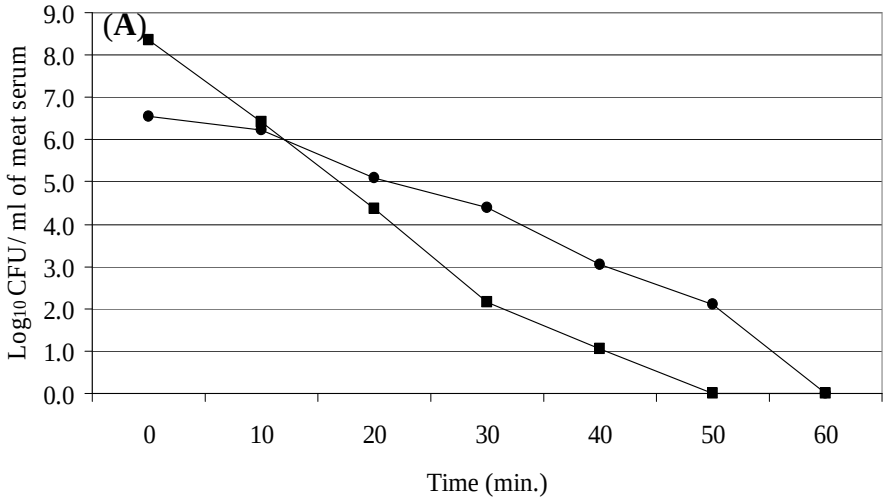

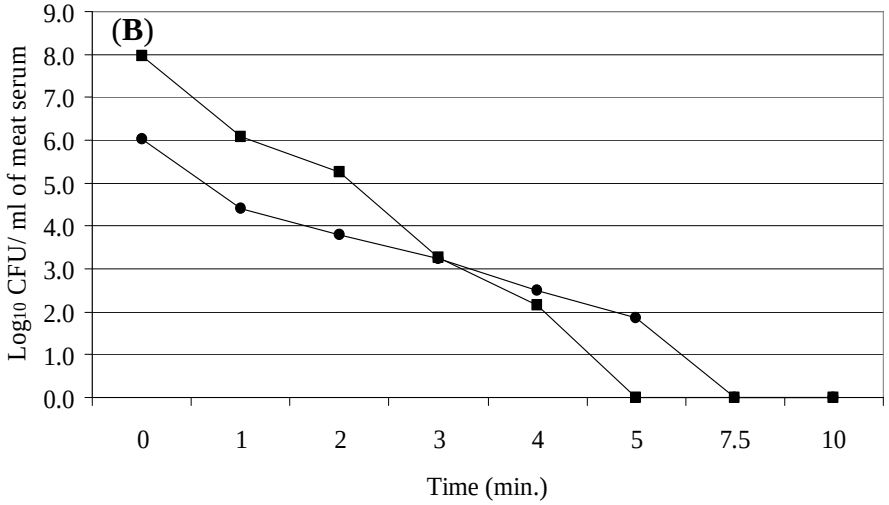

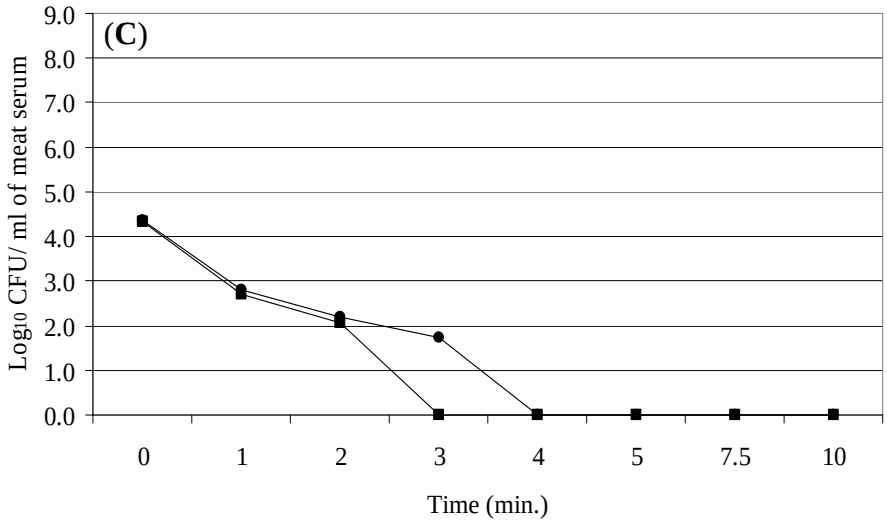

Thermal tolerance of acid adapted and non-adapted *Escherichia coli* O157:H7 and *Salmonella enterica* in ground beef during storage

A paper to be submitted to the

JOURNAL OF FOODBORNE PATHOGENS AND DISEASE

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Abstract

Thermal tolerance of acid adapted *Escherichia coli* O157:H7 and *Salmonella* was evaluated in ground beef stored at 4 and -20 °C. Both pathogens were adapted to acidic conditions by growing in Tryptic Soy Broth with 1 % glucose (TSB+ 1%G). Five-strain cocktail of both bacteria were grown separately in TSB and TSB+1%G for 24 h at 37 °C to provide cells with or without acid adaptation. Irradiated ground beef was inoculated with either acid adapted or non-adapted *E. coli* O157:H7 and *Salmonella*; subjected to heat treatment at 62 and 65 °C on day 1, 7, 14, 21, and 28 (4 °C) and on day 1, 30, 60, 90, and 120 (-20 °C). Decimal reduction time (D-values) of the pathogens was determined as an indicator of thermal tolerance. Significantly higher ($P<0.05$) D_{62}-values of non-adapted *E. coli* O157:H7 were observed on day 21 and 28 at 4 °C and on day 90 and 120 at -20 °C. Higher ($P<0.05$) D_{62}-values were observed on day 21 and 28 at 4 °C amongst non-adapted strains and on day 28 for acid adapted strains of *Salmonella*. Higher ($P<0.05$) D_{62}-values of acid adapted strains of *Salmonella* were observed on day 30, 60, and 90 when stored at -20 °C while no differences ($P>0.05$) were observed in the D_{65}-values of acid adapted and non-

adapted strains of *E. coli* O157:H7 and *Salmonella* throughout storage at both temperatures. This suggests that acid adaptation of foodborne pathogens provides cross-protection against heat treatment at lower cooking temperatures while this phenomenon is not observed at higher temperatures.

Introduction

Since its first recognition as a foodborne pathogen in the US in 1982, *Escherichia coli* O157:H7 has emerged as one of the most important foodborne pathogens. The Centers for Disease Control and Prevention (CDC) has reported that *E. coli* O157:H7 causes an estimated 73,000 infections resulting in more than 2,000 hospitalizations and 61 deaths annually in the United States (Mead et. al., 1999). Illness caused by *E. coli* O157:H7 can cause severe complications such as hemorrhagic colitis and hemorrhagic uremic syndrome (HUS; Riley et. al., 1983) and has been successfully isolated from specimens of foods associated with outbreaks of hemorrhagic colitis or HUS (Doyle and Schoeni, 1987). The annual cost of illness due to O157:H7 STEC was $405 million (in 2003), including $370 million for premature deaths, $30 million for medical care, and $5 million in lost productivity (Frenzen et. al., 2005). According to Doyle and Schoeni (1987) *E. coli* O157:H7 has been isolated from 3.7 % of the beef, 1.5 % of the pork, 1.5 % of the poultry, and 2.0 % of the lamb samples indicating that the bacterium is associated with foods of animal origin and not specifically beef.

An estimated 1.4 million cases of salmonellosis and 400 deaths occur annually in the United States (Mead et. al, 1999; Voetsch et. al., 2004) at a cost of $ 2.4 billion (Frenzen et. al., 1999). In 1999, the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) established lethality regulations for fully and partially cooked meat and

poultry products (USDA-FSIS, 1999). A 6.5-log units reduction of *Salmonella* in cooked beef and roast beef and a 7.0-log units reduction in certain fully and partially cooked poultry products was set as a performance standard for lethality, stabilization, and product handling (Weche et. al., 2005). Raw meat and poultry are usually considered as ideal growth media for bacteria, however all microbial contaminants have been subjected to physical, chemical, and nutritional stresses during processing (Yousef and Courtney, 2003). Bacteria can face exposure to extremes of acidity in many situations in the environment, in foods and in animal or human body (Nojoumi et. al., 1995). The USDA-FSIS supports use of a mixture of pathogenic bacterial strains containing relatively heat-resistant serovars and particularly those that have been implicated in outbreaks to verify compliance with performance standards (USDA-FSIS, 2001). Most of the cultures are prepared under optimal laboratory conditions, but a cocktail of any particular bacteria containing stressed cells that more truly represents the physiological state of an organism that may contaminate the product during or after processing is a better choice for thermal inactivation or challenge studies (Juneja and Novak, 2003).

Acidic foods have long been considered generally safe for human consumption and outbreaks related to these foods have rarely been recorded (Arvizu-Medrano, 2005). However, some acidic foods such as mayonnaise (Smittle, 2000), yogurt (Morgan et. al., 1993), apple juice (CDC, 2000), and orange juice (Merrel and Camilli, 2000) have been associated with foodborne outbreaks and in 80 % of these outbreaks *Salmonella* and *E. coli* O157:H7 have been implicated, suggesting the acid tolerance of these pathogens. The ability of bacteria to survive in acid foods is of concern for pathogens with low infective doses, such as *Salmonella typhi*. Acid adaptation response is a phenomenon that results in increased

resistance of microorganisms to severe acid shocks and provides cross protection against various other environmental stresses such as heat and surface active agents (Leyer and Johnson, 1993). Acid adaptation of *E. coli* O157:H7 has shown increased resistance to irradiation (Buchanan et. al., 1999a) and heat (Buchanan and Edelson, 1999b) in laboratory media and in liquid food systems; however varying results have been reported on the resistance of acid adapted *Salmonella* to environmental stresses.

Information on the acid tolerance response is limited for *S. typhi*. Tiwari et. al. (2004) found that *S. typhi* in exponential phase is able to adapt and increase its acid tolerance in culture medium; however, it is important to investigate the acid responses in stationary phase and in an actual food matrix because there is a possibility that the pathogen could contaminate food products when it is in its stationary phase. This study was undertaken to determine the thermal tolerance of acid adapted pathogens in a food system that would reflect current processing, storage and distribution practices of food products. The objective of this research was to investigate the effects of storage temperature on the thermal tolerance of acid adapted and non-adapted *Salmonella* spp. and *E. coli* O157:H7 in ground beef.

Materials and Methods

Preparation of bacterial cultures

A five-strain cocktail of *Escherichia coli* O157:H7 [ATCC 35150 (human feces from outbreak of hemorrhagic colitis); ATCC 43894 (human feces from outbreak of hemorrhagic colitis); ATCC 43895 (isolate from raw hamburger implicated in hemorrhagic colitis); WS 3062 (clinical isolate); and WS 3331 (clinical isolate)] and *Salmonella enterica* (*S.* Newport, *S.* Uganda, *S.* Heidelberg, *S.* Typhimurium, and *S.* Enteritidis) was used. The five strains of *E.*

coli O157:H7 were obtained from the Food Safety Research Laboratory (FSRL) and the five bovine strains of *Salmonella* spp. were obtained from the Veterinary Diagnostic Laboratory at Iowa State University, Ames. IA. All stock cultures were maintained on Tryptic Soy Agar (TSA; Difco, Becton Dickinson, Sparks, MD) slants at 4 °C. Working cultures were maintained by daily transfers in Tryptic Soy Broth (TSB; Difco, Becton Dickinson, Sparks, MD) and TSB + 1% glucose for the non-adapted and acid adapted strains respectively. Growth curves for individual strains of *E. coli* O157:H7 and *Salmonella* spp. were constructed to determine the time at which stationary phase was reached in order to make subsequent transfers (Data not shown). The non-adapted and acid adapted inoculum was prepared by inoculating bacterial cultures into 10 ml TSB and TSB + 1% glucose respectively and then incubating at 37 °C for 24 hours. Addition of glucose to the growth medium allows the production of organic acids, which lower the final pH of the culture medium significantly (Buchanan and Edelson, 1999c). Cultures (1ml) were then transferred into 25 ml centrifuge tubes containing TSB with or without 1% glucose and further incubated at 35 °C for 18h. The cultures were then centrifuged at 5,738 X g for 10 min. at 4 °C (SORVALL SUPER T21, Newton, CT). The supernatant was decanted and resultant pellet resuspended with 10 ml of 0.1% sterile peptone water (PW; Difco, Becton Dickinson, Sparks, MD). A cocktail was prepared by mixing the five cultures in a sterile bottle to get a final volume of 50 ml of the inoculum.

Inoculation of ground beef

Ground beef was obtained from the Iowa State University Meat Laboratory and irradiated at the Iowa State University Linear Accelerator Facility to an average absorbed

dose of 8.05 kGy. Ground beef was irradiated to remove any background gram negative microflora. The irradiated ground beef was divided into four parts of 350 g each and inoculated with 50 ml of either non adapted or acid adapted *E. coli* O157:H7 or *Salmonella* spp. respectively. Inoculum was hand mixed into the ground beef to obtain a homogenous distribution into the product. Two-gram portions of inoculated ground beef were then placed into plastic pouches (2" by 2"), heat sealed and stored at 4 °C for up to 4 weeks and -20 °C for up to 120 days. Thermal resistance of samples stored at 4 °C was analyzed at 7-day intervals for up to 28 days while those stored at -20 °C were analyzed at 30 day intervals for up to 120 days.

Thermal resistance in ground beef (D-value determinations)

Thermal tolerance of the non adapted and acid adapted strains was determined by calculating D-values at 62 (143.6 °F) and 65 °C (149 °F) for up to 10 minutes. At each test temperature the 2 g inoculated ground beef pouches were completely immersed in a water bath maintained at either 62 or 65 °C and removed from the water bath at 0, 1, 2, 3, 4, 5, 7.5, and 10 minutes. An additional 2 g ground beef pouch was placed in each water bath at both temperatures to monitor the temperature increase. A datalogger (LI-1000; LI-COR, Lincoln, NE) was used to monitor temperature by inserting a thermocouple into a 2 g ground beef pouch and placing in the water bath at 62 and 65 °C. The timing for each experiment was not started until the 2 g portions of inoculated ground beef had reached the same temperature as the water bath. This was designated as time "zero" in the study. The plastic pouches were removed from the water bath and immediately placed in an ice water bath at each time

interval. Each plastic pouch was allowed to cool down in the ice water bath for 10 min. prior to sampling.

Microbial sampling and enumeration

The plastic pouch containing 2-grams of ground beef that had been subjected to either 62 or 65 °C was then aseptically opened into a filter stomacher bag and diluted with 10 ml 0.1 % sterile peptone water (PW). The ground beef was homogenized with the diluent in a stomacher (Stomacher 400, Tekmar, Cincinnati, OH) for 1 minute. Samples were then serially diluted in 0.1 % sterile PW and spread plated on to Tryptic Soy Agar (TSA). Clavero and Beuchat (1996) suggested that a higher recovery of *E. coli* was achieved on TSA as compared to MacConkey agar and modified eosin methylene blue agar. The plates were then incubated at 37 °C for 48 h and colony forming units were manually counted and reported as log Colony Forming Units per gram (log_{10} CFU/ g).

Experimental Design and Statistical Analysis

Three independent replications of a randomized complete block design were used to prepare four 350-g batches of non inoculated ground beef that was then inoculated with 50 ml of either non adapted or acid adapted *E. coli* O157:H7 or *Salmonella* spp respectively. Subsequently the inoculated ground beef was divided into 2-g pouches in a completely randomized design prior to D-value testing on day one or storage at 4 °C and -20 °C for later testing. Survival curves were constructed for the organisms recovered on TSA, with the Y-axis representing log_{10} CFU/ g of each inoculum tested and the X-axis representing time in minutes. Decimal reduction time (D-value) was calculated as the negative reciprocal of the slope of the survivor curve for each inoculum type exposed to 62 °C (D_{62}) and 65 °C (D_{65}).

Three replications of the experiment were performed and the mean D-value of each inoculum was analyzed using analysis of variance (ANOVA) with the SAS PROC MIXED procedures (2002-03, SAS Institute, Cary, N.C.).

Results

Thermal tolerance of acid adapted and non-adapted *E. coli* O157:H7 and *Salmonella* in ground beef stored at refrigerated temperature (4 °C).

D-values of non-adapted and acid adapted *E. coli* O157:H7 and *Salmonella* in ground beef stored at 4 °C are shown in tables 1 and 2. From the data shown in these tables it can be observed that there was no significant ($P>0.05$) difference in D_{62}-values of non-adapted and acid adapted *E. coli* O157:H7 up to 14 days of refrigerated storage at 4 °C. Significant differences ($P<0.05$) were observed between non-adapted and acid adapted strains of *E. coli* O157:H7 on day 21 and 28 of the storage period. Amongst the non-adapted strains of *E. coli* O157:H7 a significant difference ($P>0.05$) was observed in D_{62}-values after 21 days of refrigerated storage whereas no significant differences ($P>0.05$) were observed for the acid adapted strains over the 28 day storage period. From the data shown in table 2 it can be seen that there was no statistical differences ($P>0.05$) in D_{65}-values of non-adapted and acid adapted strains of *Salmonella* throughout the 28 days of refrigerated storage. D_{65}-values of non-adapted and acid adapted strains of *E. coli* O157:H7 did not change significantly over the 28-day storage period.

Data in table 1 suggests that there were significant differences ($P<0.05$) in D_{62}-values of non-adapted and acid adapted strains of *Salmonella* on day 21 and 28 at 4 °C. Significant differences ($P<0.05$) were also observed in D_{62}-values amongst the non-adapted strains after

21 days of refrigerated storage. D_{62}-values of acid adapted *Salmonella* on day 1 and day 28 were found to be significantly different ($P<0.05$) while no statistical differences ($P>0.05$) were seen between remainder of the days (7, 14, and 21) during refrigerated storage. At 65 °C there were no significant differences ($P>0.05$) in the D-values of non-adapted and acid adapted *Salmonella* throughout the 28-day storage under refrigerated temperature. Also, there was no significant difference ($P>0.05$) in D_{65}-value as a result of storage time amongst the non-adapted *Salmonella* or their acid adapted counterparts.

D_{62}-values of non-adapted *E. coli* O157:H7 and *Salmonella* were comparable to their acid adapted counterparts during the initial phase of storage at 4 °C. D_{62}-values of non-adapted pathogens tended to increase tremendously after 14 days and were significantly higher than the acid adapted *E. coli* O157:H7 and *Salmonella* on day 21 and 28 of the storage period. Similar but not significant changes were also observed for D_{65}-values of non-adapted *E. coli* O157:H7 on day 21 and 28 of the storage period while *Salmonella* showed slight changes in D_{65}-values for both acid adapted and non-adapted strains throughout the 28-day storage at 4 °C.

Thermal tolerance of acid adapted and non-adapted *E. coli* O157:H7 and *Salmonella* in ground beef stored at –20 °C (Frozen Storage).

Tables 3 and 4 show D-values of acid adapted and non-adapted *E. coli* O157:H7 and *Salmonella* at 62 and 65 °C in ground beef stored over a 120-day period at -20 °C. From the data shown it can be observed that a significant difference ($P<0.05$) in D_{62}-values was observed between non-adapted and acid adapted strains of *E. coli* O157:H7 on day 120 of the storage period. Significant differences ($P<0.05$) were also observed between days 1 and 90

and days 1 and 120 amongst the non-adapted strains of *E. coli* O157:H7 whereas in the acid adapted strains significant differences ($P<0.05$) were observed between day 1 and the rest of the storage period up to 120 days. Statistical differences ($P<0.05$) were also observed between day 30 and 120 and day 60 and 120 amongst the acid adapted *E. coli* O157:H7 stored at -20 °C. For D_{62}-values of *Salmonella* significant differences ($P<0.05$) were observed between the non-adapted and acid adapted strains on day 30, 60, 90, and 120 of the storage period. No significant ($P>0.05$) differences were observed amongst the non-adapted or acid adapted strains of *Salmonella* throughout the 120-day storage at -20 °C.

From table 4 it can be observed that no significant differences ($P>0.05$) in D_{65}-values were observed between the non-adapted and acid adapted strain of *E. coli* O157:H7 as well as *Salmonella* throughout the 120-day storage at -20 °C. In addition to this no significant differences ($P>0.05$) were observed as a result of storage period amongst each of the non-adapted and acid adapted strains. D_{65}-values of non-adapted and acid adapted *E. coli* O157:H7 and *Salmonella* were slightly higher on day 1 and tended to be lower but fairly constant for the remainder of the 120 days storage at -20 °C.

Discussion

Carcass decontamination and further processing of meat products introduces food borne pathogens such as *E. coli* O157:H7 and *Salmonella* to environmental stresses including starvation, low water activity and low pH environments that may trigger protective responses to heat (Mazzotta, 2001). Numerous studies have reported that environmental factors such as pH can affect the thermal resistance of microorganisms (Bearson et. al, 1997; Leyer and Johnson, 1993; and Jay 2000).

D-values of non-adapted *E. coli* O157:H7 were higher at both 62 and 65 °C in our study in comparison to those reported by Juneja et. al. (1997). Their study reported D-values of 0.93 and 0.39 min. for non-adapted *E. coli* O157:H7 at 62.5 and 65 °C respectively. Differences in the D-values of the non-adapted *E. coli* O157:H7 could be attributed to the variation in the type of strains used to conduct these thermal tolerance studies. Results from our study have shown that there is no difference between the D-values of acid adapted and non-adapted *E. coli* O157:H7 at 65 °C irrespective of the storage temperature while at 62 °C the non-adapted cells had a higher D-value than the acid adapted cells after 21 days at 4 °C. The D-value of acid adapted *E. coli* O157:H7 was higher than that of the non-adapted counterpart after 120 days at -20 °C. These results were contrary to a study conducted by Buchanan and Edelson (1999b) that suggested a higher D-value for three different strains of acid adapted *E. coli* O157:H7 grown in laboratory media. D-values reported in their study were lower than those observed in our study, which can be attributed to the use of higher temperatures used in our study and ground beef being used instead of a laboratory media that could potentially provide some level of protection to the pathogens against heat. D-values at 62 and 65 °C in our study were also lower than those reported by Ahmed et. al. (1995) and Line et. al. (1991) since the authors used a lower temperature in their study. This suggests that there has been tremendous amount of research done on thermal tolerance of acid adapted and non-adapted food borne pathogens especially *E. coli* O157:H7 and *Salmonella* at low/ sub-lethal temperatures leaving a need to investigate higher temperatures that are more indicative of current cooking practices. Cheng et. al. (2002) demonstrated that acid adapted *E. coli* O157:H7 were more thermally tolerant than their non-adapted counterparts in laboratory

media and Ryu and Beuchat (1998) suggested that heat tolerance of *E. coli* O157:H7 can be substantially enhanced by acid adaptation as compared to acid shock at 52 °C in apple cider and orange juice. Comparing our study to Cheng et. al. (2002) and Ryu and Beuchat (1998) it can be concluded that acid adaptation might increase thermal tolerance in liquid foods but the same effect is not observed in a solid food matrix such as ground beef.

Calicioglu et. al. (2003) reported higher susceptibility of acid adapted *Salmonella* than the non-adapted cells on beef jerky after drying which are in agreement with results from our study that indicated higher susceptibility of the acid adapted *Salmonella* in ground beef stored at 4 °C. Results on the D-values in ground beef stored at -20 °C showed that the acid adapted strains of *Salmonella* were slightly more tolerant to heat than their non-adapted counterparts at 62 and 65 °C which were concurrent with results from a study conducted by Leyer and Johnson (1993) suggesting increased thermal tolerance of acid adapted *Salmonella* at 50 °C in cheese. Sharma et. al. (2005) reported significant differences in the D-values of *Salmonella* and *E. coli* O157:H7 grown in TSB+1% glucose in comparison to those strains grown in TSB regardless of the type of fruit juice they were heated in, whereas thermal resistance of *Listeria monocytogenes* was not affected by adaptation in acidified broth. As seen in our study this is not necessarily true in case of a food matrix such as ground beef over an extended period of refrigerated and frozen storage. Minimal or no differences in the thermal tolerance of acid adapted and non-adapted *E. coli* O157:H7 and *Salmonella* in ground beef indicate the possibility that the differences observed in laboratory media may not necessarily be extrapolated to a food system.

Conclusions

In conclusion it is apparent from the data that there was no significant difference (P>0.05) in the thermal tolerance of the acid adapted and non-adapted *E. coli* O157:H7 and *Salmonella* in ground beef throughout the 120-day storage period at 4 and -20 °C. Since there have been studies in the past that have reported differences in the thermal tolerance of acid adapted and non-adapted pathogens it is important to consider protective responses that are triggered as a result of environmental stresses that can be critical when choosing the most resistant target organism in order to calculate lethality of a heat treatment in a food matrix. A conservative approach of taking into consideration the most resistant target organisms can help in adding an extra safety factor to the minimum regulatory requirements for heating/ cooking meat and poultry products. Most of the studies have reported thermal tolerance of acid adapted pathogens in a laboratory media or a liquid food system but there is a lack of literature on the thermal behavior of these pathogens in an actual food matrix that has been subjected to refrigerated and frozen storage. In addition to this studies undertaken to determine thermal tolerance of acid adapted *E. coli* O157:H7 have been conducted at lower temperatures ranging from 52 to 58 °C in laboratory medium or in liquid foods such as juices. Our study has been conducted in an actual food system (ground beef) that was subjected to handling conditions mimicking day-to-day practices followed in the industry, grocery stores, and in the consumer households. We have shown that acid adaptation of *E. coli* O157:H7 and *Salmonella* does not pose any additional threat of this pathogen surviving in the foods if they have been cooked as per regulatory guideline for non-adapted pathogens. This study helps answer the question posed about the transient and prolonged effects of acid adaptation on

thermal tolerance of food borne pathogens. But further studies need to be conducted to determine the pathogenicity of these acid adapted pathogens to humans.

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Table 1: Decimal reduction time (D_{62}-values) of acid adapted and non-adapted *Escherichia coli* O157:H7 and *Salmonella* in ground beef stored at 4 °C for up to 28 days.

Storage time (days)	*Escherichia coli* O157:H7 D-value (min.)		*Salmonella* D-value (min.)	
	Non-adapted	Acid adapted	Non-adapted	Acid adapted
1	$1.97^{1, a}$	$2.20^{1, a}$	$1.98^{1, a}$	$1.93^{1, a}$
7	$2.46^{1, a}$	$2.53^{1, a}$	$2.00^{1, a}$	$2.24^{1, 2, a}$
14	$2.91^{1, a}$	$2.88^{1, a}$	$2.43^{1, a}$	$2.29^{1, 2, a}$
21	$4.50^{2, b}$	$2.71^{1, a}$	$3.55^{2, b}$	$2.35^{1, 2, a}$
28	$4.60^{2, b}$	$2.66^{1, a}$	$4.29^{2, b}$	$2.98^{2, a}$

Numbers in the superscript indicate significant differences between storage times for acid adapted or non-adapted strains of each pathogen.
Alphabets in the superscript indicate significant differences between acid adapted or non-adapted strains for each pathogen on a particular day.

Table 2: Decimal reduction time (D_{65}-values) of acid adapted and non-adapted *Escherichia coli* O157:H7 and *Salmonella* in ground beef stored at 4 °C for up to 28 days.

Storage time (days)	*Escherichia coli* O157:H7 D-value (min.)		*Salmonella* D-value (min.)	
	Non-adapted	Acid adapted	Non-adapted	Acid adapted
1	$1.58^{1, a}$	$1.27^{1, a}$	$1.15^{1, a}$	$1.23^{1, a}$
7	$1.05^{1, a}$	$0.89^{1, a}$	$0.83^{1, a}$	$0.79^{1, a}$
14	$0.91^{1, a}$	$0.82^{1, a}$	$0.82^{1, a}$	$0.73^{1, a}$
21	$1.30^{1, a}$	$0.80^{1, a}$	$1.16^{1, a}$	$0.92^{1, a}$
28	$1.40^{1, a}$	$0.97^{1, a}$	$1.38^{1, a}$	$1.10^{1, a}$

Numbers in the superscript indicate significant differences between storage times for acid adapted or non-adapted strains of each pathogen.
Alphabets in the superscript indicate significant differences between acid adapted or non-adapted strains for each pathogen on a particular day.

Table 3: Decimal reduction time (D_{62}-values) of acid adapted and non-adapted *Escherichia coli* O157:H7 and *Salmonella* in ground beef stored at -20 °C for up to 120 days.

Storage time (days)	*Escherichia coli* O157:H7 D-value (min.)		*Salmonella* D-value (min.)	
	Non-adapted	Acid adapted	Non-adapted	Acid adapted
1	$1.85^{1, a}$	$1.86^{1, a}$	$1.98^{1, a}$	$1.93^{1, a}$
30	$2.25^{1, 2, a}$	$2.40^{2, a}$	$1.65^{1, a}$	$2.56^{1, b}$
60	$2.29^{1, 2, a}$	$2.34^{2, a}$	$1.65^{1, a}$	$2.54^{1, b}$
90	$2.36^{2, a}$	$2.73^{2, 3, a}$	$1.85^{1, a}$	$2.58^{1, b}$
120	$2.35^{2, a}$	$2.85^{3, b}$	$2.03^{1, a}$	$2.62^{1, a}$

Numbers in the superscript indicate significant differences between storage times for acid adapted or non-adapted strains of each pathogen.
Alphabets in the superscript indicate significant differences between acid adapted or non-adapted strains for each pathogen on a particular day.

Table 4: Decimal reduction time (D_{65}-values) of acid adapted and non-adapted *Escherichia coli* O157:H7 and *Salmonella* in ground beef stored at -20 °C for up to 120 days.

Storage time (days)	*Escherichia coli* O157:H7 D-value (min.)		*Salmonella* D-value (min.)	
	Non-adapted	Acid adapted	Non-adapted	Acid adapted
1	$0.89^{1, a}$	$1.13^{2, a}$	$0.74^{1, a}$	$0.81^{1, a}$
30	$0.55^{1, a}$	$0.59^{1, a}$	$0.60^{1, a}$	$0.68^{1, a}$
60	$0.55^{1, a}$	$0.57^{1, a}$	$0.65^{1, a}$	$0.68^{1, a}$
90	$0.54^{1, a}$	$0.56^{1, a}$	$0.58^{1, a}$	$0.58^{1, a}$
120	$0.54^{1, a}$	$0.57^{1, a}$	$0.52^{1, a}$	$0.58^{1, a}$

Numbers in the superscript indicate significant differences between storage times for acid adapted or non-adapted strains of each pathogen.
Alphabets in the superscript indicate significant differences between acid adapted or non-adapted strains for each pathogen on a particular day.

Pathogenicity of acid adapted *Escherichia coli* O157:H7 in laboratory media and meat serum

A paper to be submitted to the JOURNAL OF FOOD PROTECTION

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Abstract

Toxicity of acid adapted and non-adapted *Escherichia coli* O157:H7 grown in laboratory media and meat serum on African green monkey kidney cells (Vero cells) was determined. *E. coli* O157:H7 was adapted to acidic conditions by growing in Tryptic Soy Broth with 1 % glucose (TSB+ 1%G). Five-strain cocktail of *E. coli* O157:H7 was grown separately in TSB and TSB+1%G for 24 h at 37 °C to provide cells with or without acid adaptation. Meat serum was prepared from irradiated ground beef and inoculated with a five strain cocktail of either acid adapted or non-adapted *E. coli* O157:H7. Vero cells were grown separately in complete RPMI media and toxic effects of acid adapted and non-adapted *E. coli* O157:H7 grown in laboratory media and meat serum were compared after storing for 1 and 7 days at 4 and -20 °C. No significant ($P>0.05$) differences were observed between the toxicity of acid adapted and non-adapted cells in laboratory media and meat serum irrespective of storage temperature. Higher ($P<0.05$) production of verotoxin was observed on day 1 as compared to day 7 at 4 and -20 °C. Significantly higher ($P<0.05$) verotoxin production was observed in meat serum inoculated with non-adapted cells on day 1 as compared to acid

adapted cells but this was not observed on day 7. These results suggest that acid adaptation of *E. coli* O157:H7 leads to decreased verotoxin secretion in meat serum.

Introduction

An estimated 73,000 cases of infection and 61 deaths occur in the United States each year as a result of *Escherichia coli* O157:H7 infection (CDC, 2005). *E. coli* O157:H7 has become a pathogen of concern in the food industry since its initial implication in various foodborne outbreaks (Mermelstein, 1993). *E. coli* O157:H7 has been recognized as the most important etiological agent of hemorrhagic colitis which is characterized by severe abdominal pain and bloody diarrhea (Bopp et. al., 1987). *E. coli* O157:H7 does not produce a traditional *E. coli* enterotoxin and is not enteroinvasive or enteroadherant but has been known to produce a toxin that is cytopathogenic to Vero cell cultures (Wells et. al., 1983). Because of its toxic effects on Vero cells they are also known as verotoxins. This cytotoxin has been suggested as a vehicle in the pathogenesis of hemolytic uremic syndrome (HUS) and is believed to be true for hemorrhagic colitis (Bopp et. al., 1987). Toxins produced by *E. coli* O157:H7 tend to bind to epithelial cells in the intestine, kidney, and brain leading to formation of tiny clots that can very easily damage the capillary beds (Acheson and Keusch, 1996). Prevalence rates of *E. coli* O157:H7 in cattle range from 0.3 to 19.7% in the feedlot, 0.7 to 27.3% in pastures, and 0.9 to 6.9% on the range (Hussein and Bollinger, 2005) suggesting a high potential for infection and reinfection of cattle that could lead to contamination at the time of slaughter and further processing. Concerns associated with safety of beef contaminated with Shiga toxin-producing *E. coli* (STEC) have been increasing since the first two human illness outbreaks in 1982 (Riley et. al., 1983). Some STEC

serotypes are rarely considered pathogenic but there are a few serotypes including *E. coli* O157:H7 that are frequently reported to cause human illnesses (Hussein and Bollinger, 2005).

Acidic foods have long been considered generally safe for human consumption and outbreaks related to these foods have rarely been recorded (Arvizu-Medrano, 2005). However, recently foodborne outbreaks have been caused due to some acidic foods such as mayonnaise (Smittle, 2000), yogurt (Morgan et. al., 1993), apple juice (CDC, 2000), and orange juice (Merrel and Camilli, 2000). Resistance and/ or tolerance to acidic conditions has an impact on virulence determinants that contribute to the survival and pathogenicity of certain foodborne pathogens such as *E. coli*, *Salmonella, Shigella* spp., and *Listeria monocytogenes* (Leyer and Johnson, 1995; and Miller and Kaspar, 1994). According to Buchanan et. al. (1994) these acid resistant/ tolerant pathogens have a greater likelihood of surviving in acidic foods for an extended period and increased portion of the population that can survive the gastric environment (Gorden and Small, 1993) enhancing the infectivity once these pathogens attach to the intestinal tract (O'Driscoll et. al., 1996). These studies suggest enhanced infectivity of acid tolerant pathogens but Yuk and Marshall (2005) have reported conflicting results showing that although organic acid adapted cells were more heat resistant they produced less total verotoxin than their non-adapted counterparts at a concentration of approximately 10^8 CFU/ ml.

Pathogenic properties of *E. coli* O157:H7 are associated with the ability of this pathogen to attach to intestinal cells and produce verotoxin (Benjamin and Datta, 1995). Tremendous amount of research has been done to determine responses of acid adapted *E. coli* O157:H7 to thermal and non-thermal treatments in laboratory media and juices but very limited literature is available on the pathogenicity of acid adapted *E. coli* O157:H7 in an

actual food system during storage. To help understand the ability of *E. coli* O157:H7 to respond to acid stress this study was undertaken to compare pathogenicity of acid adapted and non-adapted *E. coli* O157:H7 in laboratory media versus in meat serum during storage.

Materials and Methods

Preparation of bacterial cultures.

A five-strain cocktail of *Escherichia coli* O157:H7 [ATCC 35150 (human feces from outbreak of hemorrhagic colitis); ATCC 43894 (human feces from outbreak of hemorrhagic colitis); ATCC 43895 (isolate from raw hamburger implicated in hemorrhagic colitis); WS 3062 (clinical isolate); and WS 3331 (clinical isolate)] and *Salmonella enterica* (*S.* Newport, *S.* Uganda, *S.* Heidelberg, *S.* Typhimurium, and *S.* Enteritidis) was used. The five bovine strains of *Salmonella* spp. were obtained from the Veterinary Diagnostic Laboratory at Iowa State University, Ames. IA. Stock cultures were maintained on Tryptic Soy Agar (TSA; Difco, Becton Dickinson, Sparks, MD) slants at 4 °C. Working cultures were maintained by daily transfers in Tryptic Soy Broth (TSB; Difco, Becton Dickinson, Sparks, MD) and TSB + 1% glucose for the non-adapted and acid adapted strains respectively. Growth curves for individual strains of *E. coli* O157:H7 and *Salmonella* spp. were constructed to determine the time at which stationary phase was reached in order to make subsequent transfers (Data not shown). Non-adapted and acid adapted inoculum was prepared by inoculating bacterial cultures into 10 ml TSB and TSB + 1% glucose respectively and then incubating at 37 °C for 24 h. Addition of glucose to the growth medium allows production of organic acids, which lower the final pH of the culture medium significantly (Buchanan and Edelson, 1999a). Cultures (1ml) were then transferred into 25 ml TSB centrifuge tubes and further incubated at

35 °C for 18 h. Cultures were then centrifuged at 5,738 X g for 10 min. at 4 °C (SORVALL SUPER T21, Newton, CT). The supernatant was decanted and resultant pellet resuspended with 10 ml of 0.1% sterile peptone water (PW; Difco, Becton Dickinson, Sparks, MD). A cocktail was prepared by mixing the five cultures in a sterile bottle to get a final volume of 50 ml of the inoculum.

Preparation and inoculation of meat serum.

Ground beef was obtained from the Iowa State University Meat Laboratory and irradiated at the Iowa State University Linear Accelerator Facility to an average absorbed dose of 8.05 kGy. Ground beef was irradiated to remove any background gram negative microflora. Meat serum was then prepared by making a 1:1 dilution of the irradiated ground beef with 0.1 % PW and homogenizing in a stomacher (Stomacher 400, Tekmar, Cincinnati, OH) for 2 minutes. This was followed by centrifuging the aliquot at 5,738 x g for 30 min. at 4 °C. The supernatant was decanted into a sterile Wheaton bottle and filtered through a 0.45 µm cellulose acetate membrane filter (Corning glass works, Corning, NY). The filtered meat serum was then divided into four-40 ml parts in sterile tubes. This 40 ml sterile meat serum was then inoculated with the acid adapted or non-adapted *E. coli* O157:H7 and 5 ml of the inoculated meat serum was then measured into smaller sterile tubes and stored at 4 and -20 °C for 1 and 7 days. In order to compare the pathogenicity of acid adapted and non-adapted *E. coli* O157:H7 in laboratory media the pathogen was grown in TSB+1% glucose and TSB respectively. Inoculated meat serum and laboratory media was removed on day 1 and 7; incubated at 37 °C for 24 h prior to extraction of *E. coli* O157:H7 toxin.

Extraction of *E. coli* O157:H7 toxin/ cell free extract.

Toxins were extracted from acid adapted *E. coli* O157:H7 in laboratory media and in meat serum; and from non-adapted *E. coli* O157:H7 in laboratory media and meat serum. Inoculated meat serum and laboratory media was centrifuged at 7,000 x g for 15 min. at 4 °C post incubation. The supernatant was then decanted into a sterile test tube and filtered through 0.45 µm cellulose acetate membrane filter to remove any residual bacterial cells and stored at – 80 °C until toxicity assays were conducted.

Feeding Vero Cells.

Vero cells are African green monkey kidney cells (ATCC # CCL 81) that are an adherent cell line. These cells were grown in complete RPMI-1640 (Sigma-Aldrich, St. Louis, Mo) media at 37 °C with 5 % CO_2. Complete RPMI-1640 media was made by adding 2mM L-glutamine, 25 mM Hepes, 50 µg/ ml gentamicin, and 10 % fetal bovine serum (FBS) to RPMI-1640 media. Cell culture flasks (75 cm^2; Costar, Cambridge, MA) were used to grow these cells. Vero cells were split when they were approximately 80-90 % confluent. Cells were split and maintained by removing the old media from the culture flasks and washing with 8 ml Hanks balanced salt solution (HBSS) supplemented with 25 mM Hepes to remove all the fetal bovine serum containing trypsin inhibitors. 3 ml of 0.25 % trypsin was added to the flask and incubated at 37 °C for 8 min. During the incubation period the flask was removed and rocked intermittently in an effort to get the cells in a single cell suspension. At the end of incubation flasks were observed under an inverted microscope to ensure single cell suspension of the loose cells. Flasks were vortexed if large clumps were observed. 10 ml of complete RPMI media was added and pipetted up and down to break up the clumps.

Following this, cells were counted using a hemacytometer and the cell concentration and volume of cells was recorded. 1.5 ml of the trypsinized cells were transferred to 13.5 ml of fresh complete RPMI media and incubated at 37 °C. This procedure of feeding Vero cells was repeated every 72 to 96 h.

Toxicity Assay.

Vero cells were harvested when 80-90 % confluent and centrifuged at 300 X g (CR-312 Jouan Inc., Winchester, VA) for 8 minutes. The supernatant was decanted and the pellet resuspended in 7 ml of complete RPMI media. The cells were counted using a hemacytometer after staining with 0.4 % Trypan blue and cell concentration was determined. Appropriate dilutions with complete RPMI media were made in order to obtain a cell concentration of 10^4/ 25 µl (10^4 cells/ well in a 96 well micro titer plate; Costar, Corning Inc., NY). 75 µl of complete RPMI media was put into the first row of wells along the length (12 wells) of the micro titer plate and 50 µl of the complete RPMI media was put in the remaining 84 wells of the plate. Following this 25 µl of the toxin from different samples was put into the first row of wells and subsequent 1:3 dilutions were made along the width (8 wells) of the plate. Results are shown at the most concentrated form of the *E. coli* O157:H7 toxin, medium concentration (obtained as a result of four 1:3 dilutions of the most concentrated toxin), and low concentration (obtained as a result of seven 1:3 dilutions of the highly concentrated toxin). Comparison of toxicity between different sources of toxin is reported at the highest concentration. The plates were shaken well in order to mix the toxin with complete RPMI media and then 25 µl of Vero cells were added to each well. The plates were then incubated at 37 °C for 48 h. After 48 h, 15 µl of MTS dye [3-(4, 5 dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] was added to each

well and optical density was determined at 490 nm in an automated micro plate reader (EL 340, Bio-tek instruments Inc., Winooski, VT). Absorbance was measured at 0, 1, 2, 3, and 4 h post incubation at 37 °C. Higher absorbance indicated lower toxicity of a toxin to Vero cells.

Experimental Design and Statistical Analysis.

Three independent replications of randomized complete block design were used to prepare the filtered meat serum which was then inoculated with either non-adapted or acid adapted *E. coli* O157:H7. The inoculated meat serum was divided into 5 ml sterile tubes in a completely randomized design prior to storage at -20 and 4 °C for 1 and 7 days. Optical densities were measured and graphs were constructed for comparing toxicity of acid adapted and non-adapted *E. coli* O157:H7 in laboratory media and meat serum, with Y-axis representing optical density of the vero cells at 490 nm and X-axis representing different samples. Three replications of the experiment were performed and the mean optical densities of each sample were analyzed using analysis of variance (ANOVA) with the SAS PROC MIXED procedures (2002-03, SAS Institute, Cary, N.C.). Significant differences were reported at $P < 0.05$.

Results

Analysis of variance (ANOVA) suggested that there was no interactive effect of acid adaptation, storage day and temperature on the pathogenicity of *E. coli* O157:H7. Figure 1 shows the optical densities of Vero cells that have been subjected to toxin produced by non-adapted and acid adapted *E. coli* O157:H7 in laboratory media and meat serum. Overall significant differences ($P<0.05$) were observed in the optical densities on day 1 and 7

irrespective of the source from which the toxin was obtained. The different sources of *E. coli* O157:H7 toxin were; (1) meat serum inoculated with non-adapted cells stored at 4 °C, (2) meat serum inoculated with acid adapted cells stored at 4 °C, (3) non-adapted cells in TSB stored at 4 °C, (4) acid adapted cells grown in TBG+1% glucose and stored at 4 °C, (5) meat serum inoculated with non-adapted cells stored at -20 °C, (6) meat serum inoculated with acid adapted cells stored at -20 °C, (7) non-adapted cells in TSB stored at -20 °C, and (8) acid adapted cells grown in TBG+1% glucose and stored at -20 °C.

Comparing least squares means (LSmeans) of the optical densities of Vero cells indicated that there was a significant difference ($P<0.05$) in the pathogenicity of acid adapted and non-adapted cells inoculated in meat serum and stored at 4 °C on day 1, but this difference was not observed after one week of storage. This suggests that there could possibly have been a transitory change in the acid adapted cells which did not persist until one week of storage. Figure 1 suggests that toxin produced by acid adapted cells was less toxic to Vero cells as compared to that produced by non-adapted cells. Toxicity was observed to be lower after 7 days of storage at 4 °C as compared to those stored the -20 °C (fig. 1).

Figure 2 A and B show toxicity of varying concentrations of toxin produced by non-adapted and acid adapted *E. coli* O157:H7 on Vero cells after 1 day of storage at 4 and -20 °C respectively. Toxic effects of different concentrations of the toxin produced by non-adapted and acid adapted *E. coli* O157:H7 on Vero cells after 7 days of storage at 4 and -20 °C are shown in figure 2 C and D respectively. From figures 2 A, B, C, and D it can be seen that Vero cells exposed to highest concentration of the toxin had the lowest optical density indicating maximum toxicity.

Discussion

Implication of numerous acidic foods such as pasteurized apple juice and fermented meats in recent outbreaks associated with survival of *E. coli* O157:H7 have emphasized the need for studies on acid resistance and pathogenicity of these strains. Several studies in the past have suggested higher thermal tolerance of acid adapted *E. coli* O157:H7 in laboratory media (Buchanan and Edelson, 1999b) and fruit juices (Sharma et. al., 2005). But there is lack of evidence about the ability of acid adapted *E. coli* O157:H7 to produce toxin and cause human illnesses that are comparable to those cause by their non-adapted counterparts. In this study the toxicity of acid adapted and non-adapted *E. coli* O157:H7 was compared in an actual food system mimicking industrial and consumer handling practices of refrigerated and frozen storage in addition to subjecting the meat serum to abusive temperatures by incubating at 37 °C for 24 h. Our study has shown that there is no difference in the cytotoxicity of acid adapted and non-adapted *E. coli* O157:H7 in laboratory media. In meat serum acid adaptation caused the cells to be less toxic as compared to the non-adapted cells when stored under refrigeration (4 °C). But this effect was short term and no differences in the cytotoxicity were observed after 7 days of refrigerated storage. The results on cytotoxicity in this study are contrary to those reported by O'Driscoll et. al. (1996) suggesting higher virulence of certain acid adapted bacterium. Findings from our study showing less toxicity of acid adapted *E. coli* O157:H7 than their non-adapted counterparts are in agreement with earlier studies done by Yuk and Marshall (2005) showing that although organic acid adapted cells were more heat resistant they produced less total verotoxin than their non-adapted counterparts at a concentration of approximately 10^8 CFU/ ml.

Weeratna and Doyle (1991) suggested that pH of growth medium could be a factor influencing higher production of verotoxin in meat. In our study the pH of meat serum inoculated with acid adapted strains of *E. coli* O157:H7 was lower (pH ~4.6) than that of the meat serum inoculated with non-adapted cells (pH ~ 6.5) after 24 h incubation at 37 °C. Lower toxicity of acid adapted cells in our study could be attributed to low pH as suggested by Weeratna and Doyle (1991). Lower toxicity of the acid adapted cells in our study was in agreement with findings by Yuk and Marshall (2004) that suggested decrease in membrane fluidity in acid adapted cells may increase the acid resistance but decrease verotoxin secretion. Sufficient evidence has been published to prove higher survival rates of acid adapted *E. coli* O157:H7 in laboratory media and in juices. This information, combined with the results from our study suggesting that acid adapted cells have the capability to produce toxin indicates that these acid adapted pathogens are a major concern during processing and storage of foods.

The reasons for less toxin production by the acid adaptation are not clear but could be attributed to diversion of energy involved in toxin production to other metabolic activities for growth and survival under acid stress or partial repression of the verotoxin producing gene as a result of extreme pH. Although the ability of acid adapted *E. coli* O157:H7 to survive when challenged with various organic acids (Goodson and Rowbury, 1989) and its increase thermal tolerance have been extensively reported the inability of these acid adapted cells to produce toxins comparable to their non-adapted counterparts as shown in our study remains a question that needs to be further investigated.

Conclusions

Organic and inorganic acids are widely used in the food industry as a means of preservation and more recently interventions to control bacteria of public health concern. Based on our findings in this study the ability of pathogenic bacteria to adapt to low pH can pose a threat to safety of foods. Although our results indicated less toxicity of acid adapted *E. coli* O157:H7 in meat serum the ability to survive in extremely low pH conditions could provide cross-protection against thermal and osmotic stresses. Results presented in this study can be helpful in further understanding the relationship between acid adaptation and pathogenicity of *E. coli* O157:H7 as well as long term effects under various storage conditions of meat.

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Figure Legends

Figure 1: Relative toxicity of acid adapted and non-adapted *Escherichia coli* O157:H7 in laboratory media and meat serum determined by optical densities of samples stored at -20 and 4 °C for 1 and 7 days. Each data point is an average of three independent replications performed in duplicates. Error bars represent standard error. Higher optical densities represent lower toxicity.

Figure 2: Toxicity of acid adapted and non-adapted *Escherichia coli* O157:H7 at different concentrations in laboratory media and meat serum determined by optical densities of samples stored at (A) 4 °C for 1 day; (B) -20 °C for 7 days; (C) 4 °C for 1 day; and (D) -20 °C for 7 days. ■ = Toxicity of non-adapted *E. coli* O157:H7 in meat serum; □= Toxicity of acid adapted *E. coli* O157:H7 in meat serum; ●= Toxicity of non-adapted *E. coli* O157:H7 in laboratory media; ○= Toxicity of acid adapted *E. coli* O157:H7 in laboratory media.

Fig. 1, top ↑

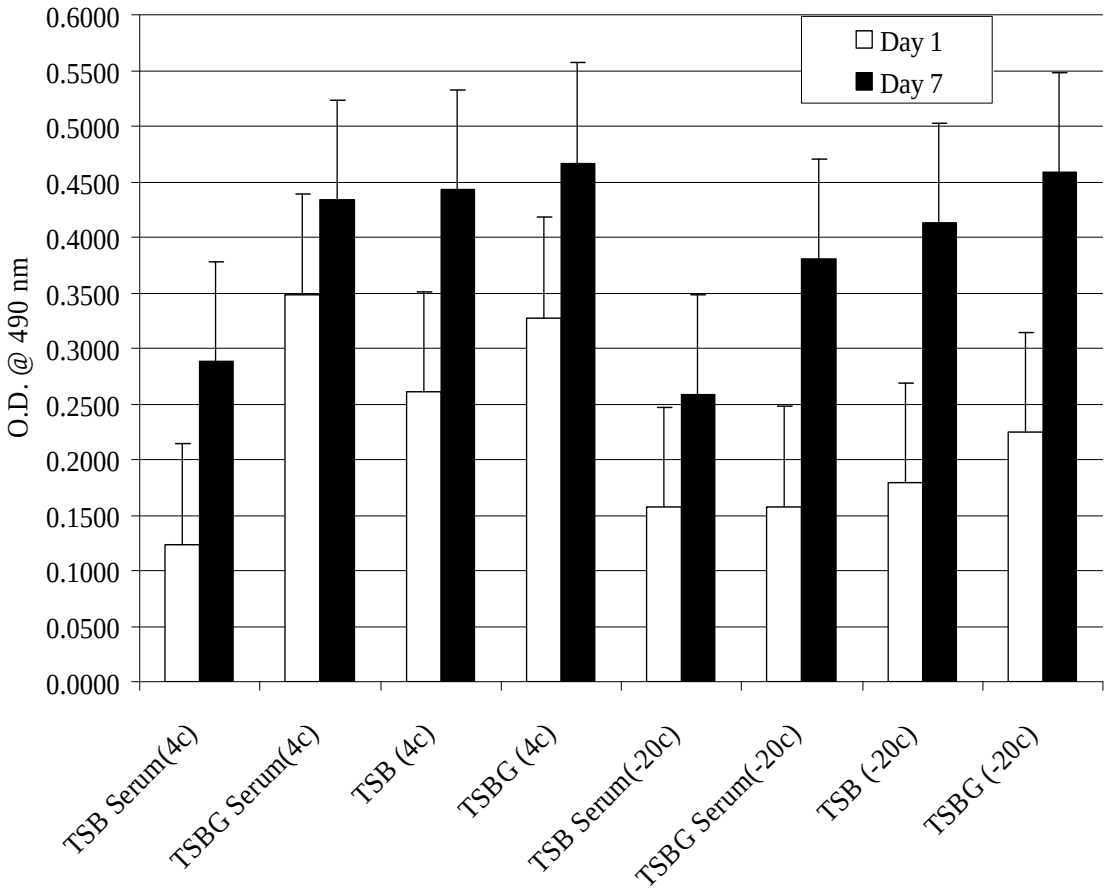
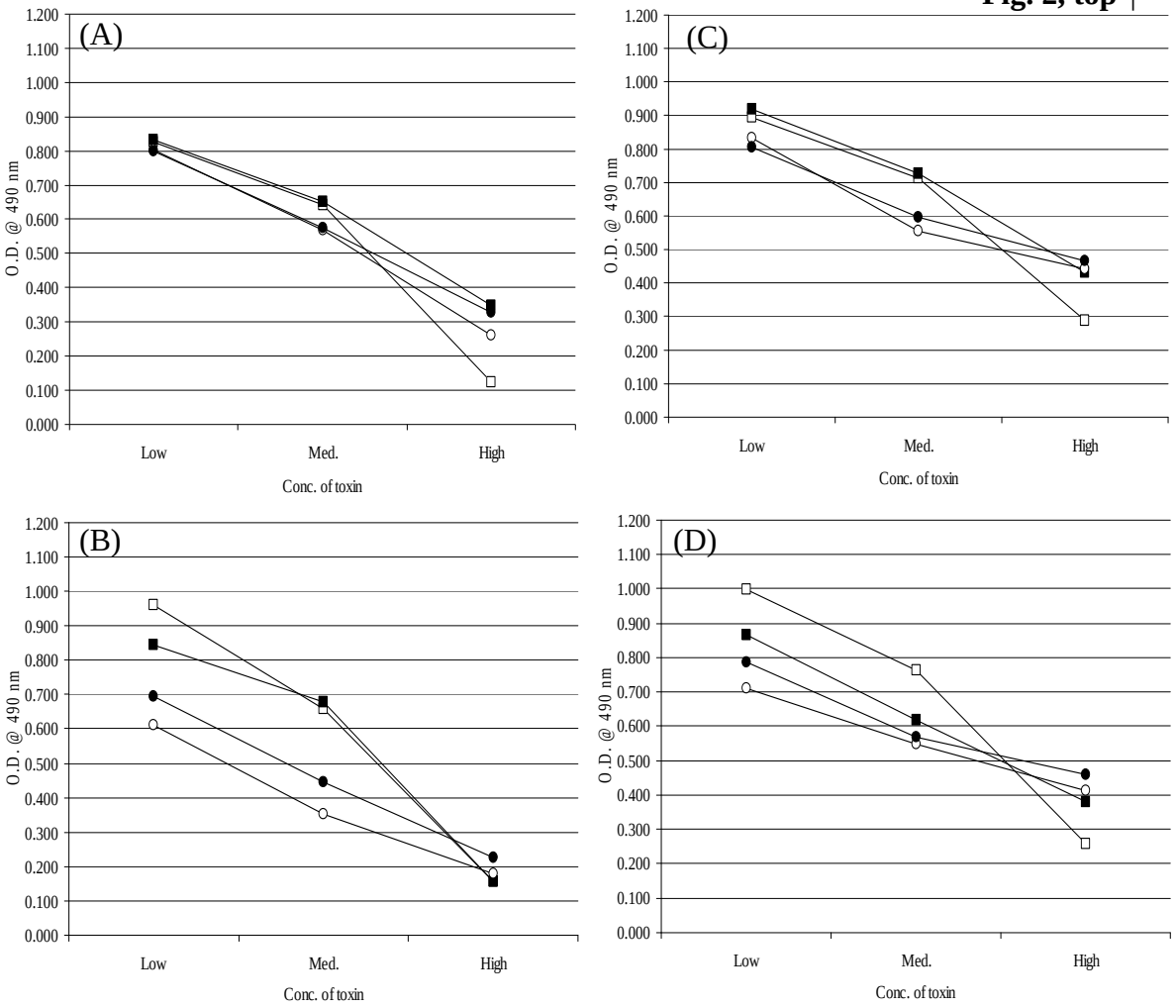


Fig. 2, top ↑



GENERAL CONCLUSIONS

In conclusion this research has provided insight on the thermal tolerance of acid adapted *Escherichia coli* O157:H7 and *Salmonella* in meat serum, effect of storage at 4 and -20 °C on the thermal resistance of these acid adapted pathogens in ground beef, and pathogenicity of acid adapted *E. coli* O157:H7 in meat serum stored for up to 7 days at 4 and -20 °C. Results have shown that there is increased thermal tolerance of acid adapted pathogens initially but over time this difference in the resistance to thermal stress is depleted in ground beef. Hence, these acid adapted cells respond to thermal stress in a manner similar to their non-adapted counterparts. Data from the evaluation of cytotoxicity of acid adapted and non-adapted cells of *E. coli* O157:H7 suggested that although there could be increased short term resistance to thermal stress in acid adapted cells it does not persist for an extended period and the amount of verotoxin produced by these cells is less or in same amounts as that produced by the non-adapted *E. coli* O157:H7.

The ability of these pathogens to survive acidic conditions is an important aspect that needs to be considered when implementing regulatory guidelines for the use of organic and inorganic acid as decontamination strategies. Subsequent influence of acid adaptation on thermal tolerance and other environmental stresses poses the need to review regulatory guidelines for cooking meat and poultry products in order to destroy these pathogens.

FUTURE STUDIES

Results from our study have indicated that there is a need for more studies to be conducted on the behavior of acid adapted food borne pathogens specifically *Escherichia coli* O157:H7 and *Salmonella*. Further studies need to be conducted to also determine pathogenicity of acid adapted *Salmonella* by conducting invasiveness assays. Also, studies to determine the actual toxin content produced by the acid adapted and non-adapted *E. coli* O157:H7 need to be developed as a reference for further shelf life studies and effects of storage temperatures on the *E. coli* O157:H7 toxin.

Another important aspect that would be useful in determining the hazard posed by acid adapted food borne pathogens would be to conduct studies to evaluate the efficacy of organic and inorganic acid such as citric, lactic, and acetic acid to reduce such acid adapted bacteria. At the same time these studies need to be conducted on other meat sources such as pork and poultry products to get a better idea of contamination issues concerned with acid adapted pathogens. Following thermal tolerance studies there is a need to also conduct research on the resistance of these acid adapted pathogens to other stresses such as irradiation, osmotic stress, and other non-thermal intervention methods.