## Abstract

**Reha Onur Azizoglu:** Influence of Antibiotic, Acid, and Salt Stress on Resistance of *Escherichia coli* O157:H7. (Under the direction of Dr. MaryAnne Drake).

The objective of this study was to understand the impact of antibiotic, acid, and salt on the stress response of *Escherichia coli* O157:H7. *E. coli* cells were exposed to sublethal levels of each stress for 4 hours and post stress tolerance and virulence factor production were evaluated. Heat tolerance (56$^{o}$C) was determined using the capillary tube method, and simulated gastric fluid (SGF, pH 1.5) survival was used to assess acid tolerance. Stressed and control cells were evaluated in triplicate. In the first part of the study, the impact of sublethal levels of antibiotic on virulence factor production, survival in SGF, and heat tolerance (56$^{o}$C) were studied. The minimum inhibitory concentration (MIC) for three antibiotics (trimethoprim, ampicillin, and ofloxacin) was determined for two *E. coli* O157:H7 strains using the dilution series method. Subsequently, virulence factor production (Stx, Hly, EaeA) was evaluated by the creation of *lacZ* gene fusions followed by use of the Miller assay (a β-galactosidase assay). Heat tolerance and SGF survival following antibiotic exposure decreased compared to control cells ($p<0.05$). Exposure to ofloxacin increased Stx and Eae production ($p<0.05$). Exposure to ampicillin or trimethoprim increased Eae production ($p<0.05$). Hly production decreased when cells were stressed with ampicillin, but increased following trimethoprim stress ($p<0.05$). Antibiotics can increase *E. coli* O157:H7 virulence factor production, but do not produce a cross-protective response to heat or decreased pH.

In the second part of the study, the impact of acid and salt exposure on SGF (pH 1.5) survival, heat tolerance (56$^{o}$C), survival in 14% salt, and survival in 14% salt at pH 5.0 (acidified with lactic acid or HCl) of two *E. coli* O157:H7 strains (EHEC1, EHEC2) and a nonpathogenic *E. coli* (NPEC) strain was studied. Acid adaptation of the *E. coli*

cells was achieved by overnight growth in TSB with 1% glucose to a final pH of 4.8. Acid exposure was done in TSB acidified to pH 5.0 with malic, lactic, citric, or hydrochloric acid (HCl). Sub-lethal salt exposure was conducted at 4% (w/v) salt concentration. Acid adaptation of *E. coli* O157:H7 enhanced SGF survival, heat tolerance, and NaCl survival of the cells with and without additional acid or salt stress ($p<0.05$). Among the acids used in acid stress studies, lactic acid had the highest impact on the development of resistance to post stress tolerance ($p<0.05$). Acidification of 14% NaCl TSB to pH 5.0 increased the resistance of cells following sublethal stresses, especially when acidified with lactic acid ($p<0.05$). Salt stress enhanced SGF tolerance of acid adapted cells of EHEC2 and NPEC ($p<0.05$) but not EHEC1 ($p>0.05$). Heat tolerance of EHEC2 and NPEC decreased following salt stress, regardless of acid adaptation ($p<0.05$). Acid adaptation and acid and salt stress of *E. coli* produce protection against further challenges, which should be taken into consideration when producing minimally processed foods.

**Keywords:** *E. coli* O157:H7, Antibiotic Stress, Acid Stress, Salt Stress, Heat Tolerance, Acid Challenge, Virulence Gene Expression

# INFLUENCE OF ANTIBIOTIC, ACID, AND SALT STRESS ON RESISTANCE OF *ESCHERICHIA COLI* O157:H7

by

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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

**FOOD SCIENCE**

Raleigh

2005

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## BIOGRAPHY

Reha Onur Azizoglu was born on 20th February 1979 in Yigilca, Turkey. He grew up in Ankara, the capital of Turkey, with his parents Sunduz and Halit Azizoglu and elder brother Suha. He proudly attended TED Ankara Private High School. After graduation he pursued his Bachelor of Science degree in the Department of Food Engineering at Middle East Technical University. He graduated in June 2002, and in Fall 2002 he was honored by the scholarship by the Turkish Republic Ministry of National Education to pursue his graduate studies in U.S. In summer 2003, he began his graduate studies in Department of Food Science at North Carolina State University. During his graduate studies he married his love of life Eda. Upon completion of his Masters degree, Reha is planning to pursue Ph.D. in Food Science.

## ACKNOWLEDGEMENTS

I would first like to thank to my advisor, Dr. MaryAnne Drake, for all her support, understanding, and encouragement throughout my study. I am thankful for her endless inspiring guidance and encouragement. I would like to acknowledge the Turkish Republic Ministry of National Education for financially supporting and giving me the opportunity to pursue my graduate studies in U.S. I would also like to thank members of my committee, Dr. LeeAnn Jaykus, and Dr. Donn Ward for their help to attain my degree.

I wish to thank all my friends both in U.S. and Turkey, especially to Zeynep for helping me to adjust to Raleigh when I first came here.

My parents and my brother always support and encourage me throughout my life. Without them I would never be able to achieve my degree. Thank you for always being on the other end of the phone when I was in Raleigh.

Finally, I would like to thank my beloved wife Eda. She always supported and encouraged me during the most depressed days of my studies. It is a great feeling to now that she is always there for me. I love you from the very depth of my heart. I believe with the power of our love nothing could ever stand in front of us.

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## Introduction

*Escherichia coli* O157:H7 is a Gram-negative, facultative anaerobe, and since the first reported outbreak in 1982, this organism has been recognized as the major cause of hemorrhagic colitis and hemolytic uremic syndrome (Arnold et al., 1995; Sussman, 1997). The gastrointestinal tract and the kidneys are the target organs of *E. coli* O157:H7. Bloody diarrhea (hemorrhagic colitis) is the primary disease symptom, as well as other disease complications including hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Doyle et al., 2001).

There is no animal model for enterohemorrhagic *E. coli* (EHEC) infection, thus all virulence factors are putative (Doyle et al., 2001). The ability of EHEC strains to cause severe disease in humans is related to their capacity to secrete Shiga toxins (Griff et al., 1998). The two main types of EHEC Shiga toxin are Stx1 and Stx2 (Muhldorfer et al., 1996; O'Brien et al., 1987). Stx2 producing strains of EHEC are more virulent than Stx1 or Stx1 and Stx2 producing EHEC (Donnenberg, 2002), and thus are more frequently associated with human disease complications (Boerlin et al., 1999) Other putative virulence factors of EHEC are intimin which is encoded by the *eae* gene, and enterohemolysin (EHEC-*hlyA*) encoded by a large plasmid-borne *ehxA* gene (Kumar et al., 2004).

In food preservation and processing, foodborne bacteria are subjected to different kinds of stresses (Yura et al., 1999). Stress is any deleterious factor or condition that adversely affects the growth or survival of microorganisms. The stress response system of a microorganism can be activated by several different stresses and may protect against multiple stresses (Yousef et al., 2002). In food processing, pH, sodium chloride, and heat are common applications to control microbial populations. Antibiotics are used in the food production and processing for the treatment, or prevention of disease, or to increase the efficiency of meat or milk production (Teuber, 1999).

Previous studies indicated that sublethal stress conditions can affect the virulence expression/production of *E. coli* O157:H7 as well as increase the resistance of the cell to subsequent challenges. Yuk et al. (2005) indicated that Shiga toxin production by *E. coli* O157:H7 was dependent on both pH and organic acid type. They found that organic acid adaptation (acetic, citric, or lactic acid) at pH 6.4 or pH 5.4 generally decreased Shiga toxin production. In a similar study, Duffy et al. (2000) reported that *E. coli* O157:H7 grown at pH 5.6 had lower Shiga toxin production than did cells grown at pH 7.4. These studies all addressed toxin production.

Foodborne bacteria are subjected to organic or inorganic acids in foods or in the gastrointestinal tract of the host (Yousef et al., 2002). Lin et al. (1996) indicated that it is important for Enterohemorrhagic *E. coli* strains to pass through the acidic environment of the gastric fluid in order to cause gastrointestinal diseases. Organic acids are more effective against foodborne pathogens than inorganic acids as they have the ability to diffuse into the cell in their nonpolar protonated form and dissociate. This action decreases intracellular pH and inhibits essential cell processes (Buchanan et al. 2004; Abee et al. 1999). Cheville et al. (1996) documented that subjection of *E. coli* O157:H7 to sublethal levels of acid stress enhanced its survival in foods and plays a key role in its pathogenesis by increasing its resistance to subsequent challenges. Cheng et al. (2002) indicated that cells acid-adapted at pH 5.0 acidified with HCl were more heat tolerant than the non-adapted cells

Application of salt to control microbial growth is common in the food industry. Cheng et al. (2002) indicated that the inhibitory effect of sodium chloride is related to a plasmolytic effect in addition to dehydration, oxygen removal, interference of enzymes and the toxicity of the sodium and chloride ions when they are in solution. Casey et al. (2002) studied the effect of 4% sodium chloride in fermented meat model systems on the survival of *E. coli* O157:H45 (an enteropathogenic *E. coli* (EPEC)) at pH 4.2. Cells

subjected to sodium chloride and acid survived better in fermented meat than cells subjected to only acid.

In order to adequately control pathogens during food processing, it is important to understand how different sublethal stresses impact the survival and stress response of *E. coli* O157:H7. The objectives of this study were;

- ✓ to determine the effect of antibiotic exposure on virulence factor production, simulated gastric fluid (SGF, pH 1.5) survival, and heat tolerance (56$^{o}$C) of *E. coli* O157:H7.
- ✓ to determine the effect of acid and NaCl exposure on simulated gastric fluid (SGF, pH 1.5) survival, heat tolerance (56$^{o}$C), NaCl (14%) survival and NaCl at pH 5.0 (14%, by HCl or lactic acid) survival of *E. coli* O157:H7.

## Literature Review

### *Escherichia coli*

*Escherichia coli* (*E. coli*) was first described in 1885 by the German pediatrician Theodore Escherich. The organism was discovered while studying the fecal flora of neonates and infants, and was also found in healthy individuals (Donennberg, 2002). The organism was first described by Escherich under the name *Bacterium coli commune* (Sussman, 1985). *E. coli* is a member of the normal commensal intestinal flora of humans and colonization takes place soon after birth. The sources of colonization are the mother and the environment. It appears rapidly in the saliva but does not appear to colonize the normal mouth or pharynx (Sussman, 1985).

The genus *Escherichia* is a member of the family *Enterobacteriaceae* whose principle habitat is the intestines of humans and animals. *Escherichia* has close genetic relations with a number of other genera in the family, most particularly *Shigella*. *E. coli* is a short, straight Gram-negative rod. It is non-spore-forming, usually motile with peritrichous flagella, often fimbriate, and occurs singly, or in pairs. A capsule or microcapsule is often present. Also, some strains produce polysaccharide slime (Sussman, 1997).

*E. coli* is a facultative anaerobe capable of fermentative and respiratory metabolism. Optimum growth temperature of *E. coli* is 37$^{o}$C and it can grow on a wide range of simple culture media and on simple synthetic media. Under anaerobic growth conditions it requires fermentable carbohydrate. It ferments glucose to pyruvate, which is converted into lactic, acetic and formic acids. Part of the latter is converted into hydrogen and carbon dioxide by formic hydrogenlyase, but some strains do not produce gas (anaerogenic) (Sussman, 1997).

Most strains of *E. coli* are nonpathogenic, living in harmony with their host. The relationship may be symbiotic, in which the bacteria benefits from the host and synthesizes cofactors that contribute to colonization resistance against pathogenic organisms for the host. On the other hand, some strains of *E.coli* are pathogenic and are capable of causing a wide variety of illnesses. Susceptible hosts to *E.coli* include birds, pigs, cattle, rabbits, sheep and humans. The gastrointestinal tract and the kidneys are the target organs that are affected by pathogenic *E. coli*. The diseases or disease complications resulting from *E. coli* infections include diarrhea, dysentery, overwhelming sepsis, hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP).

The pathogenicity of *E. coli* is strain-dependent. The differences in the ability of strains to cause disease and the diverse syndromes caused by different strains can be described by the presence of specific genes encoding virulence factors and the capacity of *E. coli* for genetic exchange. Tatum and Lederberg (1947) first described bacterial recombination in *E. coli*, and genetic transfer via conjugation remains an important mode by which the species acquires new genes, as evidenced by the large number of pathogenic factors present on plasmids in various strains of *E. coli*. Other critical virulence determinants are encoded within the genomes of bacteriophages or within pathogenicity islands, mysterious blocks of foreign DNA encoding virulence factors. Pathogenic *E. coli* strains may have hundreds of blocks of DNA that are not present in nonpathogenic *E.coli* strains (Donnenberg, 2002). On the other hand, all strains have many integrated bacteriophage genomes. In the light of this, the pathogenic potential of a particular *E.coli* strain depends on the repertoire of specific virulence genes present within its genome (Donnenberg, 2002).

It is difficult to assess the function of *E. coli* in the fecal flora. It has been suggested that it has a nutritional significance by providing a source of vitamins in some animals. In nature it is found in soil, water or at any other site it can reach from its primary habitat, usually by fecal contamination. The prevalence of certain serogroups in

the human fecal flora is greater than that of others and this may in some way be due to their capacity to persist in the intestine (Sussman, 1997). A variety of antigens may be used to type *E.coli* strains (Sussman, 1985). *E.coli* isolates are serologically differentiated based on three major surface antigens. These are O (somatic), H (flagella), and K (capsule) antigens. A total of 174 O antigens, 56 H antigens, and 80 K antigens have been identified (Doyle et al., 2001).

Pathogenic *E.coli* strains are categorized into specific groups based on their virulence properties, their mechanisms of pathogenicity, clinical syndromes, and distinct O:H serogroups. These categories are:

- Enteropathogenic *E.coli* strains (EPEC)
- Enterotoxigenic *E.coli* strains (ETEC)
- Enteroinvasive *E.coli* strains (EIEC)
- Diffuse-adhering *E.coli* strains (DAEC)
- Enteroaggregative *E.coli* strains (EAggEC)
- Enterohemorrhagic *E.coli* strains (EHEC) (Doyle et al., 2001)

**Enteropathogenic *E.coli* (EPEC)**

Enteropathogenic *E.coli* strains have the ability to cause diarrhea and produce a histopathology in the intestinal epithelium known as attaching and effacing (A/E)

lesions (Donnenberg, 2002). The major O serogroups associated with EPEC include; O55, O86, O111ab, O119, O125ac, O126, O127, O128ab, and O142. The main reservoir for EPEC is humans (Doyle et al., 2001). DNA probe hybridization and polymerase chain reaction (PCR) have become standard methods to identify EPEC-specific genetic material (Donennberg, 2002).

**Enterotoxigenic *E.coli* (ETEC)**

Enterotoxigenic *E.coli* strains have the ability to cause watery diarrhea. It is mainly associated with tropical and developing countries (Sussman 1997). Also ETEC are the most frequent cause of traveler's diarrhea (Doyle et al., 2001). These bacteria possess adhesions, known as colonization factor antigens (CFA), which enable them to bind to the small intestine mucosa, where *E. coli* does not normally occur in substantial numbers (Browne et al., 2002). ETEC is distinguished from other *E. coli* pathotypes by the ability to produce heat-labile and heat-stable enterotoxins. In order to classify a strain as ETEC, it must produce at least one of the heat-labile or heat-stable enterotoxins. Another common characteristic of ETEC strains is their ability to adhere to the intestinal epithelium. Colonization factor-mediated adherence allows for the delivery of enterotoxins and the subsequent host response that is experienced as diarrhea (Donennberg, 2002). The most frequent ETEC serogroups include O6, O8, O15, O20,

O25, O27, O63, O78, O85, O115, O128ac, O148, O159, and O167. Humans are the principle reservoir of ETEC that cause human illness (Doyle et al., 2001).

**Enteroinvasive *E.coli* (EIEC)**

The symptoms of EIEC are similar to *Shigella* spp. in that they cause non-bloody diarrhea and dysentery by invading and multiplying within colonic epithelial cells. As with *Shigella* spp., the invasive capacity of EIEC is associated with the presence of a large plasmid which encodes several outer membrane proteins (OMPs) involved in invasiveness. The antigenicity of these OMPs and that of the O antigens of EIEC are closely related. The site of bacterial localization is the colon, where EIEC invade and proliferate in epithelial cells and cause cell death. Humans are the major reservoir. The serogroups most frequently associated with illness are O28ac, O29, O112, O124, O136, O143, O144, O152, O164, and O167. The O124 serogroup is the one most commonly encountered (Doyle et al., 2001).

**Diffuse-adhering *E. coli* (DAEC)**

Diffuse-adhering *E. coli* strains have the ability to cause mild diarrhea without blood or fecal leukocytes. They are identified by a characteristic diffuse-adherent pattern of adherence to HEp-2 and HeLa cell lines. DAEC cover the cell surface

uniformly. DAEC generally do not produce heat-labile or heat-stable toxins. They also do not possess EPEC adherence factor plasmids or invade epithelial cells.

**Enteroaggregative *E. coli* (EAggEC)**

Enteroaggregative *E. coli* strains are associated with persistent diarrhea in infants and children in developing countries worldwide. It has also emerging as an etiological agent of acute intestinal infection in industrialized countries (Neto et al., 2003). These *E. coli* strains are defined as *E. coli* that do not secrete the enterotoxigenic *E.coli* (ETEC) heat-labile or heat-stable enterotoxins and which adhere to HEp-2 cells in an aggregative pattern (Donnenberg, 2002). The pathogenesis of EAggEC infection is thought to involve the adherence of the bacterium to the intestinal mucosa, possibly in both the small and large intestines, followed by secretion of one or more enterotoxins (Nishi et al., 2003). Typically, EAggEC adheres to cultured epithelial cells in a distinct staggered configuration known as the aggregative adherence (AA) pattern which differs from the localized or diffuse adherence patterns displayed by other diarrheagenic *E. coli* (Neto et al., 2003). The serotypes characteristic of EAggEC are not well characterized, but several serotypes are found commonly. Most characteristic serogroups have the flagellar antigens H18, H2, and H33 (Neto et al., 2003)

**Enterohemorrhagic *E. coli* (EHEC)**

Enterohemorrhagic *E. coli* strains were first identified as human pathogens in 1982, when *E. coli* serotype O157:H7 was associated with two outbreaks of hemorrhagic colitis (HC). Since then, certain strains and serogroups of *E.coli*, including O26:H11, O103, O104, O111, and sorbitol-fermenting O157-$H^-$, have been associated with cases of bloody diarrhea (hemorrhagic colitis) and also have been identified as EHEC strains. However, serotype O157:H7 is the predominant cause of EHEC-associated disease in the United States and many other countries (Doyle et al., 2001). The inability of the O157:H7 serotype to ferment sorbitol has facilitated the differentiation and hence detection of this serotype from other *E. coli* strains in routine testing (Donnenberg, 2002). All EHEC strains produce factors cytotoxic to African green monkey kidney (Vero) cells. These toxins had been described as verotoxins (VTs) or Shiga-like toxins (SLTs) because of their similarity to the Shiga toxin (Stx) produced by *Shigella dysenteriae* type 1. Caldwell et al. (1996) defined that these *E. coli* cytotoxins (SLTs and VTs) were related to the Shiga toxin of *Shigella* spp. as they share the following properties: DNA sequence homology and operon structure; stoichiometry of five B subunits to one A subunit in the mature holotoxin; identical enzymatic activity of the A subunit; binding to specific eukaryotic cell membrane glycosphingolipid

receptors; and identical biological properties. As a result of these relations, SLT-I and VT1 were changed to the nomenclature *stx1* (Shiga toxin 1) and SLT-II and VT2 changed to *stx2* (Shiga toxin 2). Many *E. coli* strains possess Shiga toxins and are called Shiga toxin producing *E. coli* (STEC). However, only those strains that cause HC (in humans) are considered to be EHEC strains.

Enterohemorrhagic *E. coli* can cause diarrhea and hemorrhagic colitis (HC) (bloody diarrhea) and rarely, the complications thrombotic thrombocytopenic purpura (TTP) and hemolytic uremic syndrome (HUS). HUS is a complication in children and thrombotic thrombocytopenic purpura (TTP) is an infrequent complication in adults. In addition to patient age, a high white cell count and treatment with antibiotics are also predictive for the development of HUS (Kehl, 2002). Patients with thrombotic thrombocytopenic purpura (TTP) exhibit symptoms consisting of fever, hemolytic anemia, thrombocytopenia, and impaired renal and neural functions. HUS is a disorder consisting of a cluster of clinical features: hemolytic anemia, thrombocytopenia and decreased renal function. As the clinical spectrum of HUS is similar to TTP, many physicians lump them together as one clinical entity called TTP-HUS (Liu et al., 2001). The result of this ‘‘lumping together’’ strategy is that many practitioners tend to not only regard TTP and HUS as a single entity, but also use the same therapeutic protocols

to treat patients with both conditions. TTP is more common in women than in men (ratio of 2:1) (Liu et al., 2001). Delayed diagnosis and treatment of TTP may result in a mortality rate as high as 90%. The two types of clinical courses that have been described for TPP are single acute and chronic relapsing episodes (Liu et al., 2001).

The ability of EHEC strains to cause severe disease in humans is believed to be related to their capacity to secrete Shiga toxin (Stx) as well as other virulence factors including *hly*A, and *eae*A (Griff et al., 1998). EHEC strains are both similar and dissimilar to EPEC strains. They are similar to EPEC in their possession of the chromosomal gene *eaeA* and in the production of attachment-effacement lesions. In contrast to EPEC, EHEC strains affect only the large intestine and produce large quantities of Shiga toxins (Stx) (Jay, 2000).

**Shiga Toxins**

EHEC is considered an important cause of gastrointestinal disease in developed countries (Kumar et al., 2004). *Shigella dysenteriae* produces a potent toxin that is referred to commonly as Shiga toxin. The toxins of EHEC are referred to as Shiga toxins (Stx) as they share many similarities with the toxin produced by *Shigellae* (Jay, 2000). Among several virulence determinants associated with EHEC, the production of one or more Shiga toxins is considered to be most important (Kumar et al., 2004).

The genes for Stx1 and Stx2 are encoded by temperate bacteriophages. Stx1 differs from Shiga-toxin produced by *Shigella* by three nucleotides and one amino acid, and is neutralized by antibodies to Stx. Stx1 and Stx2 are differentiated by a lack of cross-neutralization using homologous polyclonal antisera, and by a lack of DNA-DNA cross-hybridization of their genes under conditions of high stringency (Jay, 2000). Variants of Stx2 have been reported which include Stx2c, Stx2d, Stx2e and Stx2f. Though originally believed to be a non-variant toxin, subtypes of Stx1 namely Stx1c and Stx1OX3, have recently been reported (Donennberg, 2002). Stx2-producing strains are potentially more virulent than strains that produce Stx1 only or that produce both Stx1 and Stx2 (Donnenberg, 2002). In humans, epidemiologic data suggest that *E.coli* O157:O7 strains that express Stx2 are more important than Stx1 in the development of HUS and may result in increased disease severity (Donennberg, 2002). Other virulence factors of EHEC include production of a 94-kDa outer membrane protein called intimin, which is encoded by the *eae* gene present on a 34-kb chromosomal pathogenicity island termed the locus for enterocyte effacement (LEE). An enterohemolysin (EHEC-*hlyA*) encoded by a large plasmid-borne *ehxA* gene is also an EHEC virulence factor (Kumar et al., 2004).

**Antibiotics**

Food preservatives are chemical compounds added directly to food for the purpose of extending shelf life and improving food safety (Yousef et al., 1999). Antibiotics are used in the food industry for the treatment of disease (therapeutic), prevention of disease (prophylactic), or to increase the efficiency of meat (i.e., to increase weight gain) or milk production (sub-therapeutic). It is estimated that 40% of the antibiotics produced in the United States are used in the agriculture industry and among these, only 20% are used for therapeutic purposes in animals (Stevenson et al., 2003).

Antibiotics are low-molecular weight substances. They are secondary metabolites produced by microorganisms, especially *Streptomyces* spp. and fungi, that inhibit or kill a wide spectrum of other microorganisms (Jay, 2000). Antibiotics may have a cidal (killing) or a static (inhibitory) effect. The range of bacteria or other microorganisms affected by a certain antibiotic is referred to as the spectrum of activity. Antibiotic spectrum of activity is the most important property for empirical therapy. Gram-negative organisms differ from Gram-positives in having an extra cell membrane exterior to the peptidoglycan cell wall. Because of this, they are more generally resistant to antibiotics than Gram-positives (Scott et al., 2001). Antibiotics that are effective

against most prokaryotes, kill or inhibit a wide range of Gram-positive and Gram-negative bacteria, are said to be broad spectrum. If they are effective mainly against Gram-positive or Gram-negative bacteria, they are called narrow spectrum. If effective against a single organism or disease, they are referred to as limited spectrum (Russell, 1996).

A useful antibiotic that is used in food should have the following characteristics (Jay, 2000):

- a wide spectrum of activity with the ability to destroy or inhibit many different species of pathogenic organisms.
- nontoxic to the host and without undesirable side effects.
- nonallergenic to the host.
- limited impact on the normal flora of the host.
- inexpensive and easy to produce.
- chemically-stable (have a long shelf-life).
- limited production of resistance.
- rapid destruction by heat for products that require cooking.
- stable in the presence food components or products of microbial metabolism.

**Microorganisms that produce antibiotics**

Almost 10,000 antibiotics have been isolated and described: the chemical structures of the majority have been determined. This large variety of antibiotics is produced by widely diverse microorganisms (Lancini et al., 1995). *Penicillium* and *Cephalosporium* (molds) produce Beta-lactam antibiotics (penicillin, cephalosporin, and their relatives) (Scott, 2001). More than 50% of antibiotics in use today are produced by members of *Actinomycetes*, particularly the genus *Streptomyces* (Lancini et al., 1995). *Actinomycetes*, mainly *Streptomyces* species, produce tetracyclines, aminoglycosides (streptomycin and its relatives), macrolides (erythromycin and its relatives), chloramphenicol, ivermectin, rifamycins, and most of the other antibiotics that are not beta-lactams. *Bacillus* species, such as *B. polymyxa* and *B. subtilis,* produce polypeptide antibiotics (e.g. polymyxin and bacitracin), and *B. cereus* produces zwittermicin (Scott, 2001). The production of antibiotics is not always specific to species, as evidenced by the fact that the same antibiotic can be produced by different organisms that are from different species or genera (Lancini et al., 1995).

The reason that some organisms produce antibiotics is not known, but it may give them some nutritional advantage in their habitat by antagonizing the competition, or by acting as some sort of hormone or signal molecule associated with sporulation or

dormancy or germination. Most of the microorganisms that produce antibiotics are resistant to the action of their own antibiotic. On the other hand, the same organism may be affected by antibiotics produced by other organisms, and in many instances, antibiotics are most effective against closely-related strains (Russell, 1996).

**Mode of Action**

Almost all antibiotics currently used were identified between 1940-1960 (Chopra et al., 2002). Over the last 25 years, bacterial resistance to antibiotics has emerged and this now constitutes a serious threat to global public health (Chopra et al., 2002).

The molecular basis of antibiotic action is well understood and the targets of the principle agents are given in Table 1.1.

| Drug/class | Function inhibited | Molecular target |
|---|---|---|
| B-Lactams | Peptidoglycan synthesis | Transpeptidases and carboxpeptidases |
| Bacitracin | Peptidoglycan synthesis | Undecaprenyl pyrophosphate |
| D-Cycloserine | Peptidoglycan synthesis | D-alanine racemase and D-alanyl-D-alanine synthetase |
| Fosfomycin | Peptidoglycan synthesis | UDP-*N*-acetylglucosamine enolpyruvyl transferase |
| Glycopeptides | Peptidoglycan synthesis | Cell wall peptidoglycan |
| Quinolones | DNA replication/transcription | Gyrase and topoisomerase IV |
| Rifamycins | Transcription | RNA polymerase |
| Aminoglycosides | Protein synthesis | 30S ribosomal subunit |
| Chloramphenicol | Protein synthesis | 50S ribosomal subunit |
| Fusidic acid | Protein synthesis | Elongation factor G |

| | | |
|---|---|---|
| Macrolides | Protein synthesis | 50S ribosomal subunit |
| Oxazolidinones | Protein synthesis | 50S ribosomal subunit |
| Streptogramins | Protein synthesis | 50S ribosomal subunit |
| Tetracyclines | Protein synthesis | 30S ribosomal subunit |
| Mupirocin | Charging of isoleucyl tRNA | Isoleucyl tRNA synthetase |
| Sulphonamides | Folate synthesis | Dihydropteroate synthetase |
| Trimethoprim | Folate synthesis | Dihydrofolate reductase |

**Table 1.1.** Mode of action of the principal established antibiotics. (Chopra et al., 2002)

**Peptidoglycan Synthesis**

Peptidoglycan is an essential bacterial cell polymer found in the structure of the bacterial cell wall. Interference with its synthesis or structure leads to loss of cell shape and integrity followed by bacterial death (Chopra et al., 2002). Since peptidoglycan is not found in mammalian cells, antibiotics that inhibit peptidoglycan activity can be very useful as chemotherapeutic agents (Russell et al., 1996).

**DNA gyrase**

DNA gyrase is one of the enzymes that is involved in bacterial DNA replication. DNA gyrase controls the topological state of DNA in the bacterial cell by introducing negative supercoils into the molecule. Mainly quinolone antibiotics target DNA gyrase (Russell et al., 1996). The indispensable nature of gyrase and the absence of a direct

counterpart in mammalian cells make DNA gyrase an ideal bacterial drug target (Chopra et al., 2002).

**RNA polymerase**

Bacterial DNA-dependent RNA polymerase (RNAP) mediates the transcription cycle and represents a major point of regulation for prokaryotic gene expression. In addition to being essential, this multisubunit enzyme possesses other features that render it an attractive target for antibacterial inhibitors. The RNA polymerases of prokaryotic and eukaryotic cells are different. As a result, antibiotics usually are selective and inhibit the growth of bacteria but not that of fungi or mammalian cells (Lancini et al., 1995).

**Protein Synthesis**

Selective inhibition of bacterial protein synthesis by interference with functions of the 70S ribosome has yielded many clinically important antibiotics. These antibiotics, which exhibit a varied spectrum of antibacterial activity and can be either bacteriostatic or bactericidal, provide important proof of principle that inhibitors of bacterial protein synthesis can become effective antibacterial agents for clinical use (Chopra et al., 2002). Aminoglycosides are one class of antibiotics which inhibit bacterial protein synthesis (Scott et al., 2001).

### Aminoacyl tRNA synthetases

There are only a few antibiotics that interfere with the aminoacyl tRNA synthesis (Lancini et al., 1995). Prior to incorporation into polypeptides, amino acids are attached to specific tRNA molecules. In most organisms, each amino acid is converted by a specific aminoacyl tRNA synthetase to an aminoacyladenylate-enzyme complex which then interacts with an amino acid-specific tRNA to form an aminoacyl tRNA molecule. Aminoacyl tRNA molecules are then linearly ordered by interacting with mRNA bound to the ribosome. Validation of the aminoacyl tRNA synthetases as potential drug targets has been provided by the discovery and development of mupirocin (pseudomonic acid), an inhibitor of bacterial isoleucyl tRNA synthetase (Chopra et al., 2002).

### Folic acid biosynthesis

Folic acid is required for growth by both bacterial and mammalian cells. Because animals and man are unable to synthesize folate, it must be supplied in the diet. Folate is accumulated by mammalian cells, but not by bacteria, which must synthesize the compound intracellularly. This difference between the biochemistry of bacterial and mammalian cells is the basis of the selective toxicity of the sulphonamides (Chopra et al., 2002). Sulfonamides are structurally similar to *p*-aminobenzoic acid and compete in

the synthesis of folate. Sulfonamides are not active in the cells of higher organisms as they do not have the ability to synthesize folic acid (Lancini et al., 1995).

### Use of Antibiotics in Food

Antibiotics are widely used for veterinary purposes. Teuber (2001) stated that globally, 50% of all antimicrobials produced are used for veterinary purposes. Besides combating pathogens, antibiotics are also used to improve animal performance (Docic et al., 2003). Of the 22.7 billion kg of antibiotics produced each year in the United States, it is estimated that 40% are used in the agriculture industry and among these only 20% are used for therapeutic purposes in animals (Stevenson, 2003). Antimicrobial feed additives benefit production by increasing profitability, reducing environmental animal wastes, and diminishing pathogen carriage (Docic et al., 2003).

The National Committee for Clinical Laboratory Standards (NCCLS) has defined terms regarding the use of antibiotics in herds or flocks. Therapy is the application of an antimicrobial to an animal, or group of animals, which exhibit frank clinical disease. Control is the application of an antimicrobial to animals, usually as a herd or flock, in which morbidity and/or mortality has exceeded baseline norms. Prevention/prophylaxis is the application of an antimicrobial to exposed healthy animals considered to be at risk, but before expected onset of disease. Growth promotion is the

application of an antimicrobial, usually as a feed additive, to growing animals that results in improved physiological performance (Phillips et al., 2004).

When antibiotic treatment is necessary for the purposes of therapy, control and prevention, it is applied to feed or water (Phillips, 2004). The antibiotics that are used for therapeutic purposes are sometimes the same ones employed in human clinical medicine (penicillins, cephalosporins, tetracyclines, chloramphenicols, aminoglycosides, spectinomycin, lincosamide, macrolides, nitrofuranes, nitroimidazoles, sulfonamides, trimethoprim, polymyxins and quinolones) (Teuber, 1999). In the 1940s, when chickens fed by-products of tetracycline fermentation were found to grow faster than those that were not fed with those by-products, the growth promoting effects of antibiotics were recognized (Phillips, 2004). After that, many antimicrobials have been found to improve average daily weight gain and feed efficiency in livestock in a variety of applications. These benefits coupled with demonstrable target animal safety, edible tissue clearance and residue avoidance, and environmental safety are the basis for regulatory approval of growth promoting applications of antibiotics in livestock production (Phillips et al., 2004). The application of sublethal levels of antibiotics as growth promoters is specifically prone to select and enrich for resistant bacteria (Teuber, 1999). Antibiotics are widely used in poultry production to promote growth or to control infectious disease.

This practice is reported to have caused resistance of bacteria in the gut of animals, including some pathogenic bacteria. These resistant microorganisms may also act as a possible source for the transfer of antimicrobial resistance genes among bacterial populations (Al-Ghamdi, 1999).

In 2001, 23 products with antibacterial activity had US regulatory approval and were marketed for feed additive applications (Phillips et al., 2004). Fifteen of those 23 antibacterial compounds had growth promotion label claims. Among those 15, only two (bambermycins and laidlomycin) did not have additional claims for use as therapeutic feed additives. Thus, distinctions between growth promotion and prophylactic applications are sometimes difficult.

Many products used for growth promotion and prophylaxis in animals such as bacitracin, bambermycins and carbadox have little or no application in human medicine. However, many used for prophylaxis and therapy are often closely related to antibiotics used in human medicine (Phillips et al., 2004). The classes used for both humans and animals include: β-lactams (penicillins and cephalosporins); sulphonamides with and without trimethoprim; tetracyclines; macrolides, lincosamides and streptogramins; and quinolones (including fluoroquinolones) (Phillips et al., 2004). These have a variety of therapeutic and preventive applications in food animals. In pigs for example, therapeutic

antibiotics are used in the weaning period for the treatment of gastrointestinal disorders and later in life for the treatment of pneumonia (penicillins and fluoroquinolones for *Actinobacillus pleuropneumoniae*), and for intestinal infections such as those caused by *Listeria intracellularis* (macrolides, pleuromutilins) and swine dysentery (pleuromutilins).

**Pharmacodynamics of antibiotic use**

The principal goal in the use of antimicrobial agents for the treatment of infections is destruction of the pathogen as quickly as possible with minimal adverse effects on the host. In order to accomplish this goal, three basic conditions must exist. First, the antibiotic must bind to a specific target-binding site or 'active site' on the microorganism. Although the active sites are different for different classes of antibiotics, the principle is the same, namely to disrupt a critical biochemical reaction of the bacterial cell. The second condition is that the concentration of the antimicrobial be sufficient to occupy a critical number of specific active sites on the microorganism. Finally, it is important that the agent occupies a sufficient number of active sites for an adequate period of time (Phillips et al., 2004).

The activity of an antibiotic is defined and measured in terms of its ability to inhibit microbial growth (bacteria, fungi, and protozoa) (Lancini et al., 1995). The

relationship between the antibiotic concentration and the time that the antibiotic remains at these active sites is termed the area under the concentration-time curve (AUC). Pharmacodynamics is simply the indexing of the total drug exposure in the serum or other body sites (AUC) to a measure of microbiological activity of the agent against the organism. Measures of microbiological activity that are commonly used are minimum inhibitory concentration (MIC) and minimum bacteriocidal concentration (MBC). MIC is the minimum concentration of antibiotic needed to completely inhibit the growth of a given bacterial strain and MBC is the lowest concentration of antibiotic that kills 99.9% of the microorganism (Lancini et al., 1995). Therefore, the AUC/MIC is the fundamental pharmacodynamic parameter. This parameter represents the degree to which the serum concentration and time exposure of the antimicrobial exceed the minimum needed to interfere with the bacterial life cycle. The higher the AUC/MIC ratio, the greater the probability of maximum destruction of the organism. Resistance can occur as a result of using low antibiotic doses which select organisms in a population that have higher MIC values. As a result, the use of higher AUC/MIC ratios not only maximizes destruction but can also minimize the risk of selection of resistant organisms (Phillips et al., 2004). The MIC, found by using dilution tests, tells the concentration of an antimicrobial agent needed at the site of infection to inhibit the

infecting organism. On the other hand, the MIC does not represent an absolute value. The real MIC is somewhere between the lowest test concentration that inhibits the organism's growth and the next lower test concentration (NCCLS, 2000)

These basic pharmacodynamic principles can be applied to practices involving the use of antibiotics in animal food production. As discussed above, there are four major practices in animal food production that involve the use of antibiotics: therapy, control, prevention/prophylaxis and growth promotion. It is necessary to determine for each use whether sufficient AUC/MIC ratios are obtained to achieve maximum effectiveness and prevent the development of resistance. It is important to consider that the doses used must not cause toxicity in the animals. Finally, a withdrawal period (length of time needed to allow the antibiotic to be removed from edible tissue) is necessary (Phillips et al., 2004).

**Antibiotic Resistance**

Antibiotic resistance is an important issue because it appears to be accumulating and accelerating, while methods for combating it have not increased. For example:

- More than 90 percent of *Staphylococcus aureus* strains are resistant to penicillin and other related antibiotics (Workshop Report, 1998).

- There is a rise in the incidence of *Enterococcus* spp. resistant to the antibiotic vancomycin, often terminal drug of choice to treat clinical infections (Workshop Report, 1998).
- As many as 40 percent of strains of *Pneumococcus* spp. in some parts of the United States are now partly or completely resistant to penicillin and a number of otherantibiotics (Workshop Report, 1998).

The cost of these dynamics, especially multidrug resistance, is also rising in terms of mortality, disability, and financial costs. Antibiotic-resistant bacteria generate a minimum of $4 billion to $5 billion in cost to US society and individuals yearly. In 1992, 19,000 deaths directly caused by hospital-acquired infections made them the eleventh leading cause of death in the US population.

There are many issues to overcome with respect to antimicrobial resistance. These include:

- Answering questions about the use of antibiotics in food production.
- Emphasizing ways to prolong the effectiveness of existing antibiotics.
- Pursuing key areas of basic research and seeking incentives for developing new antibiotics.

- Exploring legal and regulatory mechanisms in key areas of need. (Workshop Report 1998)

Antimicrobials are essential for the prevention and treatment of bacterial infections in humans and animals (Torrence, 2001). Because of the medical and veterinary usage of antibiotics, a heavy and varied selection pressure has been imposed on the microbial world. Because of the rapidity of bacterial multiplication and exchange of genetic material among bacteria, evolution has been both rapid and complicated (Hawkey, 2000). Fifty years of increasing applications of antimicrobial agents have created a situation leading to an ecological imbalance, i.e., the enrichment of multiple-antibiotic-resistant bacteria, both pathogenic and commensal, in human and animal habitats (Teuber, 1999). It is widely recognized that the extensive use of antibiotics in agricultural animal production contributes to the development of antibiotic-resistant pathogens and that these microbes can infect both humans and domesticated animals (Holstrom et al., 2003). Antibiotic resistance in bacteria is a result of classical Darwinian selection (Normark et al., 2002). The primary impact of resistance to antimicrobials in terms of veterinary medicine is the failure of empiric therapy against bacterial infection, which causes an increase in morbidity and mortality and hence prolonged suffering of infected animals (Chopra et al., 2001).

The phenomenon of antibiotic resistance is well-known; on the other hand the factors defining it are rarely considered. For example, *Escherichia coli* produce the enzyme β-lactamase that confers resistance to ampicillin. This means that a patient with a urinary tract infection caused by such a strain cannot be treated with this drug. In the laboratory, such strains can easily be killed by ampicillin concentrations of 2,000 mg/L. However, it is not possible to achieve these concentrations in the blood or urine of patients. Thus, the concept of clinical resistance is dependent on both pharmacodynamics and clinical outcome. Clinical resistance is a complex concept in which the type of infecting bacterium, its location in the body, the distribution of the antibiotic in the body, its concentration at the site of infection and the immune status of the patient all interact (Hawkey, 2000).

**Mechanisms of Antibiotic Resistance**

Acquired resistance to antibiotics occurs either by mutations (point mutations, deletions, inversions, insertions, etc. within the bacterial genome) or by horizontal gene transfer. For each class of antibiotics, there are usually a number of mechanisms that can cause resistance. These mechanisms may also differ depending on the bacterial species and its genetic composition (Normark et al., 2002). Bacteria can resist the action of antibiotics in many ways, including the following:

(1) the presence of an enzyme that inactivates the antibiotic;

(2) modification of the target of the antibiotic, thus reducing binding of the antibiotic to the target;

(3) reduced uptake of the antibiotic;

(4) active efflux of the antibiotic (Higgins et al., 2001).

**Antibiotic inactivation or modification**

Antibiotic inactivation or modification is probably the most commonly recognized mode of resistance and was the first mechanism to be characterized (Hawkey, 2000). In this mechanism, the resistant strain produces an enzyme capable of chemically transforming the antibiotic into an inactive product (Lancini et al., 1995). Many different Gram-positive and Gram-negative bacteria produce enzymes capable of destroying β-lactam antibiotics. Those enzymes preferentially hydrolyzing penicillins are referred to as penicillinases and cephalosporinases, if the preferred substrates are cephalosporins (Hawkey, 2000). These enzymes destroy the antibiotics. The β-lactamases produced by different resistant strains are not all identical. For instance, the β-lactamase produced by *Staphylococcus* spp. hydrolyzes penicillin but is not active on cephalosporins. On the other hand, the β-lactamase produced by *E. coli* is active on both of these antibiotics (Lancini et al., 1995). In addition to antibiotic-destroying enzymes,

some enzymes inactivate the antibiotics by the substitution of chloramphenicol transacetylase. This is an example of antibiotic-inactivating enzymes (Gale et al., 1972). The acetyl-transferases inactivate antibiotics by transferring an acetyl group from an acetyl donor to a functional group of the antibiotic. The phosphoryl-transferases covalently link the phosphate group to the antibiotic. The adenyl-transferases transfer an adenyl group to the antibiotic (Lancini et al., 1995).

**Alteration of the target site**

In this case, the antibiotic enters the bacterial cell, and although it reaches the target site, it is unable to inhibit the activity of the target because of structural changes in the molecule (Hawkey, 2000). The modification mechanism often results in an altered structure of the original antibiotic target structure such that it now binds the antibiotic poorly or not at all (Lewis et al., 2002). The most common mechanism of resistance to macrolides involves modification of their target site on the ribosome, specifically methylation of an adenine residue in domain V of the 23S rRNA (Poole, 2002). Also, infections caused by *Enterococcus* spp. fail to respond to cephalosporins because enzymes (penicillin binding proteins, PBP) responsible for the synthesis of the major cell wall structural component (peptidoglycan) have a low affinity for cephalosporins and are not inhibited. Penicillins and cephalosporins are effective

against most strains of *Streptococcus pneumoniae* but by acquiring DNA sequences from other species of *Streptococcus* encoding PBP's that do not bind these antibiotics, many strains of *S. pneumoniae* are now resistant (Hawkey, 2000). The emergence of a high level cephalosporin resistance amongt penicillin resistant pneumococci by this mechanism illustrates the rapid and flexible way bacterial resistance to antibiotics can emerge. In both of these cases, DNA from the foreign streptococcal species enters the *S. pneumoniae* led by a process called transformation. Naked DNA strands are actively taken up across the bacterial cell wall and become incorporated by the process of homologous recombination (Hawkey, 2000).

**Bypass pathways**

While the antibiotic-sensitive target can remain in the bacterial cell, some resistant bacteria acquire an alternative target that is resistant to action by the antibiotic and can therefore continue to survive in the presence of the antibiotic, effectively "bypassing" the antibiotic. One of the most significant examples of this is the alternative penicillin binding protein (PBP2a) produced by methicillin resistant *Staphylococcus aureus* (MRSA), PBP2a is the altered form of the native penicillin binding site and is formed under antibiotic stress (Lewis et al., 2002). The protein is encoded by the *mecA* gene and because PBP2a is not inhibited by antibiotics such as

flucloxacillin, the cell continues to synthesize peptidoglycan and hence has a normal cell wall. The recognition of vancomycin-resistant *Enterococcus* in 1987 has aroused much interest in the bypass mechanism, as the genes involved can be transferred to *S. aureus*, and this can theoretically result in a vancomycin resistant MRSA (Hawkey, 2000).

**Decreased uptake**

By preventing an antibiotic from entering the bacterial cell or by pumping the antibiotic out as fast as it enters, the sensitive targets for antibiotic action can be protected and bacteria become resistant. The major determinant of antimicrobial resistance in Gram-negative bacteria is that they have an outer cell membrane that decreases the amount of uptake of antibiotics (Poole, 2002). The main driving force for uptake through the cytoplasmic membrane is the internal negative electric potential (Lancini et al., 1995). β-lactam antibiotics in Gram-negative bacteria gain access to the cell via a water-filled hollow membrane protein known as a porin (Hawkey, 2000). Different antibiotics may enter via different porins and therefore lack of a specific porin may confer resistance. Increased efflux of antibiotics via an energy-requiring membrane pump is a well recognized mechanism for resistance to a wide range of antibiotics (e.g.

the *mar* and *norA* genes). In the case of tetracyclines, the *tet(A)* and related genes have become very widely distributed in *Enterobacteriaeae* (Hawkey, 2000).

**Horizontal gene transfer and antibiotic resistance**

Most bacterial genomes that have been sequenced contain large segments of DNA that have been relatively recently acquired from other sources. This horizontally acquired DNA usually encodes functions that are of selective advantage to the organism such as antibiotic resistance, virulence, and biodegradation pathways (Normark et al., 2002). There are a number of different DNA elements described transferring antibiotic resistance including self replicating plasmids (that can be self transmissible by conjugation or brought into the cell by tranformation or transduction), prophages, transposons, integrons and resistance islands (Normark et al., 2002).

**Physiological resistance to antibiotics**

Resistance can be physiological mediated, meaning that it is only expressed under certain growth conditions (Normark et al., 2002). The actual mechanism of physiological resistance is not clear. Bacteria in biofilms often display physiological resistance to antibiotics. It has been argued that antibiotics may have difficulties in penetrating the organized matrix that surround bacteria in biofilms (Normark et al., 2002). Also, it may be that certain genetic systems are activated during the biofilm

mode of growth, inducing physiological resistance that is not activated during the free-living (planctonic) mode of growth. It was however, recently argued that bacteria growing in biofilms or in a free living planctonic form show the same degree of antibiotic susceptibility (Normark et al., 2002). One reason why bacteria in biofilms are difficult to treat could be that these organisms are in a stationary phase characterized by a balanced state of growth and death. It has been known for years that when bactericidal antibiotics such as the β-lactams are given to a bacterial culture, complete cell death does not usually occur. The surviving cells are called persisters. In the stationary phase and during the biofilm mode of growth, such persisters might occur in larger numbers (Normark et al., 2002).

**Further developments in antibiotics**

There is a continuous need for the development of new antibiotics because of;

- the development of resistant pathogens
- the evolution of new diseases
- the existence of naturally resistant bacteria
- the toxicity of some antibiotics (Demain, 1999)

**Antibiotic Susceptibility of *E.coli***

*E. coli* has been proposed as an indicator species in recent surveillance programs to analyze the antibiotic resistance status of the enteric microflora of both farm animals and humans (Teuber, 1999). About 90% of natural antibiotics fail to inhibit Gram-negative organisms such as *Escherichia coli*. The reasons for this include its outer cell membrane, which contains (1) narrow porin channels which retard the entry of even small hydrophilic compounds, and (2) a lipopolysaccharide moiety which slows down the transmembrane diffusion of lipophilic antibiotics. Furthermore, Gram-negative bacteria often possess a multiple-drug efflux pump which eliminates many antibiotics from the cells (Demain, 1999).

There is limited information available on the antibiotic susceptibility patterns of enterohemorrhagic *Escherichia coli* (EHEC) or Verocytotoxin-producing *E. coli* (VTEC) (Klein, 2003). Although antimicrobial-resistant *E. coli* have been recovered from various foods, including vegetables, confections, milk and milk products, the majority of resistant strains have been isolated from traditional retail meats and poultry (Schroeder et al., 2004). More recently, *E. coli* strains recovered from retail meats were found to be resistant to frontline therapeutic antimicrobials such as trimethoprim-sulphamethoxazole, cephalosporin, and fluoroquinolones (Schroeder et al., 2004). Sub-

therapeutic use of antimicrobials as feed supplements has been recognized as a potential driving force in accelerating the emergence of antimicrobial-resistant bacteria. For example, nourseothricin-resistant *E. coli* emerged after the introduction of nourseothricin as a growth promoter in farm animals in the former East Germany, and apramycin-resistant *E. coli* emerged after the introduction of apramycin for veterinary use in France and the United Kingdom (Schroeder, 2004).

**Stress Response**

Stress is any deleterious factor or condition that adversely affects the growth or survival of microorganisms. The magnitude and outcome of stresses applied to microorganisms vary. Mild stress at the sub-lethal level does not result in viability loss, but reduces or stops the growth of the microorganism. Moderate stress stops the growth of microorganism and causes some loss in cell viability. Extreme or severe stress is lethal to cells, and causes the death of the majority of the population. Stress factors may be chemical, physical, or biological (Vorob'eva, 2003). Stresses applied during the production and processing of foods include: physical treatments such as heat, pressure, etc.; addition of chemicals such as acids, salts, etc.; and biological stresses such as competition, microbial metabolites, etc. (Yousef et al., 2002). The increase in resistance

of an organism to one stress after application of a different and unrelated sub-lethal stress is known as cross-protection (Rowe et al., 1999).

Stress responses are very important to microorganisms as their habitats are subject to continuous change (Vorob'eva, 2003). When stress is applied to microorganisms, cells respond in various ways. As a result of stress, microorganisms may produce one of the following responses (Yousef et al., 2002);

- Production of proteins that repair damage, maintain the cell, or eliminate the cell, or stress agent.
- Transient increase in resistance or tolerance to deleterious factors.
- Cell transformation to a dormant state, i.e., spore formation.
- Evasion of host organism defenses.
- Adaptive mutations.

When microorganisms are stressed, an adaptive or protective response may occur. This response may increase the tolerance of the microorganism to the same or to a different type of stress. This phenomenon is called an adaptive response, or induced tolerance, habituation, acclimatization, or stress hardening (Yousef et al., 2002).

The ability of a microorganism to rapidly adapt to a changing environment is very crucial for growth and survival (Hengge-Aronis, 1999). The response of

microorganisms to stress can be immediate or longer term adaptation. Various stress related proteins may be induced that protect the cell from stress. Hengge-Aronis (1999) showed that *E. coli* has large numbers of signal transduction systems and regulatory mechanisms that allow it to respond to various environmental stresses. Under different stress conditions, two sigma subunits of RNA polymerase, $\sigma^S$(*rpoS*) and $\sigma^{70}$(*rpoD*), coexist in *E. coli* cells. Different than $\sigma^{70}$, $\sigma^S$ is subject to intricate regulation and regulates an emergency reaction in addition to long term stress adaptation. Although they are very similar both structurally and functionally, these two sigma factors control different genes. The *rpoS* gene is a regulator for stationary phase gene expression, and it is responsible for the induction of a specific subset of genes. This gene is only expressed during stationary phase or under stress conditions, and increases the resistance of the cell to a range of stresses (Rowe et al., 1999). The *rpoD* gene encodes the sigma factor $\sigma^{70}$ and is a housekeeping gene. The transcription of *rpoD* is growth condition dependent (Rijpens et al., 2002). *rpoD* confers promoter-specific transcription initiation on RNA-polymerase and it is essential for cell growth (Yamamoto et al., 2000).

The stress response system of a microorganism can be activated by several different stresses and may subsequently protect against multiple stresses (Yousef et al.,

2002). A diverse range of stresses such as heat shock, salt stress, ethanol, or starvation induces the same set of proteins, called general stress proteins (Hecker et al., 1996). These proteins provide general stress protection. Arnold et al. (1996) indicated that stationary phase and starvation of the cell induced protective proteins that increased the resistance of the cell to chemical and physical challenges. In stationary phase, *E. coli* produces 30 proteins that are regulated by *rpoS*.

**Heat Stress**

In food preservation and processing, foodborne bacteria are commonly treated with heat (Yura et al., 1999). Sublethal heat stress causes the damage of the macromolecular components of the cell (Yousef et al., 2002). When cells are exposed to a sudden increase in temperature, the expression of specific proteins is increased. This response is called the heat shock response (Dyk et al., 1995). The heat shock response is a cellular protective and homeostatic response to cope with heat induced damage to proteins (Yura et al., 1999). In order to survive heat stress, all living cells increase the level of their heat shock proteins (Hsps) (Hecker et al., 1996). Most of the Hsps in *E. coli* and other bacteria are molecular chaperones or ATP-dependent proteases and play a major role in protein folding, assembly, transport, repair and turnover under stress and non-stress conditions (Yura et al., 1999). Arsené et al. (2000) indicated that increasing

the temperature from 30$^{o}$ to 45$^{o}$C induced *E. coli* to synthesize more than 20 Hsps followed by an adaptation period in which the synthesis of Hsps decreased to reach a new steady-state level. In *E. coli,* a heat stress response induced by mild heat stress provides subsequent resistance to previously lethal heat stresses (Arsené et al., 2000). This response is controlled at the transcriptional level by the product of the *rpoH* gene, the heat shock promoter-specific $\sigma^{32}$ subunit of RNA polymerase (Arsené et al., 2000). Its activity increases after heat stress, leading to induction of a heat shock regulon (Hecker et al., 1996). $\sigma^{32}$ is found at low levels when there is no heat stress applied (Yousef et al., 2002). In addition to a sudden increase in temperature, viral infection, antibiotics, methylating and alkylating agents, hydrogen peroxide and various pollutant molecules may induce the production of Hsps in *E. coli* (Dyk et al., 1995).

A change in membrane lipid composition plays an important role in bacterial response to heat stress, which is known as 'homeoviscous adaptation'. Yuk et al. (2003) stated that high growth temperature adaptation increased heat resistance of cells due to a decrease in membrane fluidity. These researchers investigated the influence of heat adaptation at 42 and 45$^{o}$C on changes in membrane lipid composition and Shiga toxin production of *E. coli* O157:H7. Heat adapted strains of *E. coli* O157:H7 were more heat resistant than control or heat-shocked cells. Heat adaptation decreased membrane

fluidity, which might have increased Shiga toxin secretion in *E. coli* O157:H7. They also found that the presence of *rpoS* increased the heat resistance of *E. coli* O157:H7.

Buchanan et al. (1999) investigated the role of pH-dependent, stationary phase acid resistance of cross protection of *E. coli* O157:H7 when subsequently subjected to heat challenge at 58$^{o}$C. Three strains of *E. coli* O157:H7 were grown in acidogenic (TSB with glucose, final pH 4.6-4.7) and non-acidogenic (TSB without glucose, final pH 7.0-7.2) media in order to provide both with and without pH-dependent acid resistant cells in stationary phase, after which heat challenge was applied at 58$^{o}$C. The results indicated that the pH-dependent acid resistant *E. coli* O157:H7 cells were cross protected against heat challenge.

Lin et al. (2003) studied the effect of heat shock at 45$^{o}$C for 1 hour and at 48$^{o}$C for 10 minutes on the thermal tolerance and susceptibility of *L. monocytogenes* to other environmental stresses. Their study indicated that the heat shock response of *L. monocytogenes* differed with strain, the condition of heat shock treatment and the type of subsequent stress. Cells that were subjected to 45$^{o}$C heat stress showed increased survival at 55$^{o}$C. Conversely, cells subjected to 48$^{o}$C did not show increased thermal tolerance compared to non-heat shocked cells.

Rocelle et al. (1996) studied the survival of *E. coli* O157:H7 in broth and processed salami as influenced by different stresses. The cells were inoculated in TSB that was adjusted to various pHs with lactic acid and various water activities, and then the heat tolerance (52$^{o}$C) of control and stressed cells was determined. Survival of *E. coli* O157:H7 was affected by pH and water activity; specifically, survival of cells decreased when the water activity was reduced from 0.99 to 0.95.

Moro et al. (2000) investigated the effect of heat stress at 34$^{o}$C for 24 hours on the antimicrobial resistance of *E. coli* in the intestinal flora of swine. The antimicrobial resistance of heat stressed *E. coli* was higher than the non-heat stressed cells for amikacin, ampicillin, cephalothin, neomycin and tetracycline. Also, the resistance for the antimicrobials, except ampicillin and neomycin, was highest immediately after the heat stress.

**Cold Stress**

Under decreased temperature, cell membrane fluidity decreases. In order to overcome this stress, microorganisms decrease the degree of saturation in cell membrane phospholipids to attain more fluidity. Also, lower temperatures facilitate the stabilization of the hydrogen bonds in nucleic acid secondary structure. This causes a reduction of efficiency in translation, transcription and DNA replication (Phadtare et al.,

1999). The sensitivity of cells to cold stress is dependent on several factors such as temperature, freezing rate, thawing rate, culture medium, microbial strain, and storage duration. (Mihoub et al., 2003). The cell overcomes this situation throughthedeleterious case by the help of cold-shock proteins (Csps) (Phadtare et al., 1999). When *E. coli* is cold shocked, it stops growing and adapts to the new temperature (acclimation phase) (Polissi et al., 2003). In *E. coli* there are two types of Csps: class I proteins are present at very low levels at 37$^{o}$C and when the temperature drops, the number of them rapidly increases to very high levels. On the other hand, class II proteins are present at 37$^{o}$C and their number does not increase very rapidly when the temperature drops (Phadtare et al., 1999).

Elhanafi et al. (2003) investigated the effect of cold and cold-acid stress on the post-stress tolerance of *E. coli* O157:H7. Their studies showed that both of these stresses decreased acid tolerance of cells and that the application of cold stress alone decreased acid tolerance more than stressing the cell with both cold and acid. Buncic et al. (1998) studied the effects of cold storage and heat-acid shock on growth and toxin production *E. coli* O157:H7. In their studies, the cells were cold-stored at 4$^{o}$C either in nutritious media or starved at pH 7.0 or 5.5. After stressing the cells at 4$^{o}$C for 4 weeks, they were heat shocked at 45$^{o}$C for 5 minutes and acid shocked at pH 2.5 for 30 minutes

at 37 $^{o}$C and then moved to optimal conditions at 37$^{o}$C. The results of this study showed that both lag-phase duration and growth rate of cells at 37$^{o}$C increased after cold-storage with starvation, but were not changed after cold-storage in nutritious media. Also, heat-acid shock increased the growth rate of both cold stressed and non-cold stressed cells at 37$^{o}$C. Leenanon et al. (2001) studied the post stress behavior of *E. coli* O157:H7 and nonpathogenic *E. coli* after acid stress, starvation, and cold stress. Their study showed that after cold stress, heat resistance of both *E. coli* O157:H7 and nonpathogenic *E. coli* decreased. On the other hand, freeze-thaw resistance increased.

Moorehead et al. (2004) studied the effect of the *sigB* gene, which plays role in the accumulation of compatible solutes during stress conditions, on the cold stress survival and subsequent recovery of *Listeria monocytogenes*. Wild type and *sigB* mutant serotypes of *L. monocytogenes* were inoculated into buffer and onto beef steaks at 4$^{o}$C for 8 weeks during which samples were removed and cell counts were determined. Prechilling cells before growth increased the lag phase of both strains. The *sigB* gene was involved in survival and recovery from cold stress. The substrate, serotype, and culture history were determining factors in the survival characteristics of *L. monocytogenes* in Phosphate Buffered Saline and on meat at 4$^{o}$C.

Mihoub et al. (2003) studied the effect of cold adaptation of *E. coli* at 4°C for 3 hour on the survival of two *E. coli* strains, followed by freezing at -20°C for 24 hours and thawing at 37°C for 45 min. This study showed that *E. coli* cells previously adapted to cold temperatures were more resistant to freezing than cells that were not cold adapted. Also, changes in the protein profiles of the cytoplasm and outer membrane of the cells were observed. The proteins were separated by two-dimensional electrophoresis and identified by mass spectrometry or Edman sequencing. In one strain after cold adaptation, an over-expression of the EF-TU elongation factor in the outer membrane, and an under-expression of flagellin (FLIC) in the cytoplasm were observed. On the other hand, in the other strain, few changes in protein profiles were observed.

**Acid Stress**

Foodborne bacteria are faced with organic and inorganic acids in foods or in the gastrointestinal tract of the host (Yousef et al., 2002). Enterohemorrhagic *E. coli* strains must pass through the acidic gastric fluid in order to cause gastrointestinal disease (Lin et al., 1996). Acid tolerance of *E. coli* O157:H7 promotes its survival in foods and plays a key role in its pathogenesis by promoting survival during passage through the stomach (Cheville et al., 1996). Microorganisms respond to acid stress in many ways such as

membrane composition change, increase in protein efflux, increase in amino acid catabolism, and induction of DNA repair enzymes (Yousef et al., 2002). Growth in a moderately acidic environment triggers the synthesis of proteins that protect the cell from more extreme acidic conditions. Depending on the growth phase, medium, and type of acid, different systems provides resistance to microorganisms. *E. coli* possesses log and stationary phase acid tolerance response (ATR) mechanisms. It is generally considered that organic acids are more effective against foodborne pathogens than hydrochloric acid. This effectiveness of organic acids is because of the anion portion of the molecule and it differs among the organic acids (Buchanan et al., 2004). Buchanan et al. (2004) studied five different acidulants (lactic, acetic, citric, malic and HCl) at pH range between 4.0 and 5.5 and found that lactic acid had the highest and HCl had the lowest activity against enterohemorrhagic *E. coli*.

In *E. coli* O157:H7, although acid-inducible systems are present, acid tolerance is not dependent upon adaptation. Acid tolerance is induced by stationary phase or starvation (Cheville et al., 1996). Smith (2003) indicated that there are three acid resistance (AR) systems in EHEC; AR system 1 (oxidative, glucose repressed), AR system 2 (fermentative, glutamate dependent), and AR system 3 (fermentative, arginine dependent). The system induced depends on the type of medium and the growth

conditions. AR system 1 is induced when cells are grown to stationary phase in a glucose-free complex medium at pH 5.5. These cells can survive at pH 2.5 for several hours in minimal media. AR system 2 is induced when cells are grown to stationary phase in a glucose containing complex medium at pH 5.0. These cells can survive pH 2.0 to pH 2.5 for several hours. This system provides the highest level of protection against low pH. AR system 3 is induced when cells are grown to stationary phase in a glucose containing medium. These cells can survive in arginine supplemented minimum medium at pH 2.5 for several hours. Once the AR systems are induced in *E. coli* O157:H7, activity persists at refrigeration temperature for at least 1 month (Smith, 2003).

Hydrochloric acid secreted by the stomach plays an important role in defending the body against any pathogens ingested with food. The pH of the gastric fluid is 1 to 2 which is lethal to many pathogens. In an in vitro system, 20 to 80% of ingested *E. coli* O157:H7 would reach the small intestine in a viable state. *E. coli* O157:H7 can survive at the low pH of the stomach and pass into the intestine (Smith, 2003).

Lin et al. investigated the survival characteristics of different EHEC strains during acid stress. The parameters examined were absolute pH, the presence of weak acids, and the persistence of acid resistance during cold storage. This study showed that

the acid resistance of commensal and enterohemorrhagic strains of *E. coli* was similar. They also found that stressing the cells with weak acid increased the susceptibility of the cells during acid challenge. Another result of this study was that when an acid resistance system was activated, it remained active over a prolonged period of cold storage.

Arnold et al. (1995) indicated that exposure of *E. coli* to acidic conditions or growth in a moderately acidic medium increased survival at low pH. Their study showed that stationary phase and starved *E. coli* O157:H7 strains were more acid tolerant than mid-log-phase cells. Also, the degree of acid tolerance in some strains of *E. coli* O157:H7 was greater than others indicating that multiple protective systems may take place and that acid tolerance varied among different strains. Arnold et al. (1999) studied the role of *rpoS*-regulated proteins on acid, heat and salt tolerance systems. They reported that the *rpoS* sustained the acid tolerance of *E. coli* O157:H7. In addition to that, *rpoS*-regulated proteins provided cross-protection against heat and salt stresses and promoted the survival of *E. coli* O157:H7 in fermented sausage.

Acetic acid is commonly used as a food preservative. The antibacterial activity of acetic acid against various kinds of microorganisms is known. Rhee et al. (2002) studied the combined effects of salt, acetic acid and mustard flour on the survival of *E.*

*coli* O157:H7 stored at 5 and 22°C. Various concentrations of acetic acid (0, 0.25, 0.5, 0.75, 1.0 % [vol/vol] and 2% (wt/vol) sodium chloride were studied. The combination of 0.25% to 0.75% (vol/vol) acetic acid with mustard showed no synergestic or additive effect in inhibiting *E. coli* O157:H7. On the other hand, acetic acid at concentrations over 1.0% with mustard was effective in inhibiting the survival of *E. coli*O157:H7.

Cheng et al. (2003) studied the effect of acid adaptation time and the conditions of acid challenge to the acid tolerance of *E. coli* O157:H7. Three strains of *E. coli* O157:H7 were acid stressed at pH 5.0 for 1, 2, 3, 4, and 6 hr, then acid tolerance of acid adapted cells at pH 3.0, 4.0, and 5.0, which were acidified with HCl, lactic, acetic acid, and propionic acid, were determined. The results of this study indicated that acid adaptation of cells increased acid tolerance and this increase was dependent on strain, acid adaptation time, and pH of the acid challenge. Acid tolerance was most increased after 4 hr of adaptation, regardless of strain.

Stophorth et al. (2003) studied the influence of organic acid concentration on survival of *L. monocytogenes* and *E. coli* O157:H7 in beef carcass wash water and on the surfaces of model equipment. They inoculated the meat decontamination spray-washing run-off fluids with acid-adapted cultures of *E. coli* O157:H7 and *L. monocytogenes*. Then, the cultures were exposed to a mixture of water and organic

acids (acetic acid and lactic acid). The pH effects of lower concentrations of acidic washings were found to decrease over time, due to fermentation of amine compounds by the natural meat flora, allowing resuscitation of the acid stressed pathogen survivors.

Cheng et al. (2000) studied the influence of acid adaptation on the tolerance of three strains of *E. coli* O157:H7 to subsequent stresses. Cells were subjected to acid adaptation at pH 5.0 for 4 hours. Thermal tolerance at 52$^{o}$C and survival of the acid adapted cells in the presence of sodium chloride, bile salt, and ethanol was investigated and compared to survival of non-acid adapted cells under the same stresses. Survival behavior of acid adapted *E. coli* O157:H7 during subsequent stresses differed with strain and type of subsequent stress. Adaptation at pH 5.0 for 4 hours generally increased the thermal tolerance and sodium chloride tolerance. Acid adaptation had no effect on bile salt tolerance.

Leyer et al. (1995) studied acid adaptation of *E. coli* O157:H7 in acidic foods. Five strains of *E. coli* O157:H7 were used. In order to obtain acid adaptation, the cultures were stressed in broth acidified with HCl to pH 5.0 and the cells were grown in this broth for 5 hours at 37$^{o}$C. The acid adapted cells were inoculated to salami and apple cider in order to determine if acid adaptation increased survival of the cells in

acidic foods. Acid adapted cells had an increased resistance to lactic acid, and survived better than non-adapted cells during sausage fermentation, dry salami, and apple cider.

**Osmotic Stress**

Microorganisms are subjected to osmotic stress in foods that are high in salt, sugar or dried. Under these conditions, it is important for cells to maintain turgor pressure and hydration (Yousef et al., 2002). *E. coli* has some systems responsible for regulation of turgor pressure. The main function of these systems is the accumulation of intracellular osmolytes that do not inhibit enzyme activities and enable bacterial growth at high osmotic pressures. During the initial stage of the response to osmotic shock, *E. coli* cells restore their turgor through accumulation of significant amounts of $K^+$ and glutamate. At high osmotic pressures, high levels of intracellular $K^+$ can cause disorders in activities of some enzymes (Smirnova et al., 2001). Also, there may be some changes in cell metabolism because of osmotic stress. In cells that are exposed to high salt concentrations, an increase in the ratio of *trans* to *cis* unsaturated fatty acids is also observed.

As with other stresses, $\sigma^S$ plays an important role in *E. coli* response to osmotic stress. Bianchi et al. (1999) investigated whether hyper osmotic shock induced the $\sigma^{32}$ and $\sigma^E$ stress regulons of *E. coli*, and their study indicated that in order to overcome

hyperosmotic stress, the $\sigma^{32}$, $\sigma^{E}$ and $\sigma^{S}$ regulons must closely co-operate with one another. Induction of the $\sigma^{32}$ and $\sigma^{E}$ regulons appears to be an emergency response required to repair protein misfolding.

Casey et al. (2002) studied the effect of sodium chloride (4% wt/vol) on the bacteriocidal effect of pH 4.2 of *E. coli* O157:H7 in a fermented meat model. This study showed that the cells subjected to sodium chloride and acid survived better than cells subjected to only acid. The reason for the better survival with addition of sodium chloride and acid may be due to the decrease in the $a_w$ of the media. Glass et al. (1992) studied the influence of pH and sodium chloride on the survival of *E. coli* O157:H7. The pH was adjusted with lactic acid or HCl and various concentrations of sodium chloride were analysed. The study indicated that *E. coli* O157:H7 can grow in NaCl concentration as high as 6.5%. At this NaCl concentration the lag phase is longer than normal which indicated that there was a selection for salt tolerant populations.

Faleiro et al. (2003) studied the stress response of *L. monocytogenes* to pH (5.1 to 6.2) and sodium chloride (2-3.5% wt/vol) isolated from cheese. The results indicated that some strains of *L. monocytogenes* showed an osmotolerance response after incubation in 3.5% (wt/vol) sodium chloride. Developed osmotolerance response

enabled cross protection to acid shock conditions (pH 3.5). Also, the acid tolerance response enabled cross protection to osmotic shock conditions (20% wt/vol).

Campbell et al. (2004) investigated effects of combined salt, acid, and MSG on cold storage survival and subsequent acid tolerance of *E. coli* O157:H7. They found that as NaCl concentration increased and pH decreased, the survival of the cells in cold storage decreased. Blackburn et al. (1997) studied the development of thermal inactivation models for *S. enteridis* and *E. coli* O157:H7 with temperature (54.5-64.5$^{o}$C), pH (4.2-9.6 with HCl or NaOH) and NaCl (0.5-8.5% w/w) as controlling factors. The results indicated that for *E. coli* O157:H7, increases in NaCl concentration up to 8.5% had a protective effect on heat stress. Similarly, Rowe et al. (1999) studied cross protection between acid and salt stresses in *E. coli* O157:H7. The results of this study showed that using a combination of acid and salt stress enabled the cross-protection to subsequent acid or salt stress and this phenomenon was induced by the *rpoS* gene.

**Summary**

Production of a safe food product is an important issue in the food industry because various stresses are applied in order to inhibit or inactivate foodborne pathogens and spoilage organisms. Development of adaptive stress tolerance responses in organisms is important for the safety of food products especially in minimally

processed foods. In order to control the microbial growth of organisms in foods some hurdles such as pH, sodium chloride, and storage temperature are used commonly. In this study, the effect of antibiotic stress on the virulence factor expression and the post-stress survival of *E. coli* O157:H7 and the influence of acid type and sodium chloride concentration on the survival of *E. coli* O157:H7 to different stresses were determined.

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**MANUSCRIPT I**

**Impact of Antibiotic Stress on Acid and Heat Tolerance and Virulence Factor Production of *Escherichia coli* O157:H7**

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**Keywords:** *E. coli* O157:H7, Antibiotics, Heat Tolerance, Acid Challenge, Virulence Gene Expression.

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**Abstract**

This study was conducted to determine the effect of antibiotic stress on virulence factor production, simulated gastric fluid (SGF, pH 1.5) survival, and heat tolerance (56$^{o}$C) of *E. coli* O157:H7. The minimum inhibitory concentration (MIC) for three antibiotics (trimethoprim, ampicillin, and ofloxacin) was determined for two *E. coli* O157:H7 (ATCC 43895 (raw hamburger isolate), and ATCC 43890 (fecal isolate)) strains using the dilution series method. Subsequently, cells were stressed at the MIC for each antibiotic for 4 hours and post stress tolerance and virulence factor production evaluated. Heat tolerance (56$^{o}$C) was determined using the capillary tube method, and SGF (pH 1.5) survival was used to assess acid tolerance. Virulence factor production (Stx, Hly, EaeA) was evaluated by the creation of *lacZ* gene fusions followed by use of the Miller assay (a β-galactosidase assay). Stressed and control cells were evaluated in triplicate. MICs were: 0.26 mg/l for trimethoprim, 2.05 mg/l for ampicillin and 0.0256 and 0.045 mg/l for ofloxacin each strain, respectively. Heat tolerance and SGF survival following antibiotic stress decreased compared to control cells ($p<0.05$). Exposure to ofloxacin increased Stx and Eae production ($p<0.05$). Exposure to ampicillin or trimethoprim increased Eae production ($p<0.05$). Hly production decreased when cells were stressed with ampicillin, but increased following trimethoprim stress ($p<0.05$).

Antibiotics can increase *E. coli* O157:H7 virulence factor production, but do not produce a cross-protective response to heat or decreased pH.

**Introduction**

Since the first outbreak of *Escherichia coli* O157:H7 in 1982, this organism has been recognized as the major cause of hemorrhagic colitis and hemolytic uremic syndrome (Arnold et al., 1995). The gastrointestinal tract and the kidneys are the target organs. Serotype O157:H7 is the most prevalent type of this diarrheagenic *E. coli* classified as enterohemorrhagic *E. coli* (EHEC). Bloody diarrhea (hemorrhagic colitis) is the primary disease symptom, but complications resulting from *E. coli* O157:H7 infections may include hemolytic uremic syndrome (HUS), and thrombotic thrombocytopenic purpura (TTP) (Doyle et al., 2001).

There is no animal model for EHEC infection, thus all virulence factors are putative (Doyle et al., 2001). However, the ability of EHEC strains to cause severe disease in humans is undoubtedly related to their capacity to secrete Shiga toxins (Griff et al., 1998). The two main types of EHEC Shiga toxin are Stx1 and Stx2 (Muhldorfer et al., 1996; O'Brien et al., 1987). Strockbine et al. (1986) indicated that the Stx1 is similar to the Shiga toxin of *Shigella* in amino acid sequence, but Stx2 is less related to the Shiga toxin produced by *Shigella* and is not neutralized by antibodies to either Stx1 or Shiga toxin of *Shigella.* Stx2 producing strains of EHEC are more virulent than both

Stx1 or Stx1 and Stx2 producing EHEC strains(Donnenberg, 2002), and thus are more frequently associated with human disease complications (Boerlin et al., 1999). Other putative virulence factors of EHEC include production of an outer membrane protein called intimin which is encoded by the *eae* gene, and an enterohemolysin (EHEC-*hlyA*) encoded by the large plasmid-borne *ehxA* gene (Kumar et al., 2004).

In food preservation and processing, foodborne bacteria are subjected to different kinds of stresses (Yura et al., 1999). It is important for microorganisms to rapidly adapt to changing environments for growth and survival (Hengge-Aronis, 1999). The stress response system of a microorganism can be activated by several different stresses and protects against multiple stresses (Yousef et al., 2002). Arnold et al. (1996) indicated that stationary phase of growth, starvation and possibly other sublethal stresses of bacterial cells induced protective proteins that increased the resistance of the cell to chemical and physical challenges. In food processing, pH, sodium chloride, and heat are common applications to control microbial growth.

Previous studies indicated that sublethal stress conditions can affect the virulence expression/production of *E. coli* O157:H7. Yuk et al. (2005) demonstrated that Shiga toxin production of *E. coli* O157:H7 was dependent on both pH and organic acid type. They found that organic acid adaptation (acetic, citric, or lactic acid) at pH

6.4 or pH 5.4 generally decreased Shiga toxin production. Among the organic acids studied, citric acid adapted cells at pH 6.4 had the most Shiga toxin production. In a similar study, Duffy et al. (2000) reported that *E. coli* O157:H7 grown at pH 5.6 had less Shiga toxin production than did cells grown at pH 7.4. These studies all addressed toxin production.

Leenanon et al. (2002) utilized RT-PCR and ELISA to evaluate toxin expression and production. They reported that acid adaptation and starvation enhanced *stx2* toxin mRNA levels but did not enhance subsequent Stx toxin production. Yuk et al. (2003) studied the influence of heat shock and heat adaptation on intracellular and extracellular Shiga toxin concentration for *E. coli* O157:H7 and an *rpoS*-deficient mutant. They found that heat adaptation reduced the total amount of Shiga toxin produced in both normal *E.* coli O157:H7 and *rpoS* mutants. The *rpoS* mutant strain produced slightly more Shiga toxin than did the normal *E. coli* O157:H7 strain, which suggested that *rpoS* might play a role in the production of Shiga toxin (Leenanon et al., 2002). The impact of sublethal stress on the expansion of other virulence factors in EHEC has not been well-characterized. Elhanafi et al. (2004) studied the effect of the interaction between growth phase and pH on Stx2 as well as EaeA and HlyA production. They found that production of EaeA, HlyA, and Stx2 were optimal during stationary phase. However, in

contrast to Stx2, EaeA and HlyA production was enhanced during growth in acidified media (pH 5.5, lactic acid).

Antibiotics are used in the food industry for the treatment of disease (therapeutic), prevention of disease (prophylactic), or to increase the efficiency of meat (i.e., to increase weight gain) or milk (sub-therapeutic) production (Teuber, 1999). The application of sublethal levels of antibiotics as growth promoters is specifically prone to select and enrich for resistant bacteria (Teuber, 1999). Studies have indicated that some antibiotics may increase the virulence of *Escherichia coli* O157:H7. A clinical study conducted by Wong et al. (2000) indicated that antibiotic treatment increased the likelihood of development of HUS in children with *E. coli* O157:H7 infection. Kimmitt et al. (2000) indicated that toxin synthesis of Shiga toxin producing *E. coli* (STEC) was related to the induction of the bacterial SOS response, a normal response to DNA damage. SOS-inducing antimicrobial agents, particularly trimethoprim and quinolones, were shown to activate the multifunctional RecA protein which leads to the induction of previously inactive phage-encoded genes, including *stx2A* and *stx2B*. At antimicrobial levels above those required to inhibit bacterial replication, these agents are potent inducers of the transcription of *stx2*.

Understanding the stress response of *E.* coli O157:H7 and how different antibiotics may impact this response is essential to assess the efficacy of hurdle technologies to control this pathogen (i.e. carcass acid wash). The objectives of this study were to determine the effect of antibiotic stress on virulence factor production, simulated gastric fluid (SGF, pH 1.5) survival, and heat tolerance (56$^{o}$C) of *E. coli* O157:H7.

## Materials and Methods

### Bacterial strains and culture conditions

*E. coli* O157:H7 ATCC 43895 (raw hamburger isolate), and *E. coli* O157:H7 ATCC 43890 (fecal isolate) were used in heat and acid tolerance assays. For virulence gene expression studies, *E. coli* O157:H7 MAD 237, MAD 239 and MAD 241 strains (created from ATCC 43895) were used. These strains had Δ*lacZ* gene fusions within the *stx*2, *hlyA*, and *eaeA* genes, respectively, and have been previously characterized (Elhanafi et al., 2004) (Table 2.1). Previous work indicated that the phenotypic characteristics of these strains (acid, heat, freeze/thaw tolerance) were not different from the parent strain (Elhanafi et al., 2004). Stock cultures were stored in 30% (w/w) glycerol at -80$^{o}$C. Cultures were activated two days prior to use by overnight growth in Tryptic Soy Broth (TSB) (BBL, Cockeysville, MD) (pH 7.2) at 37$^{o}$C. Strains were

examined monthly for purity by negative sorbitol fermentation on sorbitol MacConkey agar. Monthly, the presence of virulence factors (*stx1/2*, *hlyA* and *eae)* was confirmed by PCR. Growth curves of each strain indicated that stationary phase was achieved after 18 h growth ($10^9$ cfu/ml). Cell enumeration was conducted by pour plating in duplicate on Tryptic Soy Agar (TSA) after serial dilution in sterile 0.1% peptone-water.

**Preparation of antibiotic stock solutions**

Ampicillin (99%, Aldrich Chemical Co., Milwaukee, Wi) , Trimethoprim (98%, Sigma-Aldrich Inc., St.Louis, MO), and Ofloxacin (99% Sigma-Aldrich Inc., St Louis, MO) were used for antibiotic stress as they represent three different modes of action. Ampicillin interferes with peptidoglycan synthesis which leads to loss of cell shape and integrity followed by bacterial death (Chopra et al., 2002). Trimethoprim inhibits folic acid synthesis by inhibiting dihydrofolate reductase. Ofloxacin inhibits DNA gyrase, an essential component in cell division (Russell et al., 1996). In addition, previous studies indicated that application of ofloxacin or trimethoprim induced Stx2 production in *E. coli* O157:H7 (Kimmitt et al., 2000; Wong et al., 2000).

Antibiotic solutions were prepared and stored at -80$^{o}$C. The stock concentrations of each antibiotic solution were as follows; ampicillin 5.12 g/l, ofloxacin 0.64 g/l, and trimethoprim 1.28 g/l. In order to dissolve the trimethoprim in distilled water, 1 ml of

glacial acetic acid was added to 300 ml of distilled water and mixed. Each antibiotic stock solution was filter sterilized (0.22 μm filter Fischer Scientific Co., Suwanee, GA) and 1.5 ml aliquots were prepared. Antibiotic solution was then aseptically added to autoclaved and cooled TSB.

**Determination of Minimum Inhibitory Concentrations (MIC) of Antibiotics**

MICs for each strain were determined for each antibiotic (ampicillin, trimethoprim, and ofloxacin) using the National Committee for Clinical Laboratory Standards's (NCCLS) dilution series method (NCCLS, 2000). In this method, 13 TSB tubes with increasing concentrations of the antibiotics were prepared. The tubes were then inoculated with a standardized suspension of the test organism ($10^6$ cfu/ml). After overnight incubation at 37$^o$C, the tubes were examined for turbidity and the minimum inhibitory concentration (MIC) was determined. MICs were confirmed by pour plating onto TSA to ascertain that cell numbers were static across a 6 h period. The MIC for each antibiotic was determined for all five *E. coli* O157:H7 strains (control and gene fusion strains).

**Antibiotic stress**

For virulence factor production studies, 18 hr cultures of *E. coli* O157:H7 MAD 237, MAD 239 and MAD 241 were inoculated into 5 ml TSB ($10^7$ cfu/mL) with the

MIC level of each antibiotic and incubated statically at 37°C. At timepoints 0, 3, and 6 hr, the Miller assay was conducted (β galactosidase assay) to determine virulence factor production. Control cells were prepared by inoculating the 18 hr culture into TSB.

For heat tolerance (56°C) and simulated gastric fluid (SGF) (pH 1.5) survival studies, TSB with the MIC level of each antibiotic was prepared and inoculated with 18 hr culture. After inoculation, the tubes were incubated statically at 37°C for 4 hr. Following 4 hr antibiotic stress, heat and SGF challenges were applied. Control cells were prepared as described previously.

**Virulence factor production**

Virulence factor production was evaluated using the Miller assay. The Miller assay is an assay of the enzymatic activity of β-galactosidase, an enzyme which hydrolyses β-D-galactosides (Miller, 1992). Activity can easily be measured with chromogenic substrates, colorless substrates which are hydrolyzed to yield colored products. The chromogenic substrate used is o-nitrophenyl- β-D-galactoside (ONPG), a colorless compound, which in the presence of β-galactosidase is converted to galactose and o-nitrophenol. The o-nitrophenol is yellow and can be measured by its absorption at 420 nm. The amount of o-nitrophenol produced is proportional to the amount of enzyme present and to the time the enzyme reacts with the ONPG. In order for the assay

to be linear, the ONPG must be provided in excess. The reaction is stopped by adding a concentrated solution of sodium carbonate, which shifts the pH to 11. At this pH, β-galactosidase is inactive. The enzyme activity can be measured by the rate of appearance of yellow color using a spectrophotometer. Miller unit measurements are independent of initial cell numbers.

Miller units are calculated by using the following formula:

$$\text{Miller units} = 1000 * \frac{OD_{420} - 1.75 * OD_{550}}{t * v * OD_{600}} \qquad \text{(Eq.-1)}$$

where, $OD_{420}$ and $OD_{550}$ are read from the reaction mixture, $OD_{600}$ reflects the cell density, $t$ = time of the reaction in minutes, $v$ = volume of the culture used in the assay, in ml (Miller, 1992).

**Simulated Gastric Fluid (SGF) Survival**

Simulated Gastric Fluid (SGF) was prepared as described by Beumer et al. (1992). SGF consisted of 8.3 g/l protease peptone (Difco, Sparks, MD), 3.5 g/l D-glucose (Sigma, St.Louis, MO), 2.05 g/l sodium chloride (Sigma, St.Louis, MO), 0.6 g/l potassium biphosphate (Sigma St.Louis, MO), 0.11 g/l calcium chloride (Sigma, St.Louis, MO), 0.37 g/l potassium chloride (Sigma, St.Louis, MO), 0.05 g/l bovine bile (Sigma, St.Louis, MO), 0.1 g/l lysozyme (Sigma, St.Louis, MO), and 13.3 mg/l pepsin

(Sigma, St.Louis, MO). The pH of the SGF was adjusted with HCl (38% w/w, Fischer Scientific Co., Pittsburg, MA) to 1.5 using a calibrated Orion Model 250A pH meter (Orion research Inc., Taiwan). The SGF was subsequently filter-sterilized (0.22 µm filter Fischer Scientific Co., Suwanee, GA). The SGF was pre-heated to 37$^{o}$C prior to inoculation. For SGF survival, 1 ml of antibiotic stressed or control cells was inoculated to 99 ml of SGF at 37$^{o}$C at 150 rpm agitation. Samples (1ml) were taken for enumeration at appropriate time points.

**Heat Tolerance**

Heat tolerance of stressed and control cells was determined at 56$^{o}$C using the capillary tube method (Bacon et al., 2003). The capillary tubes (Kimax-51, Kimble Products, Vineland, N.J.), and the syringe (Hamilton, NE) used to fill the tubes were autoclaved in order to assure the sterility. Each capillary tube was filled with 50µl of culture. The capillary tubes were manually heat sealed using forceps, special care was taken not to heat the enclosed cell suspension. After sealing, capillary tubes were placed on ice for 2 min followed by submersion in a 56$^{o}$C water bath. At each time point, 1 tube was removed. Immediately after removal from the waterbath, tubes were cooled on ice and placed in a sodium hypochlorite solution (500 ppm, pH6.5) to sanitize them. In order to remove residual hypochlorite, tubes were then rinsed twice in sterile distilled

water. Tubes were then aseptically transferred into 5 ml of sterile 0.1% peptone water using sterile forceps. The capillary tubes were finely crushed with a sterile glass rod, and appropriate serial dilutions were made. Cell populations were enumerated by pour plating appropriate dilutions in duplicate

**Statistical analysis**

All assays were conducted in triplicate with repeated measures (duplicate platings). D-values were calculated as the time in minutes, required for one log reduction of the population. D-values were calculated by regression analysis (PROC REG). Analysis of variance of D-values was then conducted (PROC MIXED) with least square means used to determine significant differences ($p<0.05$) (Statistical Analysis Software, Version 8.2, SAS Institute, Cary, NC).

## Results and Discussion

### Minimum Inhibitory Concentration of Antibiotics (MIC)

The MIC of each antibiotic was as follows; for ampicillin 2.06 mg/l for both strains, for ofloxacin 0.0256 mg/l for one strain (ATCC 43895) and 0.045 mg/l for the other strain (ATCC 43890), for trimethoprim 0.026 mg/l for both strains. These MIC values are consistent with previous literature (EUCAST, 2000). The MIC for each antibiotic was also determined for each gene fusion strain. As expected, these values were not different from the parent strain (ATCC 43895) ($p>0.05$).

### Virulence factor production

The impact of antibiotic stress at the MIC on the virulence factor production of *Escherichia coli* O157:H7 was studied using the Miller assay (Fig 2.1-3). Previous studies have indicated that sublethal levels of antibiotic stress may affect virulence gene expression of *E. coli* O157:H7 (Kimmit et al., 2000; Yoh et al., 1997; Wong et al., 2000; Zhang et al., 2000). These studies mostly concentrated on Shiga toxin production as Shiga toxins are believed to be associated with severe complications, such as TTP or HUS (Boerlin et al., 1999; Kimmit et al., 2000; O'Loughlin et al., 2001; Yoh et al., 1997; Zhang et al., 2000). The results of our studies showed that when *E. coli* O157:H7 cells were subjected to the MIC of ofloxacin and trimethoprim for 6 h, Stx2 production

increased 346-fold in ofloxacin and 15-fold in trimethoprim ($p<0.05$) (Fig 2.1). Stx2 production following ofloxacin stress was significantly higher than production following trimethoprim stress ($p<0.05$). In a similar study, Kimmit et al. (2000) investigated the role of antibiotics and the bacterial SOS response on Stx2 production by STEC. They claimed that the highest level of Stx2 production occurred when cells were subjected to the MIC of ofloxacin and trimethoprim compared to cefuroxime and furazolidone, and that the impact of ofloxacin was more than trimethoprim. In another study, Karch et al. (1986) investigated the influence of subinhibitory concentrations (1/16 MIC) of trimethoprim-sulfamethoxazole on total Shiga toxin production during the growth of strains of EHEC, EPEC and *S. dysenteriae.* They found a dramatic increase in total Stx production in cells that were grown in the presence of trimethoprim-sulfamethoxazole when compared to control cells.

A clinical study conducted by Wong et al. (2000) suggested that the possibility of development of HUS in children infected with *E. coli* O157:H7 increased when the children were treated with antibiotics. Another clinical study done by Yoh et al. (1997) investigated an outbreak of EHEC in 1996 in Japan. They reported that the number of patients that developed HUS or TTP was 51 out of 222, and that 50 of the 51 patients that developed HUS or TTP were treated with antibiotics, suggesting that antibiotic

treatment increased the severity of EHEC infection with respect to late affects. The increased probability of virulence of EHEC after antibiotic treatment may be correlated with the increased production of Shiga toxin.

O'Loughlin et al. (2001) claimed that Shiga toxins have an important role in disease pathogenesis and are responsible for the severe complications HUS and TTP. EHEC colonize the large intestine where they cause HC, and Stx are the primary agents of systemic complications. HUS is characterized by the rapid onset of hemolytic anemia, thrombocytopenia, and acute renal injury, following HC (Kaplan et al. 1998). Vascular endothelial cells are critical for normal blood flow and the maintenance of transcapillary permeability (O'Brien et al., 1992). Kaplan et al. (1998) proposed that the five B subunits of the Shiga toxin attach to the glycolipid receptor glycosphingolipid globotriosyl ceramide (Gb3) on endothelial cells. The damage of the endothelial cells by Shiga toxin leads to a procoagulant state and provides endogenous cytokines which cause increased cell damage (O'Brien et al., 1992). Further, these Gb3 receptors are present in high amounts in the cortex endothelial cells of the human kidney. The endothelial cell injury results in platelet activation and local intravascular thrombosis which lead to kidney failure (a primary symptom of HUS).

Yoh et al. (1997) studied the effect of seven different antimicrobial agents on the production and release of Shiga toxin by *E. coli* O157:H7. The antimicrobial agents studied were streptomycin (an aminoglycoside), fosfomycin, doxycycline (tetracyclines), cefazolin (a cephalosporin), minocycline (a tetracycline), gentamicin (an aminoglycoside), and ampicillin (a β-lactam). Cultures were subjected to sub-inhibitory concentrations (1/2 MIC, 1/10 MIC and 1/50 MIC) of each antimicrobial agent for 16 h. The mode of action of the antibiotics they studied were the inhibition of peptidoglycan synthesis of cell wall (ampicillin, fosfomycin); inhibition of mucopeptide synthesis in the cell wall (cephalosporin); and inhibition of protein synthesis (streptomycin, doxycycline, minocycline, gentamicin) (Chopra et al., 2002). They stated that fosfomycin increased Stx1 production 7-fold, and there was a slight increase in Shiga toxin production following minocycline, gentamicin, cefazolin, and doxycycline stress. On the other hand, when cells were subjected to ampicillin and streptomycin stress, extracellular Stx1 production decreased. Stx2 production decreased when the *E. coli* O157:H7 cells were cultured in any of these antimicrobials for 16 h. Our results indicated that the MIC of ampicillin stress for 6 hr had no effect on Stx2 production ($p>0.05$), and exposure to ofloxacin and trimethoprim at the MIC increased Stx2

production ($p<0.05$) (Fig 2.1). These observed differences are likely due to the differences in modes of action between the antibiotics studied.

The induction of Stx2 production by *E. coli* O157:H7 when subjected to certain antibiotics is probably related to the bacterial SOS response. Zhang et al. (2000) indicated that some antibiotics are known to induce the SOS response, including trimethoprim-sulfamethoxazole, which is commonly used to treat diarrheal disease in children, and ciprofloxacin, which is commonly used to treat diarrheal disease in adults. They studied the effect of ciprofloxacin (an inhibitor of DNA gyrase) and fosfomycin (an inhibitor of peptidoglycan synthesis) on Stx2 production of *E. coli* O157:H7. They found that ciprofloxacin (fluoroquinolone) increased Stx2 production dramatically, but there was no significant increase in Stx production when cells were subjected to fosfomycin. This result was related to the induction of bacterial SOS response. As fosfomycin inhibits peptidoglycan synthesis, it does not induce the bacterial SOS response. They suggested that antibiotics that induce the bacterial SOS response result in Stx prophage induction, and that these antibiotics should not be used as therapy for STEC infection.

Kimmit et al. (2000) demonstrated that the bacterial SOS response is induced when bacterial DNA is damaged and the multifunctional RecA protein is activated.

Activated RecA causes the degradation of two key repressor proteins, LexA and CI. The repression of genes regulated by LexA leads to the temporary arrest of DNA synthesis and cell division and the activation of error-prone DNA repair. In strains carrying an integrated λ-like bacteriophage (which includes all *E. coli* strains that contain *stx* genes), cleavage of the CI phage repressor/activator protein leads to the induction of the previously silent phage-encoded genes, production of phage particles, and host bacterial cell lysis. They also stated that the most potent inducers of the bacterial SOS response are the quinolones and trimethoprim. Fuchs et al. (1999) also studied the influence of the *rec*A gene on the virulence and Stx2 production of EHEC. They reported that the RecA protein had an impact on the virulence of EHEC. They suggested that higher levels of Stx2 production in EHEC were the result of higher levels of spontaneous Stx2 specific phage induction, controlled by the RecA protein.

In addition to Shiga toxins, intimin (EaeA) and hemolysin (HlyA) are other virulence factors of *E. coli* O157:H7. Epidemiological studies indicate that most EHEC strains isolated from patients with HC and HUS produce intimin (Louie et al., 1994). EHEC strains can tightly attach to epithelial cells of the intestine through adhesive lesions. The genes related to production of these lesions, including the *eaeA* gene, are clustered in a pathogenicity island named the locus of enterocyte effacement (LEE)

(McDaniel et al., 1995). Another virulence factor of EHEC is hemolysin, also called enterohemolysin. Hemolysin production induces the attaching and effacing *E. coli* lesions in host intestinal epithelial cells (Beutin et al., 1995). The impact of sublethal levels of stress on the expression of these virulence factors has not been well-addressed. Elhanafi et al. (2004) reported that cold stress or cold-acid stress had no impact on virulence factor production (Stx, HlyA, EaeA). They also reported that growth in media at pH 5.5 acidified with lactic acid enhanced EaeA and HlyA production. The results of our study indicated that intimin production increased when cells were stressed with all three antibiotics, ampicillin (6.6-fold), ofloxacin (7.1-fold), and trimethoprim (16.2-fold) ($p<0.05$) (Fig 2.2). Among the antibiotics studied, trimethoprim impacted the intimin production most ($p<0.05$), followed by ofloxacin and ampicillin, respectively ($p<0.05$). When *E. coli* O157:H7 cells were stressed with the MIC of each antibiotic, *hlyA* production increased following trimethoprim stress (5.8-fold) ($p<0.05$). On the other hand, *hlyA* production decreased following ampicillin stress ($p<0.05$) (Fig 2.3). Ofloxacin had no effect on *hlyA* production ($p>0.05$).

**Survival of E. coli O157:H7 following antibiotic stress**

Foodborne bacteria are faced with organic and inorganic acids in foods and in the gastrointestinal tract of the host (Yousef et al., 2002). Buchanan et al. (2003) noted

that organic (weak) acids are more effective against foodborne pathogens than inorganic acids. The reason organic acids are more effective is that they can diffuse into the cell and dissociate into their non-protonated state in which they lower the intracellular pH and cause inhibition of essential cell processes (Abee et al., 1999). The survival of EHEC strains under low pH conditions is important for their virulence as they must pass through the acidic gastric fluid in order to cause gastrointestinal disease (Lin et al., 1996). Previous studies indicated that the acid survival of *E. coli* O157:H7 is enhanced by sublethal levels of stresses. Arnold et al. (1995) reported that exposure of *E. coli* to acidic conditions or growth in moderately acidic medium increased cell survival at low pH. Lin et al. (2003) suggested that when an acid resistance system of EHEC was activated, it remained active over a prolonged period of cold storage. Elhanafi et al. (2004) showed that prior exposure to 4$^{o}$C or to a combination of cold and acid stress for 4 weeks decreased acid tolerance of *E. coli* O157:H7. Campbell et al. (2004) demonsstrated that monosodium glutamate (MSG) increased the acid tolerance of *E. coli* O157:H7. Yuk et al. (2005) investigated the acid survival of *E. coli* O157:H7 cells grown at pH 6.4 and pH 5.4. They showed that among the organic acids studied (acetic, citric, lactic acid), lactic acid-adapted cells (pH 5.4) showed the greatest survival. These

studies all indicate that prior sublethal stresses influence the survival (positively or negatively) of *E. coli* O157:H7 under subsequent harsh conditions.

The impact of antibiotic stress on acid tolerance of *E. coli* O157:H7 has not been previously addressed. The results of our study indicated that survival of *E. coli* O157:H7 in SGF decreased following antibiotic stress. Antibiotic-stressed cells were not recovered following 1 min in SGF. The D-values for control cells were 175.4 $\pm$ 5.9**,** and 202.4 $\pm$ 6.2 min for the two strains tested. These results indicate that regardless of the mode of action of antibiotic, sublethal levels of antibiotics do not induce a protective effect on the acid tolerance of *E. coli* O157:H7, at least in SGF.

The application of heat to kill microbes and inhibit microbial growth is common in food processing and preservation (Yura et al., 1999). Yousef et al. (2002) indicated that sublethal levels of heat stress damaged the macromolecular components of the cell. When cells are exposed to a sudden increase in temperature, the expression of specific proteins is increased. These proteins are called heat shock proteins (HSPs) (Dyk et al., 1995). The heat shock response is a cellular protective and homeostatic response to cope with heat stress-induced damage to proteins (Yura et al., 1999). Arsené et al. (2000) claimed that mild heat stress of *E. coli* provided subsequent resistance to previously lethal heat stresses. Leenanon et al. (2001) studied the impact of acid shock, acid

adaptation, starvation, and cold stress on subsequent freeze/thaw and heat resistance (56$^{o}$C) of *E. coli* O157:H7. Acid adapted cells were prepared by inoculating the cells into TSB with 10g/liter glucose (final pH 4.8-4.9), and acid-shocked cells were prepared by inoculating cells into TSB acidified to pH 4.0 with HCl. They demonstrated that both heat and freeze-thaw resistance increased after acid adaptation and starvation. On the other hand, cold stressed cells were more susceptible to heat challenge, but more resistant to freeze-thaw stress.

Ryu et al. (1998) studied the effect of acid adaptation and acid-shock (lactic acid, pH 4.8) on the heat tolerance (52, 54, 56$^{o}$C) of *E. coli* O157:H7. They noticed that acid adapted cells were more tolerant to heat challenge than unadapted or acid shocked cells. They claimed that acid adaptation and acid shock initiated different protective mechanisms. The effect of sublethal antibiotic stress on the heat tolerance of *E. coli* O157:H7 has not been previously reported. The results of our studies suggest that antibiotic stress does not have a protective effect on the heat tolerance of *E. coli* O157:H7 (Table 2.2). The D-values of *E. coli* O157:H7 decreased following antibiotic stress compared to control cells ($p<0.05$).

## Conclusions

Virulence gene production of *E. coli* O157:H7 was affected by sublethal levels of antibiotic stress, depending on the mode of action of the antibiotic. The application of

antibiotic treatment in patients infected by *E. coli* O157:O7 may increase disease severity and risk of complications. However, sublethal levels of antibiotic stress had no protective effect on acid (SGF, pH 1.5) and heat (56°C) tolerance.

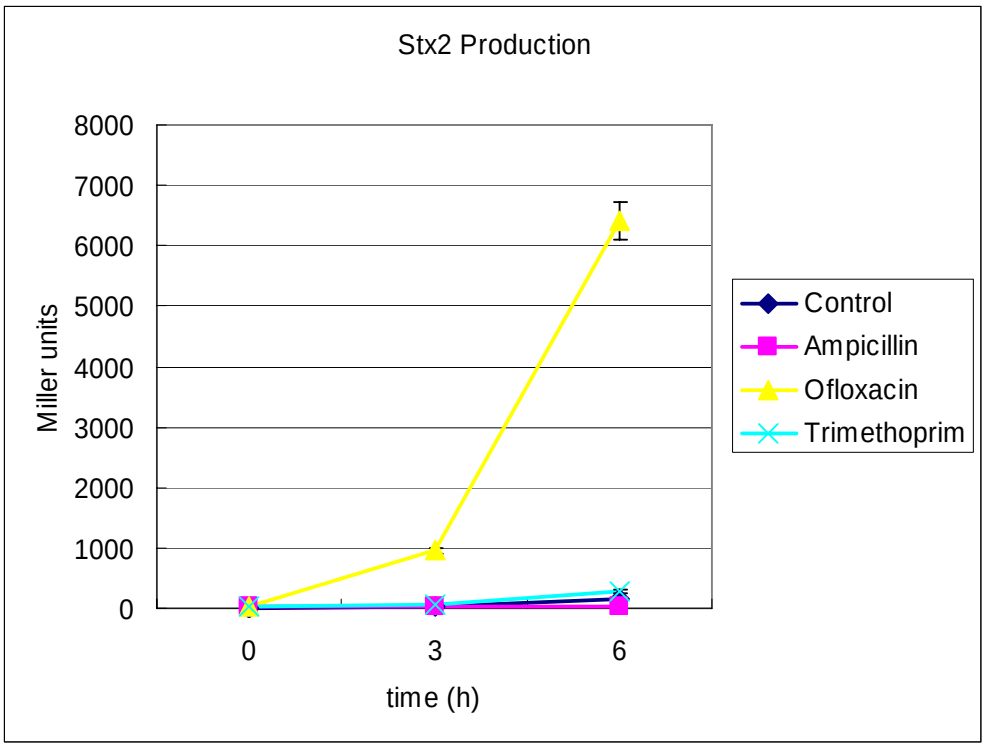


**Figure 2.1:** Stx2 production of *E. coli* O157:H7 throughout stress with ampicillin, ofloxacin, or trimethoprim at MIC (at 37°C).

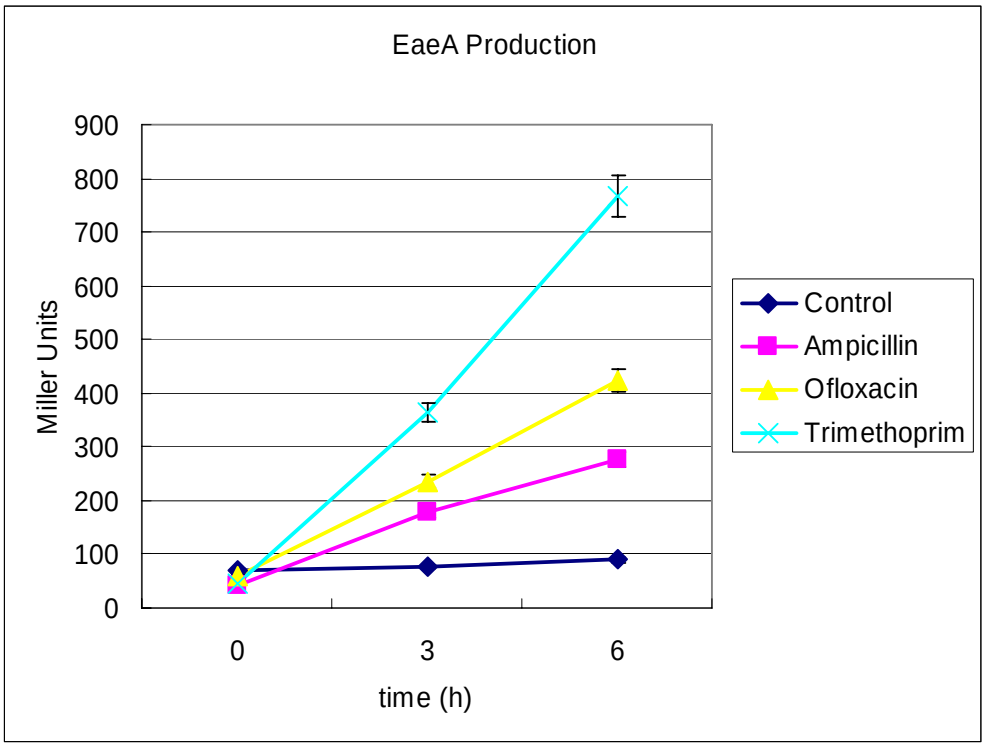


**Figure 2.2:** EaeA production of *E. coli* O157:H7 throughout stress with ampicillin, ofloxacin, or trimethoprim at MIC (at 37$^{o}$C).

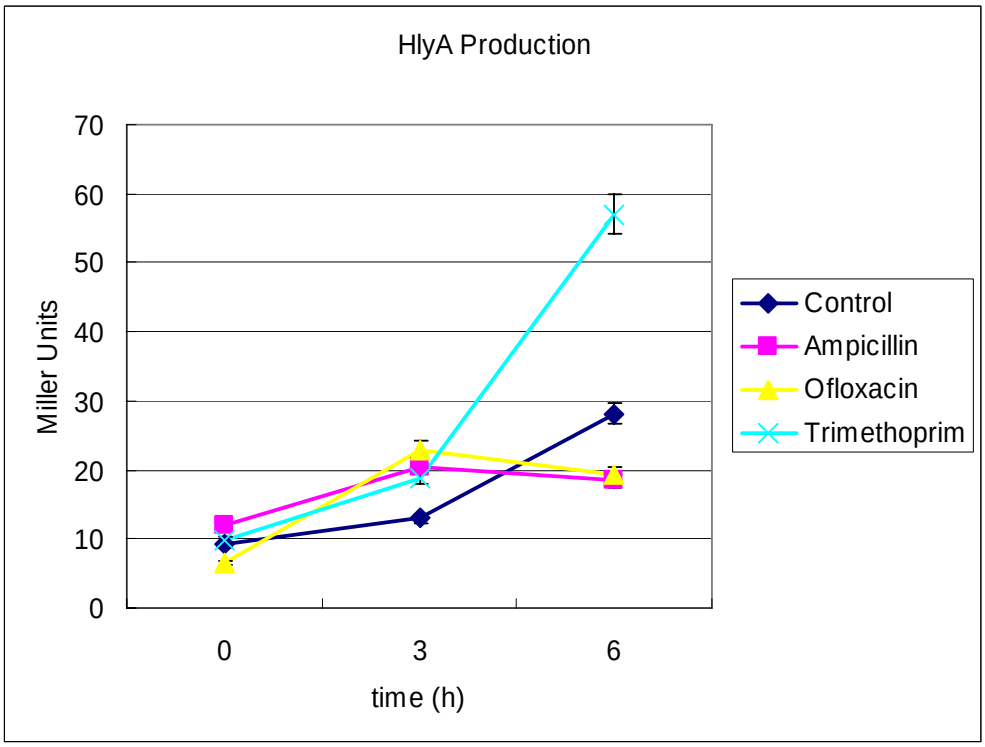


**Figure 2.3:** HlyA production of *E. coli* O157:H7 throughout stress with ampicillin, ofloxacin, or trimethoprim at MIC (at 37$^{o}$C).

**Table 2.1:** Genotypes of gene fusion strains

| | | |
|---|---|---|
| MAD 237 O157:H7 | *ΔlacZ stx2AB::lacZ* | *stx2 : lacZ* gene fusion |
| MAD 239 O157:H7 | *ΔlacZ hlyA::lacZ* | *hlyA : lacZ* gene fusion |
| MAD 241 O157:H7 | *ΔlacZ eaeA::lacZ* | *eaeA : lacZ* gene fusion |

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| *E. coli* O157:H7 Strain | Control | Ampicillin | Ofloxacin | Trimethoprim |
|---|---|---|---|---|
| ATCC 43895 | 11.6a | 0.9b | 0.8b | 0.7b |
| ATCC 43890 | 13.7a | 0.5b | 1.2b | 0.9b |

**Table 2.2:** D-values (min) of *E. coli* O157:H7 subjected to heat at 56$^{o}$C following antibiotic stress at MIC. Means in a row followed by different letters are different ($p<0.05$).

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**MANUSCRIPT II**

**Impact of Acid and Salt Stresses on Salt, Acid and Heat Tolerance of *Escherichia coli* O157:H7**

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**Keywords:** *E. coli* O157:H7, Acid Stress, Salt Stress, Heat Tolerance, Acid Challenge.

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**Abstract**

The impact of acid adaptation and acid and salt stresses on simulated gastric fluid (SGF, pH 1.5) survival, heat tolerance (56$^{o}$C), survival in 14% salt, and survival in 14% salt at pH 5.0 (acidified with organic acid or HCl) of two *E. coli* O157:H7 strains (EHEC1, EHEC2) and a nonpathogenic *E. coli* (NPEC) was studied. Acid adaptation of the *E. coli* cells was achieved by overnight growth in TSB with 1% glucose to a final pH of 4.8. Acid stress was conducted in TSB pH 5.0 acidified with malic, lactic, citric, or hydrochloric acid (HCl). Salt stress was conducted at 4% (w/v) salt concentration. Cells (acid adapted or non acid adapted) were stressed with salt or each acid for 4 hours and post stress tolerance was evaluated. Stressed and control cells were evaluated in triplicate. Acid adaptation of *E. coli* O157:H7 enhanced SGF survival, heat tolerance, and NaCl survival of the cells ($p<0.05$). Among the acids used, lactic acid had the greatest impact on the development of resistance to post stress tolerance ($p<0.05$). Acidification of 14% NaCl TSB solution to pH 5.0 increased the resistance of cells following sublethal stresses, especially when acidified with lactic acid ($p<0.05$). Salt stress enhanced SGF tolerance of acid adapted cells of EHEC2 and NPEC ($p<0.05$) but not EHEC1 ($p>0.05$). Heat tolerance of EHEC2 and NPEC decreased following salt stress, regardless of acid adaptation ($p<0.05$). Acid adaptation and acid and salt stress of

*E. coli* produce protection against further challenges, which should be taken into consideration when producing minimally processed foods.

**Keywords:** *E. coli* O157:H7, Stress Response, Acid Stress, Salt Stress, Heat Tolerance, Acid Challenge.

## Introduction

*Escherichia coli* O157:H7 is a Gram-negative, facultative anaerobe, and has been an important foodborne pathogen since the first documented outbreak in 1982 (Sussman, 1997). The gastrointestinal tract and the kidneys are the target organs for *E. coli* O157:H7 (Doyle et al., 2001). Schroeder et al. (2004) indicated that *E. coli* O157:H7 illness is most commonly associated with improperly cooked ground beef. *E. coli* O157:H7 is known as the major cause of hemorrhagic colitis (HC) (bloody diarrhea) and the disease complications hemorrhagic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP) (Arnold et al., 1995).

In food processing and preservation, foodborne pathogens are subjected to different types of stresses. Stress is any deleterious factor or condition that adversely affects the growth or survival of microorganisms. Stresses can be physical, chemical, or biological (Vorob'eva, 2003). The rapid adaptation of microorganisms to changing environments is crucial for their survival and growth. When microorganisms are

subjected to stressful conditions, their responnse can be sudden such as a response to shock, or a longer term adaptation (Hengge-Aronis, 1999). The stress response of a microorganism can be activated by different stresses and may protect against multiple often unrelated stressors (Yousef et al., 2002).

Hengge-Aronis et al. (1999) indicated that when microorganisms are subjected to stress, the number of stress induced proteins increase, which protect the cell against subsequent stress. The induction of these protective proteins that increase resistance of the cell to chemical and physical challenges can be initiated by stationary phase, starvation, and other sublethal stressors (Arnold et al., 1996). In the food industry, the understanding of the stress response of bacteria is important as bacteria are frequently subjected to sublethal levels of stress during food processing and storage. Common applications to control microbial growth in the food industry include low pH, sodium chloride, and heat.

Foodborne bacteria are exposed to organic or inorganic acids in foods or in the gastrointestinal tract of the host (Yousef et al., 2002). Lin et al. (1996) stated that it is necessary for Enterohemorrhagic *E. coli* strains to pass through the acidic environment of the gastric fluid in order to cause gastrointestinal diseases. Organic acids are more effective against foodborne pathogens than inorganic acids as they have the ability to

diffuse into the cell in their nonpolar protonated form. Once in the cell, they dissociate, decreasing intracellular pH and inhibiting essential cell processes (Buchanan et al. 2004; Abee et al. 1999). Cheville et al. (1996) documented that subjection of *E. coli* O157:H7 to sublethal levels of acids enhanced its survival in foods and plays a key role in its pathogenesis by increasing its acid resistance. There are three acid resistance (AR) systems in *E. coli* O157:H7 which depend on the type of medium and the growth conditions (Smith, 2003).

An acid stress response not only increases acid resistance but can also render cells more resistant to other sublethal stresses, an effect called cross-protection. Cheng et al. (2002) found that cells acid-adapted at pH 5.0 (HCL as acidulant) were more heat tolerant than were non-adapted cells. They also observed that thermal tolerance of *E. coli* O157:H7 differed with strain. In a similar study in model meat systems, Duffy et al. (2000) showed that exposure to pH typical to fermentation enhanced thermal tolerance of *E. coli* O157:H7 at 55$^{o}$C. Acid-adapted cells (pH 5.0 HCl) were also more resistant to 10% sodium chloride than were non acid-adapted cells. In a similar study, Yuk et al. (2004) investigated the impact of acid stress using different acid types (acetic, citric, and lactic acid at pH 5.4 and 6.4) on Shiga toxin production, membrane lipid concentration and acid survival of *E. coli* O157:H7 in SGFat pH 1.5. Among the acids

studied, lactic acid adapted cells at pH 5.4 showed the greatest and the acetic acid adapted cells showed the least survival. Acid resistance, membrane lipid concentration, and the Shiga toxin production of *E. coli* O157:H7 were dependent on both pH and the type of organic acid.

Application of salt to control microbial growth is common in the food industry. Cheng et al. (2002) demostrated that the inhibitory effect of sodium chloride is related to a plasmolytic effect in addition to dehydration, oxygen removal, interference of enzymes and the toxicity of the sodium and chloride ions when they are in solution. Casey et al. (2002) studied the effect of 4% sodium chloride in fermented meat model systems on the survival of *E. coli* O157:H45 (an enteropathogenic *E. coli* (EPEC)) at pH 4.2. Cells subjected to sodium chloride and acid survived better than cells subjected to acid only. The cross protection of NaCl to acid challenge was related to the preventive effect of NaCl to the uptake of protons from the acidic media that lowers the intracellular pH. Also, the lowered water activity of the media improved the acid survival of the cells. Blackburn et al. (1997) investigated the impact of pH 4.2-9.6 acidified with HCl or NaOH and 0.5-8.5% (w/w) NaCl on the heat tolerance of *Salmonella enterica serovar enteridis* and *E. coli* O157:H7 at 54.5$^{o}$C-64.5$^{o}$C in TSB. They found that for *E. coli* O157:H7, increases in NaCl concentration up to 8.5% (w/w)

had a protective effect on heat challenge. In another study, Faleiro et al. (2003) studied the stress response of *Listeria monocytogenes* isolated from cheese to pH 5.1-6.2 in broths acidified with lactic acid and supplemented with 2-3.5% (w/vol) sodium chloride. The results indicated that some strains of *L. monocytogenes* showed an osmotolerance response after growth in 3.5% (w/vol) sodium chloride.

In order to control pathogens in food processing, it is important to understand how different sublethal stresses impact the survival of *E. coli* O157:H7. Both decreased pH and salt are used widely in food processing. Our objectives were to determine the effect of acid and NaCl stress on survival in simulated gastric fluid (SGF, pH 1.5), heat tolerance (56$^{o}$C), 14% NaCl survival and NaCl (14%, pH 5.0 by HCl or lactic acid) survival of *E. coli* O157:H7.

## Materials and Methods

### Bacterial strains and culture conditions

*E. coli* O157:H7 ATCC 43895 (EHEC1) (raw hamburger isolate), *E. coli* O157:H7 ATCC 43890 (EHEC2) (fecal isolate), and *E. coli* ATCC 25922 (NPEC) were used in experiments. Stock cultures were stored in 30% (w/w) glycerol at -80$^{o}$C. Cultures were activated two days prior to use by overnight growth in Tryptic Soy Broth (TSB) (BBL, Cockeysville, MD) (pH 7.2) at 37$^{o}$C. Strains were examined monthly for

purity by plating on sorbitol MacConkey agar (O157:H7 strains). Growth curves of each strain indicated that stationary phase was achieved after 18 h growth ($10^9$ cfu/ml). Cell enumeration was done by pour plating in duplicate on Tryptic Soy Agar (TSA) after serial dilution in sterile 0.1% peptone-water.

**Preparation of Acidic solutions**

Acid stress was applied using 4 different types of organic and inorganic acids. Organic acids used were: Malic acid (2M) (Fischer Scientific Co., PA), lactic acid (85%) (Fischer Scientific Co., PA), and citric acid (1M) (Fischer Scientific Co., PA), and the inorganic acid tested was HCl (38% w/w) (Fischer Scientific Co., PA). TSB acidified to pH 5.0 was prepared by adjusting the pH following autoclaving and cooling to room temperature using a calibrated Orion Model 250A pH meter (Orion research Inc., Taiwan) (Table 3.1). The solutions were subsequently filter-sterilized (0.22 µm filter Fischer Scientific Co., Suwanee, GA) and stored at room temperature. Prior to application of acid stress, the solutions were pre-heated to 37$^{o}$C.

**Preparation of NaCl solutions**

Salt stress was applied using TSB supplemented with 4% NaCl (w/v). Salt challenges were conducted using TSB supplemented with 14% NaCl (w/v). TSB with 4% NaCl was prepared by the addition of 40 g NaCl (Fischer Scientific Co., NJ) to 1

liter TSB and TSB with 14% NaCl was prepared by the addition of 140 g NaCl to 1 liter of TSB. The solutions were autoclaved and cooled to room temperature. The acidified 14% NaCl solutions were prepared by adjusting the pH of these autoclaved solutions to pH 5.0 by supplementation with either lactic acid or HCl. After the addition of the acids, the solutions were filter sterilized (0.22 µm filter, Fischer Scientific Co., Suwanee, GA). The solutions were stored at room temperature and were pre-heated to 37$^{o}$C prior to experiments.

**Acid and NaCl stress**

Acid and NaCl stresses were applied by making small changes to the protocol described by Cheng et al. (2002). The cells were grown in both TSB without glucose (Becton, Dickinson and Co., MD) and TSB with 1% glucose for 18 hr to produce non-acid adapted and acid adapted cells, respectively (Buchanan et al., 2004). TSB with 1% glucose (Fischer Scientific Co., NJ) was prepared by the addition of 10 g D-glucose to 1 liter of TSB. The pH values of the TSB without glucose and the TSB with 1% glucose after 18 hr of cell growth are shown in Table 2. Overnight growth of cells in TSB with 1 % glucose resulted in acid adapted cells (Cheville et al., 1996; Buchanan et al., 2004). One ml of an18 hr culture was centrifuged at 8800 × $g$ for 12 min. The supernatant was discarded and cells were washed with 1 ml of 0.1 % peptone water. The washing step

was repeated twice. Washed cells were re-suspended in 1 ml of pre-warmed 4% NaCl TSB or pH 5.0 TSB acidified with malic acid, lactic acid, citric acid or HCl. The suspensions were incubated statically for 4 hr at 37°C. Control cells were suspended in regular TSB and incubated for 4 hr at 37°C.

**Survival in Simulated Gastric Fluid (SGF)**

Simulated gastric fluid (SGF) was prepared as described by Beumer et al. (1992). SGF consisted of 8.3 g/l protease peptone (Difco, Sparks, MD), 3.5 g/l D-glucose (Sigma, St.Louis, MO), 2.05 g/l sodium chloride (Sigma, St.Louis, MO), 0.6 g/l potassium biphosphate (Sigma St.Louis, MO), 0.11 g/l calcium chloride (Sigma, St.Louis, MO), 0.37 g/l potassium chloride (Sigma, St.Louis, MO), 0.05 g/l bovine bile (Sigma, St.Louis, MO), 0.1 g/l lysozyme (Sigma, St.Louis, MO), and 13.3 mg/l pepsin (Sigma, St.Louis, MO). The pH of the SGF was adjusted with HCl (38% w/w, Fischer Scientific Co., Pittsburg, MA) to 1.5 using a calibrated Orion Model 250A pH meter. The SGF was subsequently filter-sterilized (0.22 μm filter, Fischer Scientific Co., Suwanee, GA). The SGF was pre-heated to 37°C prior to inoculation. For SGF survival studies, 1 ml of acid or NaCl stressed or control cells was inoculated to 99 ml of SGF at 37°C with agitation at 150 rpm. Samples (1ml) were taken for enumeration at

appropriate time points. Cell enumeration was conducted by pour plating in duplicate on Tryptic Soy Agar (TSA) after serial dilution in sterile 0.1% peptone-water.

**NaCl Survival**

In these studies, TSB containing 14% NaCl (pH 7.2), and TSB with 14% NaCl acidified to pH 5.0 with HCl or lactic acid was used. One ml of acid or NaCl stressed or control cells were inoculated to 99 ml of NaCl TSB solution (pH 7.2 or pH 5.0 acidified with lactic acid or HCl) at 37$^{o}$C with agitation at 150 rpm. Samples were taken for enumeration at appropriate time points. Cell enumeration was done by pour plating in duplicate on Tryptic Soy Agar (TSA) after serial dilution in sterile 0.1% peptone-water.

**Heat Tolerance**

Heat tolerance of stressed and control cells was determined at 56$^{o}$C using the capillary tube method (Bacon et al., 2003). The capillary tubes (Kimax-51, Kimble Products, Vineland, N.J.) and the syringe (Hamilton, NE) used to fill the tubes were autoclaved in order to maintain the sterility. Each capillary tube was filled with 50µl of diluted culture. The capillary tubes were manually heat sealed using forceps; special care was taken not to heat the enclosed cell suspension. After sealing, capillary tubes were placed on ice for 2 min followed by submersion in a 56$^{o}$C water bath. At each time

point, 1 tube was removed. Immediately after removal from the waterbath, tubes were cooled on ice and placed in a sodium hypochlorite solution (500 ppm, pH6.5) to sanitize them. In order to remove residual hypochlorite, tubes were then rinsed twice in sterile distilled water. Tubes were then aseptically transferred into 5 ml of sterile 0.1% peptone water using sterile forceps. The capillary tubes were finely crushed with a sterile glass rod, and appropriate serial dilutions were made. Cell populations were enumerated by pour plating appropriate dilutions in duplicate

**Statistical analysis**

All assays were conducted in triplicate with repeated measures (duplicate platings). D-values were calculated as the time in minutes required for one log reduction of the population. D-values were calculated by regression analysis (PROC REG). Analysis of variance of D-values was done (PROC MIXED) with least square means used to determine significant differences ($p<0.05$) (Statistical Analysis Software, Version 8.2, SAS Institute, Cary, NC). Both main effects and interactions were evaluated in statistical analysis.

## Results and Discussion

### Survival of *E. coli* O157:H7 in simulated gastric fluid (SGF) at pH 1.5; heat tolerance at 56°C; and survival in 14% NaCl and 14% NaCl acidified to pH 5.0 (lactic acid or HCl) following acid stress at pH 5.0

*E. coli* O157:H7 is exposed to organic or inorganic acids in foods or in the gastrointestinal tract of the host (Yousef et al., 2002). Previous studies have indicated that organic acids are more effective against foodborne pathogens than inorganic acids, as they have the ability to diffuse into the cell in their nonpolar protonated form and subsequently dissociate, decreasing intracellular pH and inhibiting essential cell processes (Buchanan et al., 2004; Abee et al., 1999). In order to initiate infection, *E. coli* O157:H7 must survive the acidic environment of the stomach. Arnold et al. (1995) investigated the survival of *E. coli* O157:H7 in SGF and claimed that 4 of 5 strains of *E. coli* O157:H7 studied were detectable in SGF after 3 hours, which was close to transit time in the stomach, suggesting the importance of understanding the survival of EHEC in SGF.

In our study, two *E. coli* O157:H7 strain and a non-pathogenic *E. coli* strain were stressed at pH 5.0 in a solution acidified with three organic acids (malic acid, lactic acid or citric acid) or an inorganic acid (HCl), and the survival of the cells to

different challenges was investigated. For SGF tolerance, differences were observed between strains and between acid types (strain*acid type interaction, $p<0.05$) (Table 3.3). Other studies have documented differences in acid tolerance among *E. coli* O157:H7 strains and between pathogenic and nonpathogenic *E. coli* strains (Arnold et al., 1995; Cheng et al., 1998; Leenanon et al., 2001; Yuk et al., 2004). Previous studies have also documented that overnight growth in 1% glucose can be used to induce acid adaptation in *E. coli* (Berry et al., 2000; Buchanan et al., 2004; Leenanon et. al., 2001; Ryu et al., 1998; Ryu et al., 1999). The addition of glucose to the growth medium results in a lower final media pH following 18 h of growth (Table 3.2) and consequently, acid adapted cells. Acid adapted cells survived longer in SGF than did non-acid adapted cells (Table 3.3). In contrast, very little effect on SGF tolerance was observed for cells that had not been previously acid adapted, regardless of acid type used prior to SGF exposure (Table 3.3).

In general, acid adapted cells subsequently exposed to lactic acid had the greatest improvement in SGF tolerance, regardless of strain (Table 3.3). Large increases ($\geq$ 3 fold increase compared to control cells) in SGF tolerance were also observed for acid adapted NPEC following acid stress in media acidified using citric acid, malic acid, or HCl. SGF tolerance of EHEC1 was improved compared to control cells following

exposure to citric acid or HCl, but these increases were not as large as those changes observed for the NPEC strain. EHEC2 did not show any differences in SGF tolerance between acid and non-acid adapted cells except for acid adapted cells exposed to pH 5.0 acidified with lactic acid (Table 3.3). Similar to results observed in our study, Yuk et al. (2004) investigated the SGF tolerance of *E. coli* O157:H7 cells grown at pH 6.4 and pH 5.4 acidified with acetic, citric, and lactic acid. They observed that lactic acid-adapted cells at pH 5.4 showed the highest SGF survival among the organic acids studied (acetic, citric, lactic acid). Ryu et al. (1998) studied the impact of acid adaptation and acid shock on the survival of *E. coli* O157:H7 in orange juice, apple cider and TSB at pH 3.4 or 3.9 acidified with acetic acid or lactic acid. They also observed a greater acid tolerance response from cells adapted with lactic acid. They suggested that lactic acid might have a different inactivation mechanism, in which a different acid tolerance response (ATR) is activated when cells were acid-shocked with lactic acid. Buchanan et al. (1999) studied the impact of acid type on the acid resistance of EHEC at pH 3.0 following growth in TSB with and without 1% dextrose. They claimed that cells challenged at pH 3.0 with lactic acid showed the highest survival and the cells challenged at pH 3.0 with HCl showed the lowest survival among the acids studied (lactic, malic, citric, acetic and HCl).

Differences were also observed for heat tolerance based on strain, acid adaptation, and type of acid stress (strain*acid type interaction, $p<0.05$) (Table 3.4). Acid adaptation alone increased the heat tolerance for both EHEC strains but not for NPEC. Leenanon et al. (2001) also observed that acid adaptation (overnight growth in 1% glucose) increased heat tolerance of *E. coli* O157:H7. Duffy et al. (2000) reported that acid adaptation of *E. coli* O157:H7 increased heat tolerance of *E. coli* O157:H7 at 55$^{o}$C in a model fermented meat system. Leenanon et al. (2001) reported that acid shock at pH 4.0 (HCl) did not increase heat tolerance. We also observed that acid shock, regardless of acid type, did not increase heat tolerance for EHEC2 and NPEC, similar to the previously reported study (Table 3.4). However, for EHEC1, acid shock increased heat tolerance regardless of whether cells had been previously acid adapted.

Similar to SGF tolerance, lactic acid stress (pH 5.0) showed the largest and most consistent (for acid adapted and non-acid adapted cells) effect on heat tolerance (Table 3.4). Ryu et al. (1998) studied the effect of acid adaptation and acid-shock at pH 4.8 adjusted with lactic acid on the heat tolerance (52, 54, 56$^{o}$C) of *E. coli* O157:H7. They reported that acid adapted cells were more tolerant to heat challenge than acid shocked and control cells. They also indicated that acid shocked cells were more heat tolerant than control cells. According to the differences in the heat tolerance of the cells, they

claimed that acid adaptation and acid shock initiated different protective mechanisms. Kaur et al. (1998) reported that heat resistance of *E. coli* O157:H7 was dependent on conditions during the log phase of cell growth. They found that *E. coli* O157:H7 cells grown at 40$^{o}$C were more resistant to heat challenge at 55$^{o}$C and 60$^{o}$C than were cells grown at 37$^{o}$C, suggesting that general stress proteins were induced by growth at higher temperature, providing added protection to subsequent heat treatment.

The survival of *E. coli* strains in 14% NaCl and 14% NaCl at pH 5.0 acidified with lactic acid or HCl following acid stress was dependent on previous acid adaptation, strain and acid type used ($p<0.05$) (Tables 3.5-7). Three-way interactions between strain, acid stress, and acid type used for stress were observed ($p<0.05$). Due to the 3-way interaction, results are presented pairwise in three tables averaged across 1-factor (Tables 3.5-7). Previous acid adaptation increased NaCl or NaCl, pH 5.0 survival when cells were acid stressed, regardless of acid type (Tables 3.5, 6). However, previous acid adaptation had no effect on NaCl or NaCl, pH 5.0 survival for control cells (no acid stress). In general, as with previous experiments, cells stressed with lactic acid prior to NaCl exposure exhibited the largest D-values across all three strains ($p<0.05$) (Tables 3.5, 7). Acidification of 14% NaCl TSB solutions to pH 5.0 increased survival of the three strains compared to 14% NaCl TSB (Table 3.6). Also, all three strains survived

longer in 14% NaCl TSB acidified with lactic acid than 14% NaCl TSB acidified with HCl (Table 3.6). These results suggest a cross-protection phenomenon between acid adaptation and resistance to further salt or salt/acid challenge.

Leyer et al. (1993) investigated the cross protection of acid adaptation to other environmental stresses in *Salmonella typhimurium* including 2.5M (14.6% w/v) NaCl at 22$^{o}$C challenge. They observed that acid adapted cells exhibited better survival in 2.5M (14.6%) NaCl compared to nonadapted cells. They also claimed OmpC, which is an outer membrane protein responding to osmotic stress, was expressed during acid adaptation resulting in cross production to further NaCl challenge. In another study, Lou et al. (1997) indicated heat shocking at 45$^{o}$C for 1 h and 5% ethanol adaptation for 1 h increased the tolerance of *Listeria monocytogenes* to 25% NaCl. They claimed that sublethal levels of ethanol and heat adaptation induced the same set of stress proteins that protect the cells against high NaCl concentrations.

**Survival of *E. coli* O157:H7 in simulated gastric fluid (SGF) at pH 1.5; heat tolerance at 56$^{o}$C; and 14% NaCl and 14% NaCl acidified to pH 5.0 (lactic acid or HCl) survival following 4% NaCl stress**

The survival of *E. coli* strains in SGF after 4% NaCl stress was dependent on strain and previous acid adaptation (strain*acid adaptation interaction, $p<0.05$) (Table

3.8). Prior acid adaptation increased the survival of the *E. coli* cells in SGF following 4% NaCl stress, regardless of the strain with one exception. Control (no salt stress) NPEC cells that were acid adapted were not more acid tolerant than were nonadapted cells (Table 3.8). The presence of 4% NaCl enhanced SGF tolerance of acid adapted EHEC2 and NPEC strains, but not EHEC1. Cheng et al. (1998) studied the impact of the addition of 1% NaCl to the growth media on the acid tolerance of *E. coli* O157:H7 in SGF in log- and stationary phase of growth. They claimed that the addition of 1% NaCl to the growth media increased the acid tolerance of the cells in log-phase, but had no effect on the acid tolerance of cells in the stationary phase of growth. In another study, Casey et al. (2002) studied the effect of 4% NaCl in fermented meat model systems on the survival of *E. coli* O157:H45 (an enteropathogenic *E. coli* (EPEC)) at pH 4.2. Cells subjected to NaCl and acid survived longer than cells subjected to only acid. They claimed that the protective effect of the NaCl against acid challenge was related to the preventive effect of NaCl on the uptake of protons from the acidic media that lowers the intracellular pH. Faleiro et al. (2003) studied the stress response of *L. monocytogenes* isolated from cheese to pH 5.1-6.2 in broths acidified with lactic acid and 2-3.5% (w/vol) NaCl. The results indicated that some strains of *L. monocytogenes*

showed an osmotolerance response after growth in 3.5% (w/vol) NaCl. This response provided cross protection to acid shock conditions at pH 3.5.

Acid adaptation of EHEC1 and NPEC increased heat tolerance of the cells at 56$^{o}$C, regardless of salt stress (strain*salt stress interaction, $p<0.05$) (Table 3.9). Salt stress decreased heat tolerance of EHEC2 and NPEC regardless of prior acid adaptation. However, salt stress did enhance heat tolerance of acid adapted EHEC1. Blackburn et al. (1997) investigated the impact of pH 4.2-9.6 (pH adjustment with HCl or NaOH) and 0.5-8.5% (w/w) NaCl on the heat tolerance of *Salmonella enterica serovar enteridis* and *E. coli* O157:H7 at 54.5$^{o}$C-64.5$^{o}$C in TSB. They claimed that for *E. coli* O157:H7, an increase in NaCl concentration up to 8.5% (w/w) had a protective effect on heat stress.

Survival of *E. coli* in 14% NaCl was impacted by strain, salt stress, and salt challenge (strain*salt stress*salt challenge interaction, $p<0.05$). Due to the 3-way interaction, results are presented pairwise in three tables averaged across 1-factor (Tables 10-12). Survival in 14% NaCl increased when the pH of the 14% NaCl was acidified to 5.0 with lactic acid or HCl (Table 3.10). In general, as with previous experiments, cells subjected to 14% NaCl at pH 5.0 acidified with lactic acid exhibited the highest D values, regardless of strain ($p<0.05$) (Table 3.10). Also, prior acid adaptation of cells increased survival in 14% NaCl whether or not the media was

acidified. It was also observed that 4% NaCl stress had a protective effect on the survival of cells in 14% NaCl regardless of media acidification, except for acid adapted EHEC1 (Table 3.12).

Cells stressed with 4% NaCl exhibited increased resistance in 14% NaCl and 14% NaCl acidified with HCl (Table 11) ($p<0.05$). On the other hand, there was no significant improvement in bacterial cell survival for 14% NaCl acidified with lactic acid following 4% NaCl stress ($p>0.05$). Rowe et al. (1999) studied the cross protection between acid and salt in *E. coli* O157:H7. The results of their study indicated that using a combination of acid and salt provided cross-protection to subsequent acid or salt exposure and this phenomenon was induced by the *rpoS* gene.

## Conclusions

Bacteria are commonly subjected to sublethal levels of stresses in food systems. Organic acids or salt are present in many foods at sublethal levels in order to give flavor or to protect against spoilage or pathogenic bacteria, e.g. apple juice, fermented foods etc. Sublethal levels of organic acids or salt may initiate the development of stress response mechanisms in bacteria, which also results in resistance to other stresses, so-called cross-protection. The understanding of the stress response of *E. coli* O157:H7 in such systems is important to control this bacterium in food. This study showed that the SGF, heat, and salt resistance of pre-conditioned *E. coli* O157:H7 was dependent on

several factors: acid adaptation, *E. coli* strain, acid type, and salt concentration. In most cases, acid adapted *E. coli* cells exhibited increased resistance to following challenges. In some strains, cross protection phenomena were also observed. In general, acid stress with lactic acid resulted in more resistant *E. coli* cells compared to acid stress with other acidulants. Our results indicate that when *E. coli* is subjected to sublethal levels of stresses, tolerance to subsequent challenges increases, which poses an important problem in controlling this organism using various food processing and preservation technologies.

**Table 3.1:** pH, volume and molarity of each acid used in experiments

| **Acidulant** | **pH** | **Vol. (mL) of acid /L** | **Molarity** |
|---|---|---|---|
| **Lactic acid** | 5.0 | 540 | 0.0027 |
| **Citric acid** | 5.0 | 2500 | 0.0125 |
| **Malic acid** | 5.0 | 2090 | 0.0104 |
| **Hydrochloric acid** | 5.0 | 497 | 0.0025 |

Initial pH of TSB was 7.4; final pH varied by ± 0.05

**Table 3.2:** Final pH of cells grown in TSB without glucose and TSB with 1% glucose after 18 hr of growth at 37C.

The initial pH of TSB without glucose was 7.29 and TSB with 1% glucose was 6.68.

| ***E. coli* Strain** | EHEC1 in TSB with 1% glucose | EHEC1 in TSB without glucose | EHEC2 in TSB with 1% glucose | EHEC2 in TSB without glucose | NPEC in TSB with 1% glucose | NPEC in TSB without glucose |
|---|---|---|---|---|---|---|
| **pH** | 4.89±0.01 | 7.03±0.03 | 4.80±0.01 | 7.31±0.03 | 4.84±0.02 | 6.97±0.01 |

EHEC 1 and 2 refers to the 2 strains of *E. coli* O157:H7 evaluated in this study.

NPEC – nonpathogenic *E. coli*

**Table 3.3:** D-values in min for *E. coli* strains in SGF following 4 hr acid stress at pH 5.0 acidified with different acids ($p<0.05$).

| | EHEC1 in TSB with 1% glucose | EHEC1 in TSB without glucose | EHEC2 in TSB with 1% glucose | EHEC2 in TSB without glucose | NPEC in TSB with 1% glucose | NPEC in TSB without glucose |
|---|---|---|---|---|---|---|
| **Control** | 51.8±5.2 ef | 40.1±2.7 gh | 39.4±0.8 gh | 36.8±1.9 gh | 39.1±4.8g h | 24.6±1.9j |
| **Malic Acid** | 51.3±3.1 f | 32.6±1.0 hi | 39.0±3.2 gh | 37.3±1.2 gh | 129.1±1.3 b | 49.1±2.2f |
| **Lactic Acid** | 112.9±9. 7d | 31.6±0.8 hi | 57.6±3.1 e | 42.4±1.9 gh | 148.0±3.3 a | 27.4±3.4ij |
| **Citric Acid** | 55.9±6.2 ef | 35.6±2.7 h | 40.6±1.5 gh | 41.1±2.9 gh | 121.3±2.2 c | 38.4±1.9gh |
| **HCl** | 54.5±3.2 ef | 41.5±3.4 gh | 34.1±2.3 h | 35.3±3.1 h | 125.7±5.1 bc | 36.2±1.1h |

Values are the means of triplicate replications. Means followed by different letters are different ($p<0.05$). EHEC 1 and 2 refer to the 2 strains of *E. coli* O157:H7 evaluated in this study. NPEC – nonpathogenic *E. coli*

**Table 3.4:** D-values in min for *E. coli* strains at 56 $^{o}$C following 4 hr acid stress at pH 5.0 acidified with different acids ($p<0.05$).

| | EHEC1 in TSB with 1% glucose | EHEC1 in TSB without glucose | EHEC2 in TSB with 1% glucose | EHEC2 in TSB without glucose | NPEC in TSB with 1% glucose | NPEC in TSB without glucose |
|---|---|---|---|---|---|---|
| **Control** | 12.6±3.5 gh | 8.0±0.3n o | 21.4±2.4 c | 15.9±2.3 efg | 16.5±2.0def | 16.9±0.6def |
| **Malic Acid** | 19.8±4.2 cd | 10.5±1.1 jklmn | 12.2±2.8 hijk | 10.8±1.0 ijk | 9.1±0.9lmno | 10.6±0.1jklm n |
| **Lactic Acid** | 30.8±3.2 a | 16.4±0.4 ef | 11.6±0.6 hijklm | 10.7±1.0 ijklmn | 10.8±1.8ijkl mn | 9.1±0.5lmno |
| **Citric Acid** | 26.3±4.3 b | 9.6±0.9k lmno | 13.7±3.2 fghi | 12.0±0.5 hijkl | 7.5±0.9o | 14.4±1.8fgh |
| **HCl** | 18.6±2.3 cde | 11.9±0.7 ijkl | 12.7±1.6 hij | 11.5±1.5 hijklm | 6.9±0.7o | 8.6±0.4mno |

Values are the means of triplicate replications. Means followed by different letters are different ($p<0.05$). EHEC 1 and 2 refer to the 2 strains of *E. coli* O157:H7 evaluated in this study. NPEC – nonpathogenic *E. coli*

**Table 3.5:** Average D-values in min for *E. coli* strains in 14% NaCl and 14% NaCl at pH 5.0 acidified with lactic acid or HCl following 4 hr acid stress at pH 5.0 acidified with different acids ($p<0.05$) averaged across NaCl stress.

| | EHEC1 in TSB with 1% glucose | EHEC1 in TSB without glucose | EHEC2 in TSB with 1% glucose | EHEC2 in TSB without glucose | NPEC in TSB with 1% glucose | NPEC in TSB without glucose |
|---|---|---|---|---|---|---|
| **Control** | 203.4±57.8 fg | 238.6±75.7b c | 203.1±93.8 fg | 195.7±114.3g h | 186.8±86.3hij | 213.5±90.1 def |
| **Malic Acid** | 250.7±65.6b | 178.1±31.4i j | 192.3±65.3h | 172.0±56.6jk | 190.0±52.3ghi | 164.4±42.3 k |
| **Lactic Acid** | 275.9±66.5a | 218.5±35.3d efg | 281.8±93.5a | 211.5±103.7ef | 279.1±85.9a | 239.0±50.7 bc |
| **Citric Acid** | 227.4±35.0c d | 187.9±25.4h i | 225.8±63.6c de | 204.7±57.0fg | 215.1±36.7def | 177.5±42.3i j |
| **HCl** | 187.3±31.3h i | 159.6±19.4. 3k | 167.5±57.6j k | 158.4±52.8k | 175.3±37.1ijk | 157.8±20.9 k |

Values are the means of three stresses with triplicate replications. There was a 3-way interaction for strain*NaCl stress*acid stress ($p<0.05$), thus results are presented averaged across strain*acid stress (Table 5) and strain*NaCl stress (Table 6) and acid stress*NaCl stress (Table 7). Means followed by different letters are different ($p<0.05$). EHEC 1 and 2 refer to the 2 strains of *E. coli* O157:H7 evaluated in this study. NPEC – nonpathogenic *E. coli*

**Table 3.6:** D-values in min for *E. coli* strains in 14% NaCl and 14% NaCl at pH 5.0 acidified with lactic acid or HCl following 4 hr acid stress at pH 5.0 acidified with different acids ($p<0.05$) averaged across acid type.

| | EHEC1 in TSB with 1% glucose | EHEC1 in TSB without glucose | EHEC2 in TSB with 1% glucose | EHEC2 in TSB without glucose | NPEC in TSB with 1% glucose | NPEC in TSB without glucose |
|---|---|---|---|---|---|---|
| **14% NaCl** | 165.8±23.9f | 167.9±17.5 f | 140.4±28.9 g | 128.9±18.1gh | 117.2±36.2hi | 106.3±19.3i |
| **14% NaCl acidified with Lactic Acid** | 283.8±28.9a | 240.3±18.1 c | 268.4±64.7 b | 232.3±67.9c | 277.7±45.9ab | 278.6±42.6ab |
| **14% NaCl acidified with HCl** | 237.2±40.5c | 181.4±37.3 e | 233.5±3.8c | 204.2±20.2d | 232.9±33.1c | 186.4±26.5e |

Values are the means of triplicate replications. There was a 3-way interaction by strain*NaCl stress*acid stress ($p<0.05$), thus results are presented averaged across strain*acid stress (Table 5) and strain*NaCl stress (Table 6) and acid stress*NaCl stress (Table 7). Means followed by different letters are different ($p<0.05$). EHEC 1 and 2 refer to the 2 strains of *E. coli* O157:H7 evaluated in this study. NPEC – nonpathogenic *E. coli*

**Table 3.7:** D-values in min for *E. coli* strains in 14% NaCl and 14% NaCl at pH 5.0 acidified with Lactic acid or HCl following 4 hr acid stress at pH 5.0 acidified with different acids ($p<0.05$) averaged across bacterial strain.

| | **Control** | **Malic Acid** | **Lactic Acid** | **Citric Acid** | **HCl** |
|---|---|---|---|---|---|
| **14% NaCl** | 109.6±23.9i | 128.0±29.1h | 168.4±21.8fg | 157.9±31.4g | 124.9±22.9h |
| **14% NaCl acidified with Lactic Acid** | 299.2±31.0b | 236.6±40.8d | 331.4±55.1a | 248.9±22.9c | 201.7±19.7 |
| **14% NaCl acidified with HCl** | 211.7±28.8e | 209.2±37.5e | 253.1±40.5c | 212.4±30.6e | 176.6±24.4f |

Values are the means of triplicate replications. There was a 3-way interaction for strain*NaCl stress*acid stress ($p<0.05$), thus results are presented averaged across strain*acid stress (Table 5) and strain*NaCl stress (Table 6) and acid stress*NaCl stress (Table 7). Means followed by different letters are different ($p<0.05$). EHEC 1 and 2 refer to the 2 strains of *E. coli* O157:H7 evaluated in this study. NPEC – nonpathogenic *E. coli*

**Table 3.8:** D-values in min for *E. coli* strains in SGF following 4 hr salt stress at 4% NaCl .

| | EHEC1 in TSB with 1% glucose | EHEC1 in TSB without glucose | EHEC2 in TSB with 1% glucose | EHEC2 in TSB without glucose | NPEC in TSB with 1% glucose | NPEC in TSB without glucose |
|---|---|---|---|---|---|---|
| **Control** | 51.9±5.2c | 40.1±2.7d | 39.4±0.8d | 36.9±1.9de | 39.2±4.8de | 24.7±1.9f |
| **4% NaCl** | 47.2±2.1c | 9.6±0.7g | 76.7±4.8b | 33.4±1.3e | 90.2±4.4a | 34.9±1.4de |

Values are the means of triplicate replications. Means followed by different letters are different ($p<0.05$). EHEC 1 and 2 refer to the 2 strains of *E. coli* O157:H7 evaluated in this study. NPEC – nonpathogenic *E. coli*

**Table 3.9:** D-values in min for *E. coli* strains at 56 $^{o}$C following 4 hr salt stress at 4% NaCl.

| | EHEC1 in TSB with 1% glucose | EHEC1 in TSB without glucose | EHEC2 in TSB with 1% glucose | EHEC2 in TSB without glucose | NPEC in TSB with 1% glucose | NPEC in TSB without glucose |
|---|---|---|---|---|---|---|
| **Control** | 12.4±3.5c | 7.9±0.3d | 21.2±2.4a | 15.8±2.3b | 16.3±2.0b | 16.7±0.6b |
| **4% NaCl** | 15.9±1.3b | 9.1±0.8d | 12.6±0.9c | 7.5±0.7d | 12.3±0.6c | 13.0±0.5c |

Values are the means of triplicate replications. Means followed by different letters are different ($p<0.05$). EHEC 1 and 2 refer to the 2 strains of *E. coli* O157:H7 evaluated in this study. NPEC – nonpathogenic *E. coli*

**Table 3.10:** D-values in min for *E. coli* strains in 14% NaCl and 14% NaCl at pH 5.0 acidified with lactic acid or HCl following 4 hr salt stress at 4% NaCl, averaged across strain and NaCl challenge.

| | EHEC1 in TSB with 1% glucose | EHEC1 in TSB without glucose | EHEC2 in TSB with 1% glucose | EHEC2 in TSB without glucose | NPEC in TSB with 1% glucose | NPEC in TSB without glucose |
|---|---|---|---|---|---|---|
| **14% NaCl** | 156.0±28.7j | 146.7±3.4k | 160.7±89.3ij | 130.9±53.1l | 172.9±73.2i | 139.4±36.7kl |
| **14% NaCl acidified with Lactic Acid** | 302.2±53.8 ab | 292.3±28.8 cd | 302.2±12.4 ab | 288.3±51.2 de | 309.0±27.3 a | 299.3±12.9 bc |
| **14% NaCl acidified with HCl** | 280.1±60.9 e | 256.5±13.1 f | 291.2±100.3 cd | 221.0±37.1 h | 259.7±61.5 f | 235.7±35.4g |

Values are the means of triplicate replications. Means followed by different letters are different ($p<0.05$). There was a 3-way interaction for strain*NaCl stress*NaCl challenge ($p<0.05$), thus results are presented averaged across strain*NaCl challenge (Table 10), NaCl stress*NaCl challenge (Table 11), and strain*NaCl stress (Table 12). EHEC 1 and 2 refer to the 2 strains of *E. coli* O157:H7 evaluated in this study. NPEC – nonpathogenic *E. coli*

**Table 3.11:** D-values in min for *E. coli* strains in 14% NaCl and 14% NaCl at pH 5.0 acidified with Lactic acid or HCl following 4 hr salt stress ($p<0.05$) averaged across NaCl stress and NaCl challenge.

| | Control | 4% NaCl |
|---|---|---|
| **14% NaCl** | 109.1±23.9d | 193.1±48.1c |
| **14% NaCl acidified with Lactic Acid** | 298.7±31.0a | 299.0±37.6a |
| **14% NaCl acidified with HCl** | 211.2±28.8b | 303.6±51.4a |

Values are the means of triplicate replications. Means followed by different letters are different ($p<0.05$). There was a 3-way interaction for strain*NaCl stress*NaCl challenge ($p<0.05$), thus results are presented averaged across strain*NaCl challenge (Table 10), NaCl stress*NaCl challenge (Table 11), and strain*NaCl stress (Table 12). Means followed by different letters are different ($p<0.05$). EHEC 1 and 2 refer to the 2 strains of *E. coli* O157:H7 evaluated in this study. NPEC – nonpathogenic *E. coli*

**Table 3.12:** Average D-values in min for *E. coli* strains in 14% NaCl and 14% NaCl at pH 5.0 acidified with lactic acid or HCl following 4 hr NaCl stress ($p<0.05$) averaged across NaCl stress and strain.

| | EHEC1 in TSB with 1% glucose | EHEC1 in TSB without glucose | EHEC2 in TSB with 1% glucose | EHEC2 in TSB without glucose | NPEC in TSB with 1% glucose | NPEC in TSB without glucose |
|---|---|---|---|---|---|---|
| **Control** | 202.8±57.8g | 238.1±75.7d | 202.6±93.8g | 195.1±114.3g | 186.1±86.3h | 213.0±90.1f |
| **4% NaCl** | 289.4±81.1c | 225.5±61.7e | 300.2±42.9b | 231.7±37.6de | 308.1±55.7a | 236.6±52.1d |

Values are the means of triplicate replications. There was a 3-way interaction for strain*NaCl stress*NaCl challenge ($p<0.05$), thus results are presented averaged across strain*NaCl challenge (Table 10), NaCl stress*NaCl challenge (Table 11), and strain*NaCl stress (Table 12). Means followed by different letters are different ($p<0.05$). EHEC 1 and 2 refer to the 2 strains of *E. coli* O157:H7 evaluated in this study. NPEC – nonpathogenic *E. coli*

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