ABSTRACT

KRESKE, AUDREY CHRISTINA. Effects of Atmosphere and Organic Acids on the Survival of *Escherichia coli* O157:H7 Under Conditions Similar to Acidified Foods. (Under the direction of Fred Breidt, Jr.).

The ability of *Escherichia coli* O157:H7 to survive in acidified vegetable products is of concern because of previously documented outbreaks associated with fruit juices. A study was conducted to determine the survival of *E. coli* O157:H7 in organic acids at pH values typical of acidified vegetable products (pH 3.2 and 3.7), under different atmospheres, and a range of ionic strengths (0.086 to 1.14). Determination of internal pH and catalase activity in acid solutions can explain how dissolved oxygen or D- versus L-lactic acid reduces the survival of *Escherichia coli* O157:H7 when the organism is exposed to low pH (3.2) values typical of fermented and acidified foods. All solutions contained 20 mM gluconic acid, which was used as a non-inhibitory low pH buffer to compare the individual acid effect to that of pH alone on the survival of *E. coli* O157:H7. *E. coli* O157:H7 cells challenged in buffered solution with ca. 5 mg/L dissolved oxygen (present in tap water) over a range of ionic strengths at pH 3.2 exhibited a decrease in survival as the ionic strength was increased over 6 h. Overall, under oxygen limiting conditions in an anaerobic chamber, there is no significant difference in the loss of viability of *E. coli* O157:H7 cells regardless of pH, acid type, concentration, or ionic strength.

For lactate concentrations up to 40 mM, there was no significant difference in internal pH values when cells were incubated in D- versus L-lactic acid solutions. Unexpectedly, cells incubated under aerobic conditions maintained a significantly higher internal pH (ca.

5.8) than cells incubated under anaerobic conditions (ca. 5.4), regardless of the isomer of lactic acid used. Overall, catalase activity was higher when cells were in the presence of L-lactic acid versus D-lactic acid however there is a decrease in survival when cells are incubated in L-lactic acid. Under the conditions tested, differences in survival of *E. coli* O157:H7 between isomers of lactic acid and atmospheres is not a result of internal pH or catalase activity. However, the lower internal pH maintained by cells incubated anaerobically (5 mM lactic acid) results in a smaller pH gradient and decreases the accumulation of acid anions inside the cell, which may contribute to increased survival under anaerobic conditions. Many acid and acidified foods are sold in hermetically sealed containers with oxygen limiting conditions. Our results demonstrate that *E. coli* O157:H7 may survive better than previously expected from studies with acid solutions containing dissolved oxygen.

Effects of Organic Acids and Atmosphere on the Survival
of *Escherichia coli* O157:H7 Under Conditions
Similar to Acidified Foods

by
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DEDICATION

To Mommy and Daddy the people who made me who I am.

Through good times and tough times, you never stopped believing in me.

BIOGRAPHY

Audrey Christina Kreske was born and raised in Miami, Fl. Growing up in the South, she spent most of her time in the pool or outside playing sports. Excelling at fast pitch softball throughout middle school and high school, she received an athletic scholarship to attend Lenoir Rhyne College, Hickory, NC. Majoring in biology and minoring in math, Audrey always enjoyed her classes geared towards microbiology. After deciding to attend graduate school for Food Science, she attended the University of Georgia and studied under the direction of Dr. Larry R. Beuchat. A scientist was born during her time in Dr. Beuchat's laboratory and Audrey decided to pursue a Ph.D. at North Carolina State University under the advisement of Dr. Fred Breidt, Jr.

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CHAPTER I

Literature Review

1.1 Introduction

Outbreaks of disease from the consumption of acid foods, such as apple cider and apple juice, have raised concerns about the safety of foods that use low pH and organic acids as the main barriers preventing the growth of pathogenic bacteria (29, 30). The U.S. Code of Federal Regulations (21 CFR part 114) defines acid and acidified foods as having a pH of 4.6 or lower. Acid foods are foods that naturally have a pH below 4.6. Acidified foods reach their equilibrated pH of 4.6 or lower through the addition of acid or acid food ingredients, such as organic acids.

Organic acids such as lactic, acetic, citric, and malic acid, are commonly used or naturally occur in several food products to extend shelf life by inhibiting spoilage microorganisms and pathogens (39). Research on organic acids has focused on bench top experiments testing the effectiveness of organic acids against pathogens (13, 17, 78) in solutions at low pH. Research has shown that *Escherichia coli* O157:H7 survives significantly better in low pH solutions containing organic acids under anaerobic conditions compared to aerobic conditions (78). This increase in survival raises concerns about the ability of organic acids and low pH to protect foods under anaerobic conditions that may become contaminated with *E. coli* O157:H7 (127). Bacteria use oxygen to produce energy but the presence of oxygen gives rise to the production of reactive oxygen species (ROS) which are harmful to the cell (127). There is research on the effects of oxidative stress on bacteria (3, 10, 22, 25-27, 57, 63, 70, 75, 126) however research to determine the effects of oxidative stress in combination with low pH is lacking.

1.2 *Escherichia coli* O157:H7

Escherichia coli are gram negative, facultative anaerobe, nonsporeforming rods that are commonly found among the gut microflora of humans and warm-blooded animals. However, there are strains that are pathogenic and can cause disease (90). Manure from cattle, sheep, goats, and swine are some of the sources of *E. coli* contamination in foods. Zhao *et al.* (136) detected *E. coli* in 38.7% of the chicken samples, 19.0% of the beef samples, 16.3% of the pork samples, and 11.9% of the turkey samples from retail stores in the Greater Washington, D.C. area. There are substantial opportunities for cross contamination of fruits and vegetables and other types of commodities with *E. coli* O157:H7 during growing, processing, and handling (1). The prevalence of *E. coli* on fresh cabbage from upper Midwest farms was 3.7% in 2003 and 7.3% in 2004 (98). In 2002, the prevalence of *E. coli* on fresh cabbage and cucumbers from Minnesota organic (noncertified) farms was 13.3% and 8.3%, respectively (97). *E. coli* was detected in 8.2% of famers' market samples consisting of lettuce, spinach, carrots, and green onions from Alberta, Canada (14). *E. coli* O157:H7 was confirmed in 4 out of 39 market samples of carrots, cucumbers, onions and spinach in South Africa (2). Samadpour *et al.* (118) detected enterohemorrhagic *E. coli* in 6% of sprouts samples and 4% of mushroom samples from retail stores in Seattle, Washington. Outbreaks of *E. coli* O157:H7 have been associated with fresh cheese curds, apple cider and juice, improperly cooked ground beef and patties, alfalfa sprouts, water, dry cured salami, yoghurt, roast beef, raw milk, lettuce, and spinach (3, 24-31, 63, 73, 96, 112, 135).

Pathogenic E. coli are classified into several categories based on a variety of properties, they are as follows: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffuse-adhering *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), and enterohemorrhagic *E. coli* (EHEC) (90). EHEC strains, particularly *E. coli* O157:H7, produce one or two verotoxins also know as Shiga toxins 1 and 2 (Stx1 and Stx2) that cause acute to severe disease in humans. *Shigella dysenteriae* type 1 toxin and Stx 1 are homologous, they differ by only one amino acid in the A polypeptide (128). *S. dysenteriae* type 1 and Stx1 show a ~55% amino acid homology with Stx2, which is known to be 1,000 times more toxic to human renal microvascular endothelial cells than the former (54, 89).

E. coli O157:H7 was first isolated from two outbreaks of hemorrhagic colitis associated with eating ground beef sandwiches in 1982 (135). *E. coli* O157:H7 can be differentiated from other EHEC strains because it typically does not ferment sorbitol, has no β-glucoronidase activity, and is acid-resistant (42). When compared to other coliforms, *E. coli* O157:H7 was not able to grow at 45°C, but grew within a temperature range of 19.3-41°C (36-48 h) in EC medium (107). The main ecological reservoir of this bacterium is the intestines of ruminants such as cattle, sheep, goats, and deer. *E. coli* O157:H7 and other *E. coli* strains are transiently present in young calves and feedlot cattle. These strains can be isolated at higher rates during the summer months than the winter months (61). Cattle are healthy carriers of *E. coli* O157:H7 because they lack the glycolipid receptor in their gastrointestinal tract for the toxin (106).

E. coli O157:H7 can cause mild diarrhea, bloody diarrhea, life-threatening hemolytic uremic syndrome (HUS) in children, and thrombotic thrombocytopenic purpura (TTP) in adults (42, 129). Mead *et al.* (88) reported that in the United States, an estimated 62,458 illnesses, 1,843 hospitalizations, and 52 deaths are caused annually by foodborne infections of *E. coli* O157:H7. From 1982 to 2002, 8,598 cases of *E. coli* O157:H7 infections reported from 49 states in the U.S. resulted in 354 (4.1%) cases of hemolytic uremic syndrome and 40 deaths (109). Hemolytic uremic syndrome is only caused by shiga toxin producing bacteria and symptoms include acute renal failure, thrombocytopenia, and hemolytic anemia. Symptoms develop one week after the onset of diarrhea (89, 129). Children and the elderly are of most concern for development of HUS. In patients infected with *E. coli* O157:H7 under the age of 10 ca. 5-10% will develop HUS and ca. 15% of those that develop HUS will result in fatalities. The infectious dose of *E. coli* O157:H7 is very low, less than 100 CFU (90). Data from outbreaks determined counts in 1993 linked to ground beef patties, to be less than 700 organisms per patty before cooking (53) and in 1994 from salami, to be less than 50 organisms (130). There is currently no good animal model for *E. coli* O157:H7 infection that mimics a human infection (79).

After surviving passage through the acidity of the stomach, EHEC must colonize the intestinal tract to be able to cause disease. The *E. coli* O157:H7 genome contains a pathogenicity island, the locus of enterocyte effacement (LEE), which encodes the proteins needed for the development of the attaching and effacing lesion (54). The pathogenicity island has a G + C content of 38.3%, which is comparably lower than the average G + C

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content of the *E. coli* genome (50.8% G + C) (72). The LEE contains genes coding for a

bacterial membrane protein, intimin, the translocated intimin receptor (Tir), and the *esc* and

sep genes which encode type III secretion systems (72) that secrete the Esp proteins, which

are involved in signal transduction (72). Intimin attaches to intestinal epithelial cells

allowing the bacterium to form attaching and effacing lesions. Attachment is important

because otherwise the bacteria would be flushed out of the intestine by peristaltic flow (79).

The formation of lesions in the intestinal tract results in the loss of microvilli, pedestal

formation, and the accumulation of cytoskeletal proteins at the surface of epithethial cells

(54). The type III secretion system is induced upon contact with host cells and functions to

export proteins directly into eukaryotic cells.

EHEC produce shiga toxins that are holotoxins composed of an enzymatic A subunit

and B subunit, which binds the holotoxin to the globotriaosylceramide receptor (Gb_3) on

epithelial cells. The toxin is taken up by epithelial cells through receptor-mediated

endocytosis and travels to the golgi apparatus and the endoplasmic reticulum, finally entering

the cytosol (54). The A subunit is nicked and reduced to generate A_1 and A_2 peptides. The

A_1 peptide noncovalently binds to the B pentamer which has N-glycosidase activity. This

results in depurination of adenine in the 28S rRNA, thus inhibiting protein synthesis in

eukaryotic cells (89). This infection is primarily localized in the intestine of the patient

without any septicemia developing. Patients develop fatal sequelae (HUS), as a result of the

shiga toxins entering the blood stream (54).

1.3 Acid resistance systems of *E. coli*

The ability of a microorganism to survive under acidic conditions depends on maintaining pH homeostasis. If the organism cannot remove the accumulation of anions the internal pH will continue to drop resulting in cell death (9). Many strains of *E. coli* O157:H7 are acid tolerant with a minimum external pH required for growth from 4.0 to 4.5. Cells can survive in a medium with a pH as low as 1.5-2.0, similar to the gastric environment of the stomach (90, 113). However, Lin *et al*. (80) noted that differences in acid tolerance and resistance systems may occur among *E. coli* strains.

Four acid resistance systems (AR) have been described in *E. coli* as well as *E. coli* O157:H7 (8, 10, 46, 105, 110). These systems contribute to the low infectious dose of *E. coli* O157:H7 because cells can survive passage through the acidic environment of the stomach. AR1 is a low pH inducible system that is expressed during oxidative metabolism. The other three systems are expressed during fermentative metabolism, and require glutamate (AR2), arginine (AR3), or lysine (AR4). AR1 is induced during stationary phase and is glucose repressed. AR1 requires an alternate sigma factor, σ^s (RpoS), cyclic AMP, and the cyclic AMP receptor protein (CRP) for its expression. The amino acid decarboxylase systems consist of a decarboxylase enzyme that consumes an intracellular proton and an antiport which exchanges exogenous amino acids for the end product of the decarboxylase reaction. The AR2 decarboxylase system requires exogenous glutamate to be present and is induced during stationary phase. Glutamate decarboxylase isoenzymes (GadA and GadB) convert glutamate intracellularly into CO_2 and gamma aminobuytric acid (GABA) which is excreted

by the antiporter (GadC). The optimal pH of the glutamate decarboxylases is 3.8 (110).

AR3 is induced by low pH, anaerobic conditions, and the presence of exogenous arginine.

The arginine decarboxylase (AdiA) converts arginine into CO_2 and agmatine while the

antiporter (AdiC) exports agmatine for extracellular arginine (111). The pH optimum of the

arginine decarboxylase is 5.2 (110). In AR4, the lysine decarboxylase (CadA) converts

lysine into CO_2 and cadaverine while the antiporter (CadB) exports cadaverine for

extracellular lysine (99). Of the systems listed above, AR2 system is the most protective,

allowing cells to survive at pH 2 in LB broth for 1 h (8, 23, 110). Lin *et al.* (81)

demonstrated that after the induction of AR systems they remain active during storage in

broth (neutral pH) at 4°C for up to 28 days. Acid-adapted *E. coli* O157:H7 required AR1 to

survive in apple cider (pH 3.5) for 3 days at 25ºC (105). Mutants lacking AR2 or AR3

survived in the apple cider (pH 3.5) for up to 3 days. Bearson *et al.* (10) demonstrated that

both AR2 and AR3 protected *E. coli* O157:H7 from oxidative stress caused by the addition of

hydrogen peroxide when challenged at pH 2.5 for 4 h. The presence of acid resistance

systems protect cells from oxidative stress by raising the internal pH, which from our

research has shown to have a significant effect on catalase activity.

  During stationary phase, strains of *E. coli* O157:H7 were able to achieve ca. 100%

survival in tryptic soy broth (pH 2) at 25°C for 4 h compared to the nonpathogenic control *E.*

coli strain had only 40% log CFU/mL of survivors after the same treatment (6). Strains of *E.*

coli O157:H7 were shown to have increased acid tolerance to pH 3 with the addition of

organic acids after cells were subjected to acid adaptation in tryptic soy broth (pH 5) for 4 h

(32). *E. coli* O157:H7 strains that survived in brain heart infusion broth at pH 2.5 for 7 h

were acid-adapted in tryptic soy broth supplemented with 1% glucose for 18 h (final pH 4.6-

5.2) (20). These data raise concerns about low acid foods or acidified foods with pH values

of 3-6 that may contribute to the adaptation of *E. coli* O157:H7, preparing cells to survive

passage in the stomach.

Arnold and Kaspar (6) demonstrated that four out five *E. coli* O157:H7 strains

survived 3 h of incubation in synthetic gastric fluid (pH 1.5) compared to other *E. coli*

serotypes. Zhao *et al*. (137) demonstrated the ability of *E. coli* O157:H7 to grow and survive

in unpasteurized apple cider (pH < 4) at 8ºC for ca. 7 to 12 days. *E. coli* O157:H7 survived

fermentation, drying and storage at 4ºC for 2 months in dry, fermented sausage with a final

pH of 4.4 (51). *E. coli* O157:H7 survived in cheese brine (pH 5.7) stored at 8 and 15ºC for

28 days (69). *E. coli* O157:H7 survived in Galotyri cheese (pH 3.9) stored at 4 (final pH 4.5)

and 12ºC (final pH 5.8) for up to 28 days. *E. coli* O157:H7 survived in apple, orange,

pineapple, and white grape juice concentrates (pH 3.6-3.7) stored at -23ºC for up to 12 weeks

(101). It is evident that *E. coli* O157:H7 has acid resistance systems that aid in the survival

through the human gut as well as long term storage in food products.

1.4 Organic acid and pH stress

Organic acids are known as weak acids and have been used for many years as food

preservatives in sauces, mayonnaises, dressings, salads, drinks, and fruit juices (115). In

addition to inhibitory effects, microorganisms can use organic acids as carbon sources, to

generate energy (33). Common inhibitory organic acids are lactic, acetic, propionic, sorbic and benzoic acids. Citric, malic, and fumaric acids are used for the flavor they impart to foods, but they also have activity as preservatives (38). Organic acids such as acetic, lactic and citric acids, are used at concentrations of 1.5-2.5% to rinse and decontaminate cattle carcasses after slaughter. These acids are thought to enter the bacterial cell in the uncharged protonated form, which facilitates transport across the non-polar cell membrane (18).

Lactic acid is a carboxylic acid that is colorless, nonvolatile, and is a typical end product of glucose or fructose fermentation by bacteria. The acid has a pK_a of 3.86 and there are two optical isomers, D- and L-lactic acid (122). Research has shown that the two isomers of lactic acid affect cells differently (13, 86, 87). Lactic acid is used in food products to improve taste, quality, and for controlling microbial growth. Products containing lactic acid include cheese curds, unsalted butter, egg whites, beer, bread dough, olives, pickles, and fermented meats. Acetic acid is a carboxylic acid and is known for the pungent smell and sour taste of vinegar. The pK_a of acetic acid is 4.75 (38). This acid is more potent against bacteria and yeasts compared to molds. Acetic acid is used as a preservative in baked goods, cheeses, condiments and relishes, gravies and sauces, and meats. Citric acid is a tricarboxylic acid that has three pK_a, 3.14, 4.77, and 6.39 and is used in soft drinks, mayonnaise, and canned tomatoes. *E. coli* O157:H7 survived at 4 and 10°C for 21 days and grew at 25°C for 56 days in acidified TSBYE (pH 4.0) with 31 mM total citric acid (36). Malic acid is a dicarboxylic acid with two pK_a, 3.40 and 5.11 (41). This acid is

nonhygroscopic and has high water solubility. Malic acid is used for its flavoring and acidification properties in sherbets, ices, fruit preserves, jams, jellies, and beverages (41).

As the pH drops below pK_a for a given organic acid, the protonated form (>50%) will be the dominate form of the acid in solution (9, 15). The uncharged protonated form of organic acids are able to pass freely across cell membranes. Once inside a bacterial cell, the acid anion cannot readily diffuse through the cell membrane and may accumulate to molar amounts in the cell cytoplasm, depending on the difference between the internal and external pH (33, 41). Studies by Eklund (43) demonstrated that protonated sorbic acid was 15 to 600 times more effective in inhibiting the growth of *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* than the anion form. Salmond *et al.* (117) demonstrated a strong correlation between growth inhibition of *E. coli* and the concentration of the protonated form of benzoic acid. Organic acids may act on cells by inhibiting metabolic reactions, disruption of the cell membranes, contributing to the stress of maintaining pH homeostasis, as well as other deleterious effects (18). The antimicrobial activity of organic acids may be affected by several factors such as the pK_a, temperature, specific type of acid, acid concentration, pH, and ionic strength (μ). Other variables that influence the effectiveness of organic acids, include physiological state of the organism, growth phase, redox potential of medium, atmosphere, storage time, and effects of processing.

Ryu *et al.* (116) demonstrated that acetic acid was most effective in inhibiting the growth of *E. coli* O157:H7 followed by lactic acid and least effective was malic and citric

acids in acidified TSB (pH 3.4, 3.9, and 4.5). However, Bjornsdottir *et al.* (13) demonstrated that protonated L-lactic acid was the most lethal acid against *E. coli* O157:H7 strains followed by acetic, malic, and citric acid in a non-inhibitory low pH buffer (pH 3.2). Viable *E. coli* O157:H7 cells were decreased by > 7 log CFU/mL when treated with 100 mM lactic acid (pH 3.8) for 4 h at 37ºC, and the inhibition observed decreased with decreasing temperature (20 and 5ºC) (87). Alakomi *et al.* (4) demonstrated that treatment of *E. coli* O157:H7, *Pseudomonas aeruginosa*, and *Salmonella Typhimurium* with 5 mM lactic acid (pH 4.0 ± 0.1) caused outer membrane permeabilization in addition to lowering the cytoplasmic pH. *E. coli* O157:H7 survived at 4°C and 10°C for 14 days and grew at 25°C for 56 days in acidified TSBYE (pH 4.7) with 33 mM total lactic acid (36). Conner and Kotrola (36) reported that *E. coli* O157:H7 survived and grew in acidified TSBYE (pH 5.2) with 27 mM total acetic acid for 35 days at 4°C and 10°C and for 35 days at 25°C, respectively. Bjornsdottir *et al.*(13) reported an increase in survival when acid-adapted *E. coli* O157:H7 cells were incubated in 5 mM protonated acetic acid (pH 3.2, μ, 0.6, 25ºC) compared to pH 3.2 in the absence of acetic acid. The discrepancies in the literature about which acid is the most effective acid may be a result of a lack of standard methods for determining efficacy. Experimental variables include: pH, culture conditions and growth media, temperature, as well as differences in methods for calculating the acid concentrations (protonated *vs.* total acid concentration).

Bjornsdottir *et al.* (13) observed less than a 1 log CFU/mL reduction in *E. coli* O157:H7 cells treated with D-lactic acid (1-20 mM protonated, 25°C, μ, 0.6) compared to L-

lactic acid at the same concentrations over 6 h. Gravesen *et al.* (52) demonstrated that

Listeria monocytogenes was more sensitive to 100 mM D-lactic acid than L-lactic acid in

brain heart infusion broth at pH 3.7 at 37°C. Benthin and Villadsen (12) reported that L-

lactic acid was more inhibitory to *Lactobacillus delbrueckii* subsp. *bulgaricus* than D-lactic

acid in batch fermentations without pH control (inoculation medium, pH 6.5 with 12 g/L

lactic acid). Depending on the organism, one isomer of lactic acid can be more inhibitory,

usually the isomer the organism produces is less inhibitory. *E. coli* (21) and *L. delbrueckii*

subsp. *bulgaricus* (12) produce D-lactic acid and *L. monocytogenes* (52) produces L-lactic

acid. Further research is needed to determine the different effects of D- and L-lactic acid on

microorganisms.

1.5 Internal pH

 E. coli and other bacteria maintain an internal pH around neutral for optimum growth

(102, 103) which allows cellular processes to occur. If the cell is unable to maintain an

internal pH close to pH 7, cellular enzymatic processes will cease and the cell cannot

metabolize or replicate. Shechter *et al.* (121) demonstrated with fluorescence dyes that 3

strains of *E. coli* maintained an internal pH of ca. 7.5 when the external pH was 5.5-7.5.

Studies by Russell (114) demonstrated that acid tolerant bacteria allowed their intracellular

pH to drop along with the extracellular pH to maintain a small pH gradient which prevented

an accumulation of volatile fatty acid anions within the cell (until the pH was < 5.2). The

internal pH of *Lactobacillus plantarum* decreased to ca. 4.5 as the external pH dropped to 3.0

in modified HHD media (85). The pH gradient of *L. plantarum* (modified HHD, pH 5.0)

decreased with the addition of sodium acetate or lactic acid up to 160 mM. Diez-Gonzalez

and Russell (40) determined that *E. coli* O157:H7 (compared to K-12) decreased internal pH

when growing anaerobically at pH 5.8, which prevented the accumulation of acetate anion

(up to 160 mM sodium acetate) in the cell. *E. coli* O157:H7 achieved a final cytoplasmic pH

of ca. 5.2 when 50 mM protonated lactic acid was added to the incubation media at pH 4.0

(71). The internal pH of *Listeria monocytogenes* was reduced to 6.2 when exposed to D- or

L-lactic acid (7.3 mM, pH 4.2) (52). Gravesen *et al.* (52) concluded that these results

indicate that the two isomers are able to penetrate the membrane identically. There was no

significant difference in the internal pH when stationary phase *E. coli* O157:H7 and non-

O157 cells were exposed to 100 mM D- or L-lactic acid at pH 3.8 for 10 min (86). The

reason for the difference in survival with D- versus L-lactic acid is not currently understood.

Acid stress decreases the survival of pathogens and spoilage organisms in food

products. Research has demonstrated that not all organic acids are similar in their

effectiveness against pathogenic microorganisms. The literature has shown that pathogens

possess acid resistance systems that alleviate acid stress and increase the likelihood of

survival. Microorganisms decrease internal pH as external pH decreases to maintain a small

pH gradient thus preventing accumulation of the acid anion.

1.6 Oxygen stress

Oxygen is used by aerobically growing organisms for respiration or oxidation of nutrients to obtain energy (22). Even though this is the most beneficial way for the cell to produce energy, the presence of oxygen means that the cell can experience oxidative stress. Oxidative stress can be defined as an imbalance in the oxidant/antioxidant equilibrium in favor of the oxidant (123). The oxygen atom has two unpaired electrons with the same spin state (29). Molecular oxygen can be reduced to water by the respiratory chain of bacteria, requiring four electrons and four protons, catalyzed by several membrane-associated respiratory chain enzymes (22). About 1-5% of the time, reactive intermediate by-products are formed (48). These reactive oxygen species (ROS), which are formed by univalent addition of electrons include: superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$) (Figure 1). ROS are able to react with cell components, causing harm to the cell (48). ROS can also be generated by gamma radiation, ozone, redox cycling molecules (such as paraquat and menadione), UV light, and transition metals (126). ROS are produced through NADPH oxidase (respiratory bursts) in activated phagocytic cells to battle infection (59, 76). The cell has several enzymes that detoxify ROS such as superoxide dismutase, catalase, peroxidase, and DNA repair enzymes.

The superoxide anion can function as an oxidant and a reductant. The superoxide anion is in the protonated form, hydroperoxyl radical (HO_2) when the pH is below 4.8 (pK_a), losing its negative charge, thus making it more reactive. The hydroperoxyl radical spontaneously disproportionates at acidic pH to form hydrogen peroxide (11, 47, 60).

Superoxide can be produced from the autoxidation of tetrahydropteridines (100), ferredoxins (92), hemoproteins (93), hydroquinones and leukoflavins (74, 82, 91). Superoxide production in glucose-fed *E. coli* cells has been estimated to be 5 µM/s (66). Xanthine oxidase (84), aldehyde oxidase (108), and flavoprotein dehydrogenases can generate superoxide enzymatically.

Superoxide attacks thiols, tocopherols, and iron-sulfur proteins. It has the ability to reduce transition metals and metal complexes. Superoxide can oxidize dehydratases that contain Fe-S clusters at their active sites, such as dihydroxy acid dehydratase, 6-phosphogluconate dehydratase, aconitase, and fumarases A and B, thus releasing Fe^{2+} (47, 59). This increases the pool of free iron available to form the hydroxyl radical (75). The ferrous iron reduced by the superoxide anion reacts with hydrogen peroxide to produce the highly reactive hydroxyl radical through the Haber-Weiss reaction (Figure 2).

When another electron reacts with superoxide anion, the peroxide ion is formed O_2^{2-} with a pK_a of 11.85. The peroxide ion is immediately protonated to form hydrogen peroxide (H_2O_2). H_2O_2 is able to cross bacterial cell membranes since it is uncharged and form the highly reactive hydroxyl radical through the Haber-Weiss reaction along with superoxide radical (58, 67). The Fenton reactions is the reduction of H_2O_2 by iron(II) to form iron(III), the hydroxyl radical, hydroxyl anion, and water (Figure 3). H_2O_2 is produced enzymatically by SOD, monoamine and D-amino acid oxidases, xanthine oxidase, urate oxidase, glucose oxidase, and lysyl oxidase (58). It will attack thiol groups on proteins and reduced glutathione. H_2O_2 is also known to react nonenzymatically with keto acids such as pyruvate.

Because of its poor reactivity compared to the hydroxyl radical, hydrogen peroxide can diffuse away from the production site and produce the hydroxyl radical in different parts of the cell (60).

Sources of hydrogen peroxide are spontaneous and superoxide-catalzyed dismutation of the superoxide (45). Cells grown on media containing glucose had intracellular levels of H_2O_2 that were ca 10^{-8} M (55, 104). *E. coli* grown on glucose produces ca. 14 μM H_2O_2 every second (120). The cytotoxic effects of H_2O_2 falls into two categories: mode one killing (< 2.5 mM) results in DNA damage and requires active metabolism and mode two killing ($>$ 12.5 mM) causes uncharacterized damage in the absence of metabolism (68). Brandi *et al.* (16) demonstrated that *E. coli* cells exposed to > 10 mM H_2O_2 were killed as a result of the production of the hydroxyl radical through the reaction of H_2O_2 and iron. Hydrogen peroxide has been used in the food industry as a sterilizing agent for aseptic packaging (131). In foods, hydrogen peroxide is used, as a food grade antimicrobial agent in milk intended for use in cheese making (up to ca. 15 mM) (77), in modified whey (up to ca. 15 mM), corn starch (up to ca. 45 mM) and dried eggs, and as an disinfectant for fruits and vegetables (44).

The hydroxyl radical is the most reactive of the ROS species because it can directly react with biomolecules (65). It can be produced by the reaction of H_2O_2 with metal ions or by UV-induced homolytic fission of the O-O bond in H_2O_2 (58). This radical reacts with any molecule in the cell at diffusion limited rates (124). The location of its formation will influence the molecules it will oxidize, typically within a few nanometers. The pK_a of this molecule is similar to that of hydrogen peroxide at 11.85 (37). At 25°C, the standard

electrode potential is 1.83 V lending to its strong oxidizing power. The dissociation of water

molecules by radiation is a source of the hydroxyl radical (64). An accumulation of the

hydroxyl radical can cause DNA and protein damage, lipid peroxidation, and disulfide bond

formation (22, 127). DNA damage such as cleavage of backbone and formation of lesions

will block replication (5, 65).

Oxygen in the environment creates a selective pressure on microorganisms to have

proper defenses against the reactive oxygen species (94). Bacterial cells produce enzymes

that include superoxide dismutase (SOD), catalase, and peroxidase, which prevent damage to

DNA, lipids, proteins, and cell membranes (126).

Cells respond to the presence of superoxide by turning on the *SoxRS* regulon, which

is a family of genes that is responsible for detoxifying superoxide and containing cell

damage. The SoxR protein is an iron-sulfur redox sensor. When oxidized, SoxR

transcriptionally activates the *soxS* gene (50). The SoxS protein in turn activates the genes

under control of the regulon, including Mn-containing SOD (*sodA*), DNA repair enzyme

endonuclease IV (*nfo*), glucose 6-P dehydrogenase (*zwf*), fumarase (*fumC*), aconitase (*acnA*),

ferredoxin reductase, and MicF regulatory RNA, which post-transcriptionally controls

expression of the outer membrane porin gene (*ompF*) (22, 127). When *E. coli* cells are

exposed to peroxide stress they respond by expressing ca. 30 proteins. A subset of these

proteins are controlled by an oxidant sensitive regulator called OxyR. OxyR expresses nine

proteins that detoxify peroxides (57). An altered redox state of the cytosol, results in a

oxidized OxyR (formation of an intramolecular disulfide bond between cysteine residues 199

and 208) which is then able to activate transcription (7). The redox potential of OxyR is -185

mV (138) compared to that of the cell which is ca. 260-280 mV. Thus under normal

conditions OxyR is reduced and does not initiate transcription. OxyR is autoregulated

because it activates transcription of its own reduction pathway (138). OxyR regulates *katG*

(HPI, functions as a catalase and as a peroxidase) (35), NADPH-dependent alkyl

hydroperoxidase (*aphCF*), fur (which controls iron uptake), glutathione reductase (*gorA*),

glutaredoxin 1 (*grxA*) and a protective DNA binding protein (Dps) (57, 132). There is no

overlap in protection between SoxRS and OxyR, these systems are apparently totally

separate.

There are three SODs known to exist in *E. coli*, Mn-containing SOD (*sodA*), Fe-

containing SOD (*sodB*), and a Cu-Zn containing SOD (*sodC*) (47). They react with the

superoxide anion, producing hydrogen peroxide (Figure 4). The rate constant for this

reaction is $k_2 \approx 2\text{x}10^9$ mol $^{-1}$ s^{-1} (47). Copper is the active transition metal in the Cu-Zn SOD

while the Zn helps stabilize the enzyme (47, 56). The Cu-Zn SOD is found in the

perisplamic space, and is inhibited non-specifically by cyanide and by

diethyldithiocarbamate (DDTC) which binds to copper and removes it from active sites (56).

Mn SOD is not usually produced during anaerobic growth it is induced under aerobic growth

and it is controlled by the *SoxRS* regulon (47). Mn SOD is more labile to denaturation by

heat, organic solvents or detergents than Cu-Zn SOD, and is not inhibited by cyanide or

DDTC (56). The Mn SOD and Fe SOD are located in the cell cytoplasm (49) and both show

decreased activity at high pH (56). Cu-Zn SOD and Fe SOD are inactivated by prolonged

exposure to H_2O_2 but Mn SOD is not affected (56). The Fe SOD is constitutively expressed in *E. coli*, and is active during the transition of cells from an anaerobic to an aerobic environment (47).

There are two chromosomally encoded catalases, hydroperoxidase I (HPI) encoded by *katG* and hydroperoxidase II (HPII) encoded by *katE*. These enzymes break hydrogen peroxide down into water and oxygen (Figure 5). Another catalase, *katP* is encoded on plasmid pO157. *katP* has catalase-peroxidase activity and is located in the periplasmic space (19). *katG* (HPI) is regulated in response to peroxide stress by the OxyR regulon. *katE* (HPII) is induced during transition into stationary phase by RpoS (134). *E. coli* B *katG* (HPI) catalase has a pH optimum of 7.5 (35). *katG* (HPI) is located in the periplasm, and is associated with the cytoplasmic membrane, and *katE* (HPII) is located in the cytosol (62). *katP* has similar catalase activity compared to *katG*, and its maximal activity was observed at pH 7.2 (19). Moore *et al.* (95) determined the optimum activity of *E. coli* catalase (*katG)* to be at pH 6.5. Singh *et al.* (125) demonstrated the pH optimum for catalase activity (*katG*) of *Archaeoglobus fulgidus, Bacillus stearothermophilus*, *Burkholderia pseudomallei*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Rhodobacter capsulatus* and *Synechocystis* PCC 6803 was between 6 and 6.5. Varnado *et al*. (133) determined the optimum catalase activity for the *katP* enzyme of *E. coli* O157:H7 to pH 7.2 and cited a sharp pH dependence. Claiborne and Fridovich (35) reported a decline in *E. coli katG* (HPI) activity when the pH was < 6 for *Escherichia coli* B.

Peroxidases are enzymes that use electron donors to reduce hydrogen peroxide to water. *E. coli* makes NADPH peroxidase (alkyl hydroperoxide reductase, Ahp), which is encoded by *ahpCF*. This enzyme reduces organic hydroperoxides (Figure 6), and is under the control of the OxyR regulatory system. Seaver and Imlay (119) demonstrated that for low concentrations (≤ 20 μM) of hydrogen peroxide the main detoxifying enzyme is Ahp. Catalase is more efficient in neutralizing millimolar concentrations of peroxides than peroxidase, because it does not require reductants such as NADH (119).

Oxygen is necessary for aerobic life. The presence of oxygen may result in the production of reactive oxygen species which leads to oxidative stress. The cell has developed defense mechanisms such as detoxifying enzymes and DNA repair enzymes to deal with the reactive oxygen species. Research has shown that cross protection can occur when microorganisms are exposed to certain stresses, Christman *et al.* (34) demonstrated that *Salmonella typhimurium* develops thermal resistance when exposed to oxidative stress. Maurer *et al.* (83) demonstrated that *Escherichia coli* K-12 induces genes in response to oxidative stress in an acidic pH environment and repressed these genes in a basic environment. They deduced that low pH may amplify the toxicity of oxygen radicals.

1.7 Proposed research

The ability of pathogens to survive in low pH fruit juices (pH 3-4) which naturally contain organic acids and cause infection (29, 30) has raised questions about the safety of acidified products that contain organic acids such as pickles, banana peppers, and olives.

These products are hermetically sealed resulting in an anaerobic environment. Results from previous research carried out with oxygen containing bench-top solutions may not be representative of an anaerobic environment. Aqueous solutions exposed to the atmosphere contain ca. 5 mg/L of dissolved oxygen, and therefore ROS can be present.

A study was conducted to determine the survival of *E. coli* O157:H7 in organic acids at pH values typical of acidified vegetable products (pH 3.2 and 3.7) under different dissolved oxygen conditions (≤ 0.05 and 5 mg/L) and a range of ionic strengths (0.086 to 1.14). The objectives were to: 1) examine the effects of pH, acid type, ionic strength, and dissolved oxygen on the survival of *E. coli* O157:H7 cells, 2) examine the effects of low pH, lactic acid isomer and dissolved oxygen on the internal pH and catalase activity of *E. coli* O157:H7 cells. Understanding how oxygen radicals and D-lactic, affect the survival of *E. coli* O157:H7 in the low pH products may aid in the development of safer acidified foods.

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$$O_2 \xrightarrow{e^-} \bullet O_2^- \xrightarrow{e^- + 2H^+} H_2O_2 \xrightarrow{e^- + H^+} \bullet OH \xrightarrow{e^- + H^+} H_2O$$

$$H_2O_2 \xrightarrow{Catalase} O_2 + 2H_2O$$

$$\bullet O_2^- \xrightarrow[2H^+]{SOD} O_2 + H_2O_2$$

$$RH_2 \xrightarrow{Peroxidase} R + 2H_2O$$
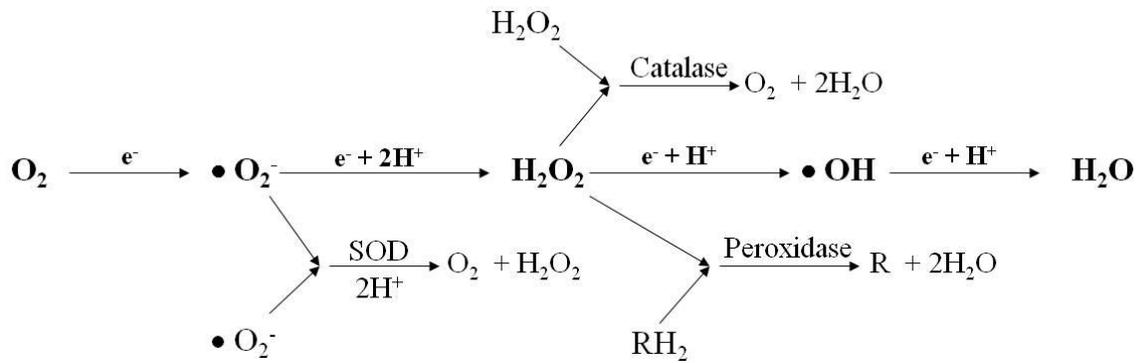
Figure 1. Univalent reduction of oxygen and detoxifying enzymes
Superoxide ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), and superoxide dismutase (SOD). Adapted from (48, 65)

$$\bullet \, O_2^- \; + \; H_2O_2 \quad \xrightarrow{\;\;Fe\;\;} \quad O_2 + \; \bullet OH + OH\text{-}$$
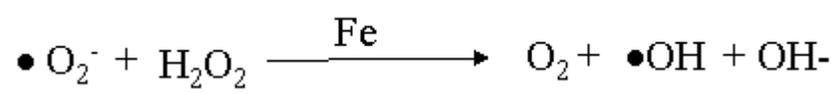
Figure 2. Haber-Weiss reaction

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + \bullet OH + OH\text{-}$$

Figure 3. Fenton reaction

Net reaction

$$\bullet\, O_2^- + \bullet\, O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$
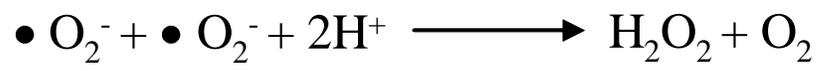
Figure 4. Superoxide dismutase

$$2H_2O_2 \longrightarrow 2H_2O + O_2$$

Figure 5. Catalase

$$H_2O_2 + RH_2 \longrightarrow 2H_2O + O_2$$
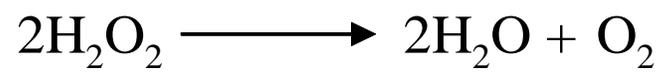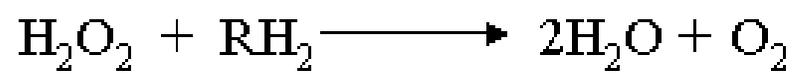
Figure 6. Peroxidase

CHAPTER II

Escherichia coli **O157:H7 acid resistance**

2.1 Abstract

The ability of microorganisms to survive in low pH and in the presence of organic acids is of significance to the food industry. Foods have been preserved throughout history by the lowering of pH and the production of lactic acid, resulting from fermentation by lactic acid bacteria. The objectives of this study were to 1) compare *E. coli* O157:H7 B202 to a cocktail of acid-adapted *E. coli* O157:H7 strains, 2) examine the effects of growth media and atmosphere on the development of acid-adapted *E. coli* O157:H7 B202 cells, 3) determine if the addition of an iron chelator would increase the survival of *E. coli* O157:H7 under aerobic conditions, 4) determine cell survival when an *E. coli* K-12 OxyR mutant is acid challenged under aerobic conditions. We have demonstrated that *E. coli* O157:H7 B202 grown in TSBG for 18 h at 30°C reduces the pH to 4.9 and predominantly produces acetic and lactic acid thus becoming acid-adapted. For the acid solutions tested, the growth atmosphere does not effect the reduction of viable cells during acid challenge in a specified atmosphere. *E. coli* O157:H7 B202 behaves similarly to a five-strain *E. coli* O157:H7 cocktail in acid solutions. The OxyR mutant, which regulates catalase production and other oxidative stress responses, and addition of 2,2'-bipyridyl which chelates iron that contributes to oxidative stress, did not alter survival of *E. coli* strains in the presence of acid solutions under aerobic or anaerobic conditions. The methodology developed in this study will aid in further research to determine the combined effects of organic acids and oxygen on *E. coli* O157:H7 under acid conditions.

2.2 Introduction

The ability of microorganisms to survive in low pH and in the presence of organic acids is of significance to the food industry. Foods have been preserved throughout history by the lowering of pH and the production of lactic acid, resulting from fermentation by lactic acid bacteria. Acid and acidified fruits and vegetables have consistently been found to be safe, because of their low pH (commonly pH 3-4) and the presence of organic acids. During the 1990s a series of outbreaks of disease were associated with apple juice and cider as well as orange juice (5, 6, 8). This raised concerns about the safety of foods preserved by low pH and the presence of organic acids.

Previous literature has shown that cells exposed to moderate pH (5) will develop acid resistance to low pH values (around pH 2) typical of stomach acid. Buchanan and Edelson (4) demonstrated that *Escherichia coli* cells develop acid resistance after growth in tryptic soy broth supplemented with glucose (final pH 5). *E. coli* O157:H7 survives significantly better in lactic acid solutions at pH 3.2 under anaerobic conditions at 30°C compared to aerobic conditions (with dissolved oxygen present) (1). The presence of oxygen in solution results in the formation of reactive oxygen species that will attack cellular components. Oxygen detoxifying enzymes are present in bacterial cells and protect cells from this oxidative imbalance. Free iron and hydrogen peroxide in bacterial cells will react to form the hydroxyl radical through the Fenton reaction. Experimentally, 2,2-bipyridyl can be added to remove iron from solutions to supress the Fenton reaction (14).

The objectives of this study were to 1) compare *E. coli* O157:H7 B202 to a cocktail of *E. coli* O157:H7 strains, 2) examine the effects of growth media and atmosphere on the development of acid-adapted *E. coli* O157:H7 B202 cells, 3) determine if the addition of an iron chelator would increase the survival of *E. coli* O157:H7 under aerobic conditions and, 4) determine cell survival when an *E. coli* K-12 OxyR mutant is acid challenged under aerobic conditions.

2.3 Materials and Methods

2.3.1 Bacterial strains

The following O157:H7 strains were used: *E. coli* B200 (ATCC 43888, human feces isolate), *E. coli* B201 (SRCC 1675, apple cider outbreak isolate), *E. coli* B202 (SRCC 1486, salami outbreak isolate), *E. coli* B203 (SRCC 206, ground beef isolate), and *E. coli* B204 (SRCC 1941, pork isolate). *E. coli* K-12 and an OxyR mutant (GS09, oxyR::kan mutant) were kindly provided by Dr. Gisela Storz (National Institutes of Health).

2.3.2 Preparation of inocula

Stock cultures of *E. coli* O157:H7 strains were stored in tryptic soy broth (TSBG, BBL/Difco, Sparks, MD) supplemented with 1% glucose (Sigma Chemical Co., St. Louis, MO) and 16% glycerol at -80°C. Each strain was prepared by loop inoculum (ca. 10 μl) onto tryptic soy agar (TSAG, BBL/Difco) supplemented with 1% glucose and incubated for 24 h

at 30ºC (to be consistent with the temperature of acid treatments). Three colonies of each

strain were then transferred into 10 mL of TSBG and incubated statically for 18 h at 30ºC to

induce acid resistance as previously described (4). Stock cultures of *Escherichia coli* K-12

and the OxyR mutant were grown in TSBG for 18 h at 30°C. For GSO9, the TSBG was

supplemented with 10 mM sodium pyruvate (TSBP) for 18 h at 30°C. Sodium pyruvate is

required for the OxyR mutant to be able to grow in the presence of oxygen.

2.3.3 Preparation of acid solutions

Calculations for pH, ionic strength, and protonated acid concentrations in the buffered

acid solutions were made using custom Matlab routines (pH$_{tools}$, available at:

http://www.mathworks.com/matlabcentral) as previously described (11). This program also

allows for the adjustment of pK$_a$ values based on ionic strength and temperature. All

solutions contained gluconic acid (Sigma) which was used as a non-inhibitory low pH buffer

(20 mM) to determine the inhibitory effects of pH 3.2 and 3.7 in the absence of added

organic acids (3). Solutions containing only 20 mM gluconic acid (buffered solution) were

used as the controls. Solutions at pH 3.2 were made up containing a range of 2,2'-bipyridyl

(Sigma) concentrations (0.05, 0.1 and 0.2 mM) to determine the optimum concentration. D-

and L-lactic and acetic acid (Sigma) were added to the buffered solution at concentrations 5

and 40 mM. The acid solutions were adjusted using HCl/NaOH to reach the desired pH and

NaCl was added, as needed, to produce the indicated ionic strength (μ). Solutions incubated

in the anaerobic chamber (COY, Grass lake, MI) were allowed to equilibrate for at least 48 h

prior to use. The mixed anaerobic gas atmosphere consisted of 5% CO_2, 10% H_2, and 85% N_2. The dissolved oxygen content of solutions was measured using the CellOx 325 dissolved oxygen sensor (WTW, Weilheim, Germany). The lower limit of detection with this instrument was ca. 0.05 mg/L.

2.3.4 Biochemical analysis

E. coli B202 (0.1% inoculum) were grown in 100 mL of tryptic soy broth without dextrose (TSBD) and TSBG for 18 h at 30°C under aerobic and anaerobic conditions Cultures referred to as GS (grown statically) are cells grown in TSBG statically for 18 at 30°C; cultures referred to as GSA (grown statically in the anaerobic chamber) are cells grown in TSBG statically in the anaerobic chamber for 18 h at 30°C. At 2 h intervals, 1 mL samples were removed and analyzed to determine the absorbance at 600 nm and the pH.

E. coli O157:H7 cells were grown in 25 mL of TSBG for 18 h at 30°C under aerobic and anaerobic conditions. After 18 h, 2 mL were removed and serially diluted for high-performance liquid chromatography (HPLC). Organic acids were measured using a Thermo Separation Products HPLC system (ThermoQuest, Inc., San Jose, CA) consisting of a P1000 pump, SCM100 solvent degasser, AS3000 autosampler, and UV6000 diode array detector (ThermoQuest). A Bio-Rad HPX-87H column, 300 mm by 7.8 mm (Bio-Rad Laboratories, Hercules, CA) was used to resolve acids and sugars. The HPLC operating conditions were: sample tray at 6°C, column at 65°C, and 0.03 N H_2SO_4 eluent at 0.9 mL/min flow rate. The UV6000 detector was set to 210 nm at a rate of 1 Hz for data collection. ChromQuest

(version 4.1) chromatography software was used to analyze the data. The peak heights were used for quantitative integration.

2.3.5 Growth on gluconic acid as primary carbohydrate source

E. coli B202 was grown in 10 mL of M9 minimal salts media prepared with glucose (pH 7.0 $\pm$ 0.1, M9G, BBL/Difco) for 24 h at 30°C. To determine growth on gluconic acid, M9 media (without glucose, M9) was prepared containing ca. 20 mM gluconic acid as well as adding incremental amounts of glucose ranging from 1-5 mM. M9 solutions were prepared with gluconic acid as the sole carbohydrate source (5 and 20 mM) at pH 7.0 and pH 3.2. Samples of cultures were taken at 12 and 24 h to determine pH, absorbance$_{600}$, and log CFU/mL by plate count on TSAG.

2.3.6 Comparison of *E. coli* O157:H7 five-strain cocktail to strain B202

The following strains were used to make the five-strain cocktail: *E. coli* B200 (strain O157:H7, ATCC 43888, human feces isolate), *E. coli* B201 (strain O157:H7, SRCC 1675, apple cider outbreak isolate), *E. coli* B202 (strain O157:H7, SRCC 1486, salami outbreak isolate), *E. coli* B203 (strain O157:H7, SRCC 206, ground beef isolate), *E. coli* B204 (strain O157:H7, SRCC 1941, pork isolate). After 18 h, cultures were harvested separately by centrifugation (3,000 X *g*, 10 min, 20 $\pm$ 2°C), washed, and resuspended in 0.85% saline solution. Suspensions of *E. coli* O157:H7 strains were combined to give equal populations of each strain. The five-strain mixture of cells was serially diluted in sterile 0.85% saline, and

plated in duplicate on TSAG using a spiral plater (Spiral Biotech). Plates were incubated for 24 h at 30°C to determine populations.

The five-strain cocktail (100 μl) or strain B202 by itself was added to acid solutions (900 μl, 30ºC) with the specified atmosphere and ionic strength, mixed thoroughly, and incubated statically for 6 h at 30°C. After incubation, 100 μl aliquots of the test suspension were diluted in 900 μl of 50 mM MOPS buffer (Sigma) and vortexed to facilitate neutralization. Suspensions were serially diluted in 450 μl saline solutions and plated in duplicate on TSAG as described above. The plates were incubated at 30ºC for 24 h to determine the reduction in viable cells. The lower limit of detection for bacterial cells was ca. 4×10^3 CFU/mL.

2.3.7 Atmosphere during growth versus atmosphere during challenge

E. coli O157:H7 cells were grown in 25 mL of TSBG for 18 h at 30°C under aerobic (bench top) or anaerobic atmosphere (equilibrated in the anaerobic chamber). Cultures were centrifuged (3,000 X *g*, 10 min, 20 ± 2°C), washed, and resuspended in 0.85% saline solution. Cell concentrations were determined as described above. The inoculum (100 μl) was added to acid solutions (900 μl, 30ºC) at the specified atmosphere, mixed thoroughly and incubated statically for 6 h at 30°C. After incubation, samples were neutralized, serially diluted and plated on TSAG.

2.3.8 Statistical analysis

Experiments were independently replicated three times. Log reduction was calculated as the difference between the initial count and the final count ($\log_{10}$ [N_0/N]). Log numbers were analyzed using the general linear models with Tukey adjustment of the Statistical Analysis Systems version 9.1 (Statistical Analysis System, SAS Institute, Cary, N.C.). Results were considered significant when the P-value was $P \leq 0.05$.

2.4 Results

2.4.1. Acid adaptation of *E. coli* O157:H7

Growth of *E. coli* O157:H7 in TSBG and TSBD for 18 h at 30°C to determine if the cells acidify the media to induce acid adaptation as shown in Figure 7, 8, and 9. The pH for TSBG and TSBD at 0 h was 6.5 $\pm$ 0.05 and 7.1 $\pm$ 0.1, respectively. Regardless of the atmosphere, *E. coli* O157:H7 cells grown in TSBG reached a final pH of 4.9 and a final absorbance$_{600}$ of ca. 0.6 in 18 h (Figure 7 & 8). Cells grown in TSBD under aerobic and anaerobic conditions reached a final pH of 6.7 and 6.6 and a final absorbance$_{600}$ of 0.49 and 0.45 respectively (data not shown). The anaerobic culture has a significantly lower pH than the aerobic culture between 4 and 12 h (Figure 9).

2.4.2 Biochemical analysis of spent media

Samples of spent TSBG media after 18 h were analyzed on the HPLC to determine the change composition (Figure 10). At time 0 h, TSBG contained glucose at 49.09 $\pm$ 2.66 mM, succinic acid at 2.29 $\pm$ 0.32 mM, and acetic acid at 13.06 $\pm$ 1.92 mM. The spent TSBG of aerobic cultures contained less glucose than the spent TSBG from anaerobic cultures (Figure 10). The amount of succinic acid in the spent TSBG was 1.80 $\pm$ 0.37 and 1.46 $\pm$ 0.42 for aerobic and anaerobic cultures, respectively. Under aerobic and anaerobic conditions, the spent TSBG contained lactic acid at 27.06 $\pm$ 3.41 and 22.87 $\pm$ 9.30, respectively. Spent media from *E. coli* O157:H7 cells grown under aerobic and anaerobic conditions produced variable amounts of ethanol.

2.4.3 Growth on gluconic acid as primary carbohydrate source

To determine if gluconic acid (which was used as a buffer in acid solutions) was able to be utilized as a carbon source, growth of *E. coli* O157:H7 in M9 minimal salts media supplemented with gluconic acid was determined at 30°C for 24 h. The results are shown in Table 1 and Figure 11. *E. coli* O157:H7 reached a population of 8.09 $\pm$ 0.59 log CFU/mL after 12 h of growth in M9 media with 20 mM gluconic acid compared to 7.78 $\pm$ 0.08 log CFU/mL in M9G (Table 1). After 24 h, *E. coli* O157:H7 cells grown in M9 media with 20 mM gluconic acid reached populations of 8.67 $\pm$ 0.04 and 8.71 $\pm$ 0.02 in anaerobic and aerobic conditions, respectively. *E. coli* O157:H7 grown in M9 media supplemented with 5 mM and 20 mM gluconic acid at pH 7 for 24 h reached populations of 8.26 $\pm$ 0.21 and 8.66 $\pm$

0.03 log CFU/mL, respectively (Figure 11). *E. coli* O157:H7 survived but no growth was

observed at pH 3.2 with 5 mM and 20 mM gluconic acid present (Figure 11).

2.4.4 Comparison of *E. coli* O157:H7 five-strain cocktail to strain B202

Log reduction of *E. coli* O157:H7 cocktail versus *E. coli* O157:H7 B202 cells

recovered from various solutions at pH 3.2 and 3.7 are shown in Figures 12 and 13. Under

the conditions tested, *E. coli* B202 and the five-strain cocktail survived similarly when tested

in acid solutions. Under aerobic conditions, there was a 2.79 ± 0.26 log CFU/mL reduction

in B202 cells treated with an ionic strength (μ) of 1.14 at pH 3.2 compared to a 3.92 ± 0.27

log CFU/mL reduction in the five-strain cocktail. There was no significant difference ($P >$

0.05) between *E. coli* B202 and the five-strain cocktail when treated with 5 mM L-lactic acid

at pH 3.2 (μ, 0.342), regardless of atmosphere. Under anaerobic conditions, *E. coli* B202

survived significantly better ($P \leq 0.05$) than the five-strain cocktail when treated with 40 mM

protonated acetic acid at pH 3.2 (μ, 0.342).

2.4.5 Atmosphere during growth versus atmosphere during challenge

Acid challenge of *E. coli* O157:H7 under aerobic and anaerobic conditions when

cultures are grown in different atmospheres is shown in Figure 14. There is no significant

difference ($P > 0.05$) in the reduction of *E. coli* O157:H7 cells grown under aerobic versus

anaerobic conditions and treated with acid solutions under aerobic conditions (Figure 14).

Regardless of atmosphere of growth or acid solution, there was no significant difference ($P >$

0.05) in the reduction of viable *E. coli* O157:H7 cells when challenged in acid solutions under an anaerobic atmosphere. There was a protective effect observed when *E. coli* O157:H7 cells are grown under aerobic or anaerobic conditions and challenged in 5mM protonated D- or L-lactic acid compared to 20 mM gluconic acid under aerobic conditions, as observed previously (2).

2.4.6 Addition of 2,2'-bipyridyl to acid solutions

Because free iron can facilitate the Fenton reaction, the log reduction of *E. coli* O157:H7 B202 cells challenged in acid solutions with the addition of 2,2'-bipyridyl, an iron chelator, is shown in Figures 14 and 16. There was no significant difference ($P > 0.05$) in the reduction of viable cells of *E. coli* O157:H7 in 20 mM gluconic acid at pH 3.2 with the addition of 0.05, 0.1, and 0.2 mM 2,2'-bipyridyl (Figure 15). *E. coli* O157:H7 cells challenged under anaerobic conditions were significantly reduced ($P \leq 0.05$) in the number of survivors compared to cells challenged under aerobic conditions in 0.05 and 0.01 mM 2,2'-bipyridyl (20 mM gluconic acid at pH 3.2). The addition of 0.2 mM 2,2'-bipyridyl to D- and L-lactic acid solutions did not have a significant effect ($P > 0.05$) on the reduction of viable *E. coli* O157:H7 cells (Figure 16). Regardless of acid solution, there was no significant difference ($P > 0.05$) in the reduction of *E. coli* O157:H7 cells in the presence of 0.2 mM 2,2'-bipyridyl under anaerobic conditions.

2.4.7 Survival of *E. coli* K-12 OxyR mutant in acid solutions

The log reduction of *E. coli* K-12 parent and the OxyR mutant (defective in the response to peroxide stress) in acid solutions under aerobic conditions at pH 3.2 is shown in Figure 17. Unexpectedly, *E. coli* K-12 cells challenged in 20 mM gluconic acid were reduced by 3.02 log CFU/mL compared to 1.21 log CFU/mL reduction for the OxyR mutant (Figure 17). *E. coli* K-12 and the OxyR mutant were not significantly different ($P > 0.05$) in reduction of viable cells when treated with L-lactic (5 and 40 mM) at pH 3.2. There was a 3.05 ± 0.46 log CFU/mL reduction in *E. coli* K-12 cells treated with 40 mM acetic acid at pH 3.2 compared to a 1.84 ± 0.02 log CFU/mL reduction in the OxyR mutant.

2.5 Discussion

2.5.1 Acid adaptation of *E. coli* O157:H7

Buchanan and Edelson (4) described a method for producing acid-adapted *E. coli* O157:H7 cells by growing cells in the presence of glucose which is fermented, resulting in the production of acid and a decrease in pH. Strains of enterohemorrhagic *E. coli* were not as resistant to acid challenge (pH 2 or 3 for up to 7 h) when the culture medium did not contain glucose (4). Our results show that as *E. coli* B202 enters into log phase, for statically grown cultures, there is a gradual reduction in pH until it reaches pH 4.9. Ryu *et al.* (15) demonstrated that when *E. coli* O157:H7 cells were grown in TSB (pH 7.0 ± 0.1) and TSBG (pH 6.9 ± 0.1) for 18 h the pH of the medium was reduced to pH 6.2 ± 0.2 and 4.9 ± 0.2,

respectively. The presence of glucose in the culture medium as a fermentable carbohydrate source induces acid tolerance in *E. coli* O157:H7 B202 as a result of the gradual decrease in pH and the production of acids.

2.5.2 Biochemical analysis of spent media

Determining the amount of exposure to acid preceding acid challenge is of importance to understanding acid resistance of *E. coli* O157:H7. Predominantly acetic and lactic acids were produced by the *E. coli* O157:H7 when grown in TSBG. This analysis shows that *E. coli* O157:H7 will be gradually exposed to levels of acetic and lactic acid during growth that allow for adaptation to these organic acids. Diez-Gonzalez *et al*. (9) reported that when *E. coli* O157:H7 strains isolated from cattle were grown in broth containing 10 mg/mL glucose, the final pH was 4.8 and acetic acid accumulated in the medium. Diez-Gonzalez *et al*. (9) showed that starch fermented in the colon of cattle fed $\geq$ 80% grain reduces the pH of the colon to 5.9 $\pm$ 0.6 and increases the concentration of volatile fatty acids (acetic, propionic, and butyric) to $\geq$ 60 mM, and subsequently increases the log cells/gram of acid-resistant *E. coli* recovered (Luria-Bertani broth, pH 2.0, 1 h). *E. coli* O157:H7 incubated anaerobically in a basal medium supplemented with glucose (ranging 10-50 mM) decreased the pH and accumulated acetic and lactic acid in the media resulting in increased acid resistance compared to cultures incubated with <10 mM glucose (10). There was a slight increase in the amount of succinic acid, regardless of atmosphere. *E. coli* O157:H7 cells grown under anaerobic conditions produced more ethanol compared to the

aerobic culture, but this was variable among replicates. Under anaerobic conditions, *E. coli* will produce acetic acid, ethanol, lactic acid, formic acid and small amounts of succinic acid (7).

2.5.3 Growth on gluconic acid as primary carbohydrate source

In our system, 20 mM gluconic acid was used as a non-inhibitory buffer as described in (3). Because of this reason, the ability of *E. coli* O157:H7 to use gluconic acid as a sole carbon source is of interest. Our data shows the utilization of gluconic acid by *E. coli* O157:H7 when grown in M9G (20 mM gluconic acid) at pH 7 (Table 1). *E. coli* O157:H7 was able to grow in M9 media with gluconic acid (5 and 20 mM) at pH 7.0 but there was no growth detected at pH 3.2. Breidt *et al*. (3) reported no change in gluconic acid concentration during incubation of *E. coli* O157:H7 in 2, 20, 200 mM gluconic acid at pH 3.1 (μ, 0.37). *E. coli* O157:H7 B202 is able to grow at pH 7 but only survive at pH 3.2 in M9G confirming the use of gluconic acid a non-inhibitory buffer unable to be used by the cell.

2.5.4 Comparison of *E. coli* O157:H7 five-strain cocktail to strain B202

The *E. coli* O157:H7 strains tested were from a variety of sources: human feces (B200), apple cider outbreak (B201), salami outbreak (B202), ground beef (B203), and pork (B204). Bjornsdottir (1) demonstrated that *E. coli* B202 was the most resistant of the strains listed above to acid challenge at 5 mM protonated D-lactic acid at pH 3.2 (ca. μ, 0.68, 25°C). Our results show that B202 survived similarly compared to the five-strain cocktail in the acid

solutions tested. The acid resistance of *E. coli* O157:H7 B202 is a good representative of the group of O157:H7 strains under our conditions.

2.5.5 Atmosphere during growth versus atmosphere during challenge

To determine whether the culture conditions (aerobic versus anaerobic) can affect the resistance of the bacterium during the acid challenge, we tested cells under an aerobic and anaerobic conditions. The resistance of *E. coli* is affected by growth phase (exponential or stationery) and presence of glucose and amino acids in culture media (13). The results show that the atmosphere the culture is grown in does not affect the survival of *E. coli* B202 during subsequent acid challenge. It is likely that cultures are grown under static conditions used up the oxygen present in the media, which eventually became anaerobic. However, *E. coli* (13) and *E. coli* O157:H7 (10) grown anaerobically were shown to require more acetic acid present in the culture media to induce acid resistance compared to aerobic grown cultures. *E. coli* O157:H7 B202 grown TSB without glucose but with aeration were significantly reduced ($P \leq 0.05$) in the ability to survive in 5 mM D-lactic plus 40 mM acetic acid compared to cells grown in TSBG statically (1).

2.5.6 Addition of 2,2'-bipyridyl to acid solutions

E. coli O157:H7 cells challenged with acid solutions in the presence of oxygen are responding to acid and oxidative stress. 2,2'-bipyridyl is a ferrous iron chelator that binds free iron in the system making it unavailable for enzymes and the Fenton reaction. Under

aerobic conditions, *E. coli* O157:H7 cells treated with 40 mM D- and L-lactic acid in the presence of 0.2 mM 2,2'-bipyridyl were not significantly different ($P > 0.05$) in the reduction of viable cells. There is no effect as a result of the presence of 2,2'-bipyridyl in the reduction of *E. coli* O157:H7 under aerobic conditions compared to Figure 14. Regardless of acid solutions, there is no significant difference ($P > 0.05$) in the reduction of viable cells acid in the presence of 0.2 mM 2,2'-bipyridyl under anaerobic conditions. Unexpectedly, 2,2'-bipyridyl had no effect on the survival of *E. coli* O157:H7 under aerobic conditions and actually decreased survival under anaerobic conditions.

2.5.7 Survival of *E. coli* K-12 OxyR mutant in acid solutions

The OxyR protein is an oxidant sensitive regulator controlling the expression of nine proteins that detoxify peroxides (12). OxyR regulates HPI (*katG*), NADPH-dependent alkyl hydroperoxidase (*aphCF*), fur (which controls iron uptake), glutathione reductase (*gorA*), glutaredoxin 1 (*grxA*) and a protective DNA binding protein (Dps) (16). *E. coli* K-12 mutant was used because it was readily available. Unexpectedly, *E. coli* K-12 OxyR mutant challenged in 20 mM gluconic acid and 40 mM acetic acid survived better than wild-type. These results did not give any information as to why *E.coli* survives significantly better under anaerobic conditions.

We have demonstrated that *E. coli* O157:H7 B202 grown in TSBG for 18 h at 30°C reduces the pH to 4.9 and predominantly produces acetic and lactic acid thus becoming acid-adapted. For the acid solutions, the growth atmosphere does not effect the reduction of

viable cells during acid challenge in a specified atmosphere. *E. coli* O157:H7 B202 behaves similarily to a five-strain *E. coli* O157:H7 cocktail in tested acid solutions. The OxyR mutant and addition of 2,2'-bipyridyl does not answer the question of increased survival under anaerobic conditions.

2.6 Acknowledgements

2.6 References

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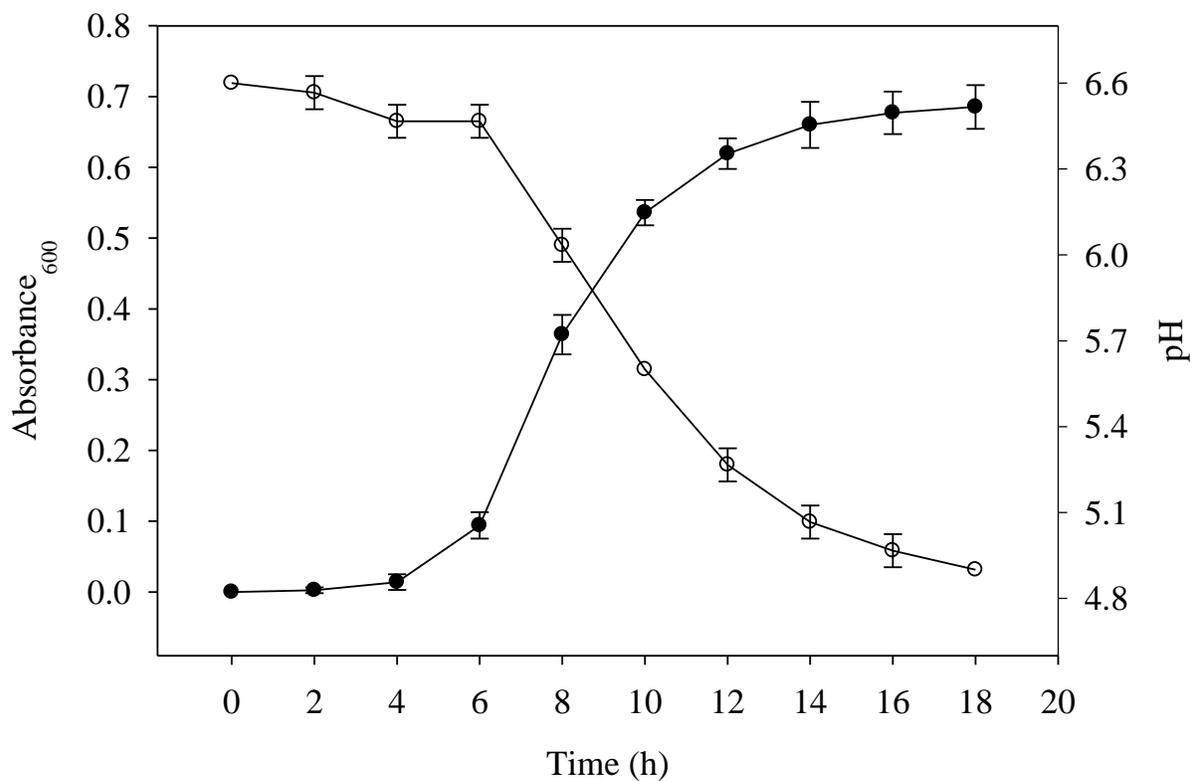
Figure 7. Growth of *E. coli* O157:H7 in TSBG under aerobic conditions for 18 h at 30°C
E. coli O157:H7 B202 was grown in TSBG under aerobic conditions for 18 h at 30ºC to determine the absorbance$_{600}$ (filled circles) and the pH (open circles). Error bars indicate the standard deviation for 3 trials.
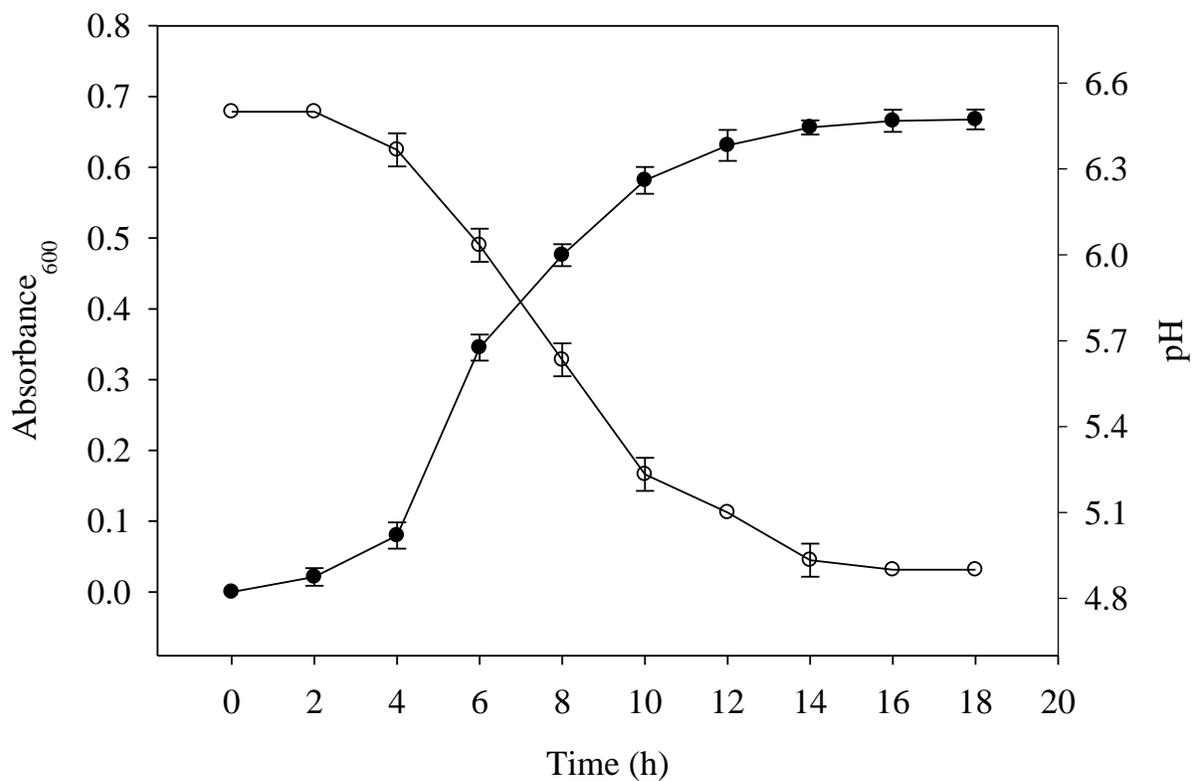
Figure 8. Growth of *E. coli* O157:H7 in TSBG under anaerobic conditions for 18 h at 30°C

E. coli O157:H7 B202 was grown in TSBG under anaerobic conditions for 18 h at 30ºC to determine the absorbance$_{600}$ (filled circles) and the pH (open circles). Error bars indicate the standard deviation for 3 trials.
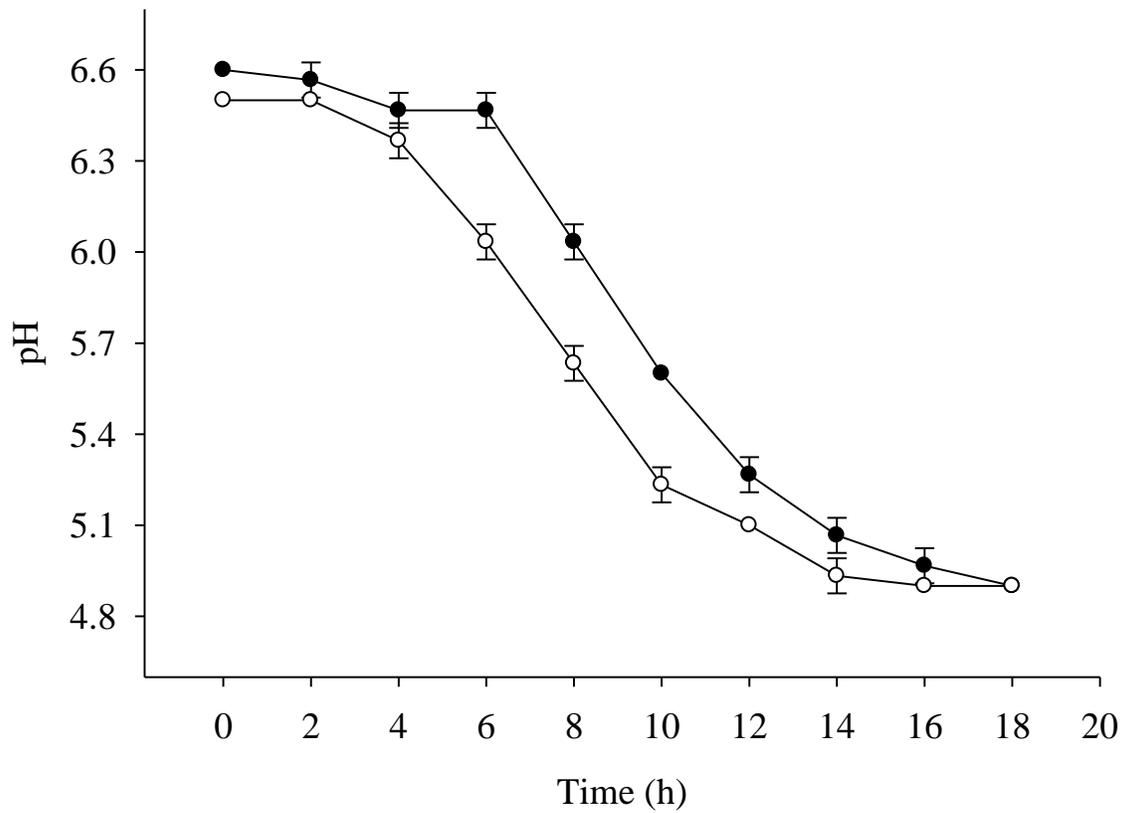
Figure 9. Decline in pH when *E. coli* O157:H7 cells are grown in TSBG under aerobic and anaerobic conditions

E. coli O157:H7 B202 was grown in TSBG under aerobic (empty bars) and anaerobic conditions (striped bars) for 18 h at 30ºC. Error bars indicate the standard deviation for 3 trials.
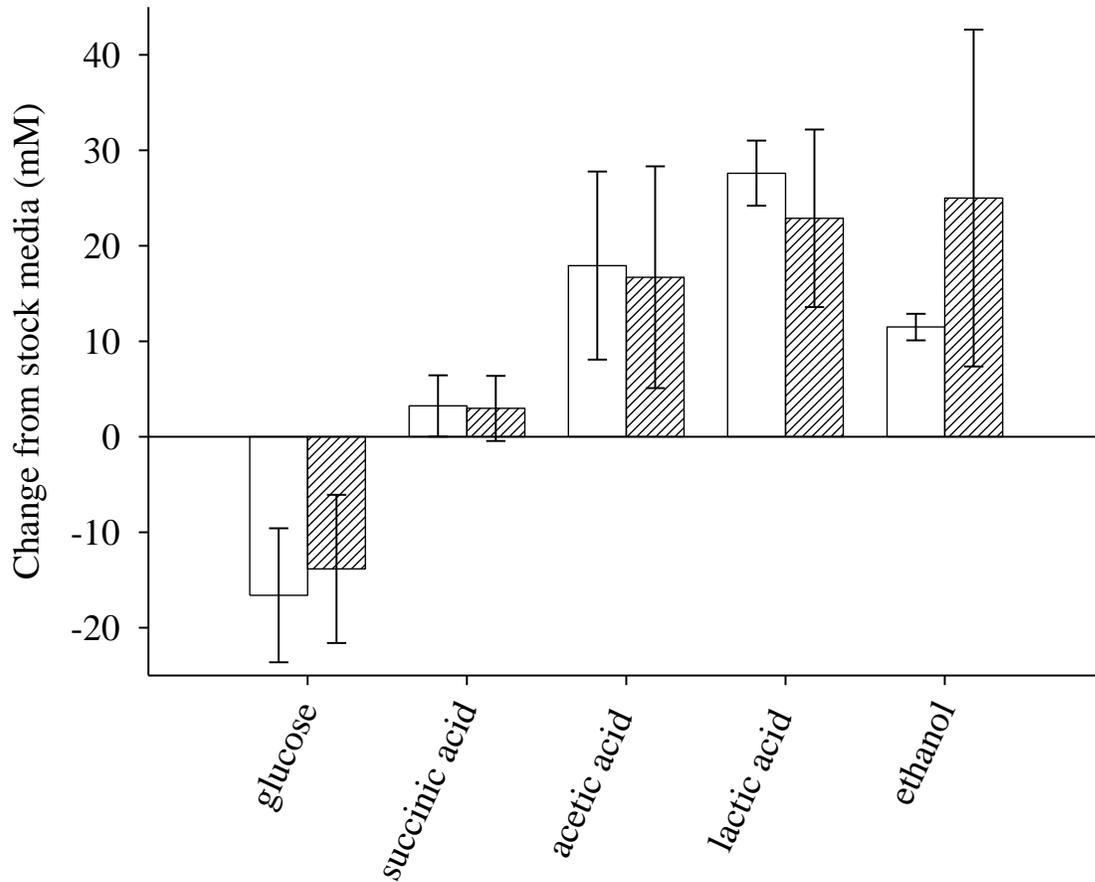
**Figure 10. End products of *E. coli* O157:H7 grown in TSBG under aerobic and anaerobic conditions**
E. coli O157:H7 B202 was grown in TSBG under aerobic (empty bars) and anaerobic conditions (striped bars) for 18 h at 30ºC. Error bars indicate the standard deviation for 3 trials.

Growth Media	12 h				24 h			
	Aerobic		Anaerobic		Aerobic		Anaerobic	
	Log CFU/mL	Std Dev	Log CFU/mL	Std Dev	Log CFU/mL	Std Dev	Log CFU/mL	Std Dev
M9G[a]	7.78	0.08	6.87	0.20	8.47	0.06	8.51	0.12
M9[b] + 20 mM gluconic acid	8.09	0.54	6.95	0.08	8.71	0.02	8.67	0.04
+ 5 mM glucose	8.56	0.04	7.79	1.03	8.68	0.04	8.60	0.35

[a]M9G is M9 minimal salts media prepared with glucose. [b]M9 is M9 minimal salts media prepared without glucose supplemented with ca. 20 mM gluconic acid.

Table 1. Growth of *E. coli* O157:H7 in M9 minimal media supplemented with 20 mM gluconic acid at pH 7
E. coli O157:H7 B202 was grown in M9 minimal media under aerobic and anaerobic conditions for 24 h at 30ºC. Standard deviations indicate the standard deviation for 3 trials. The initial population at time 0 h was 4.55 Log CFU/mL.
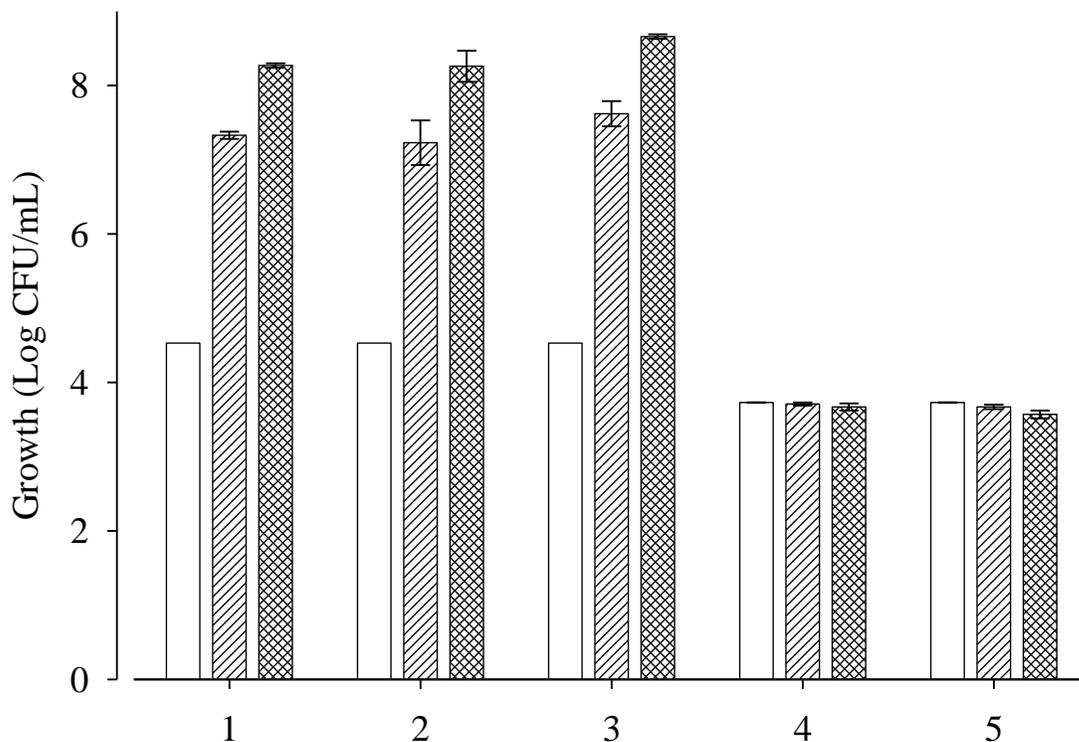
Figure 11. Growth and survival of *E. coli* O157:H7 in M9 media supplemented with gluconic acid at pH 3.2 and 7

E. coli O157:H7 B202 was grown in M9 media supplemented with gluconic acid at pH 3.2 and 3.7 under aerobic conditions for 24 h at 30ºC.   The initial population at time 0 h (empty bars) was 4.55 Log CFU/mL, 12 h (striped bars), and 24 h (crossed bars). Error bars indicate the standard deviation for 3 trials. **1**, M9 minimal salts media, pH 7.0, **2**, M9 + 5mM gluconic acid, pH 7.0, **3**, M9 + 20 mM gluconic acid, pH 7.0, **4**, M9 + 5 mM gluconic acid, pH 3.2, **5**, M9 + 20 mM gluconic acid, pH 3.2,
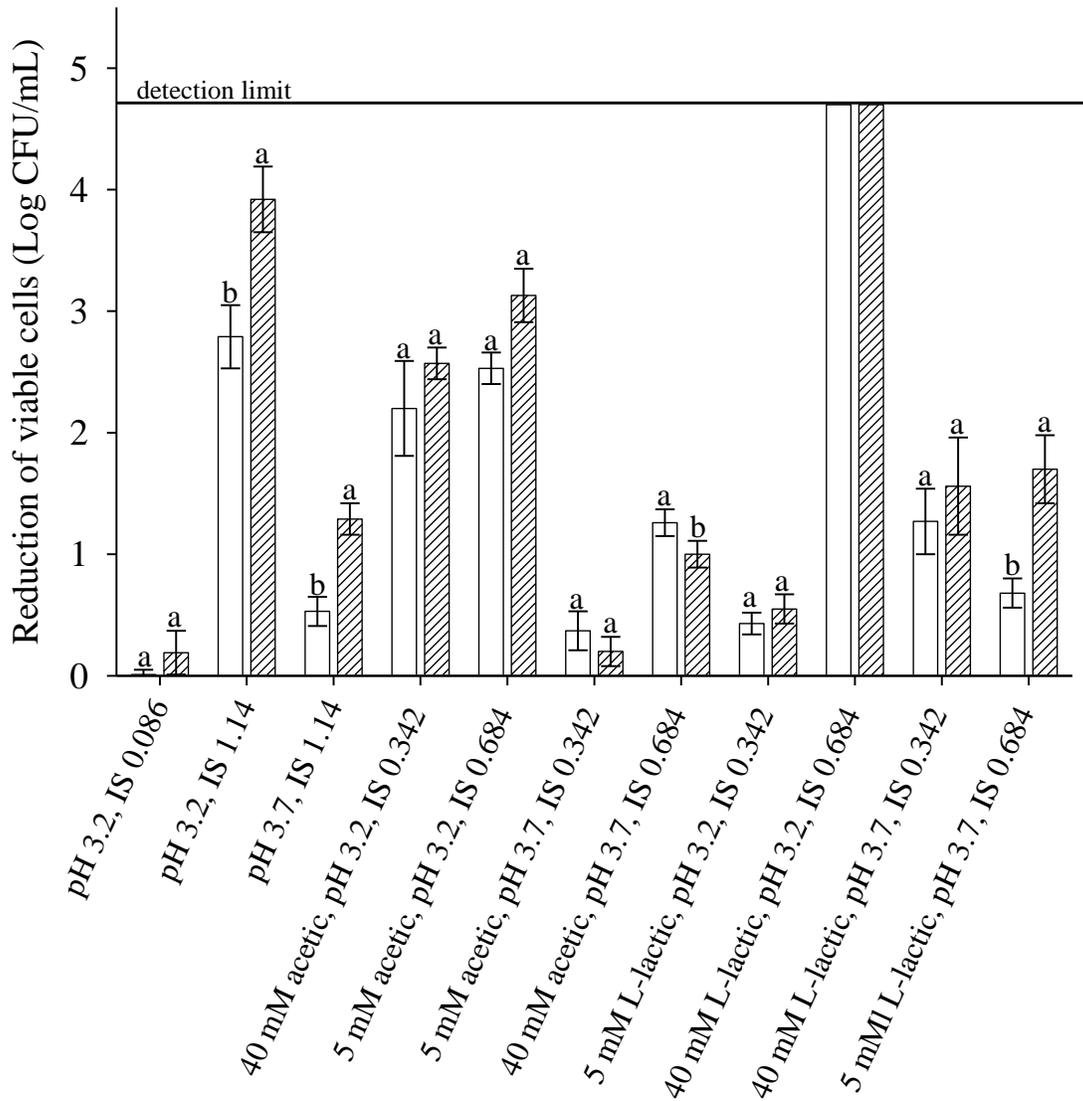
Figure 12. Comparison of log reduction of *E. coli* O157:H7 five-strain cocktail to *E. coli* O157:H7 B202 challenged in various low pH solutions under aerobic conditions
E. coli O157:H7 B202 (clear bars) *E. coli* O157:H7 five-strain cocktail (striped bars) were incubated under aerobic conditions in solutions with an ionic strength (μ) of 0.342 at pH 3.2 for 6 h at 30ºC. Error bars indicate the standard deviation for 3 trials. Within the test solution, mean values without the same lower case letter are significantly different ($P \leq 0.05$).
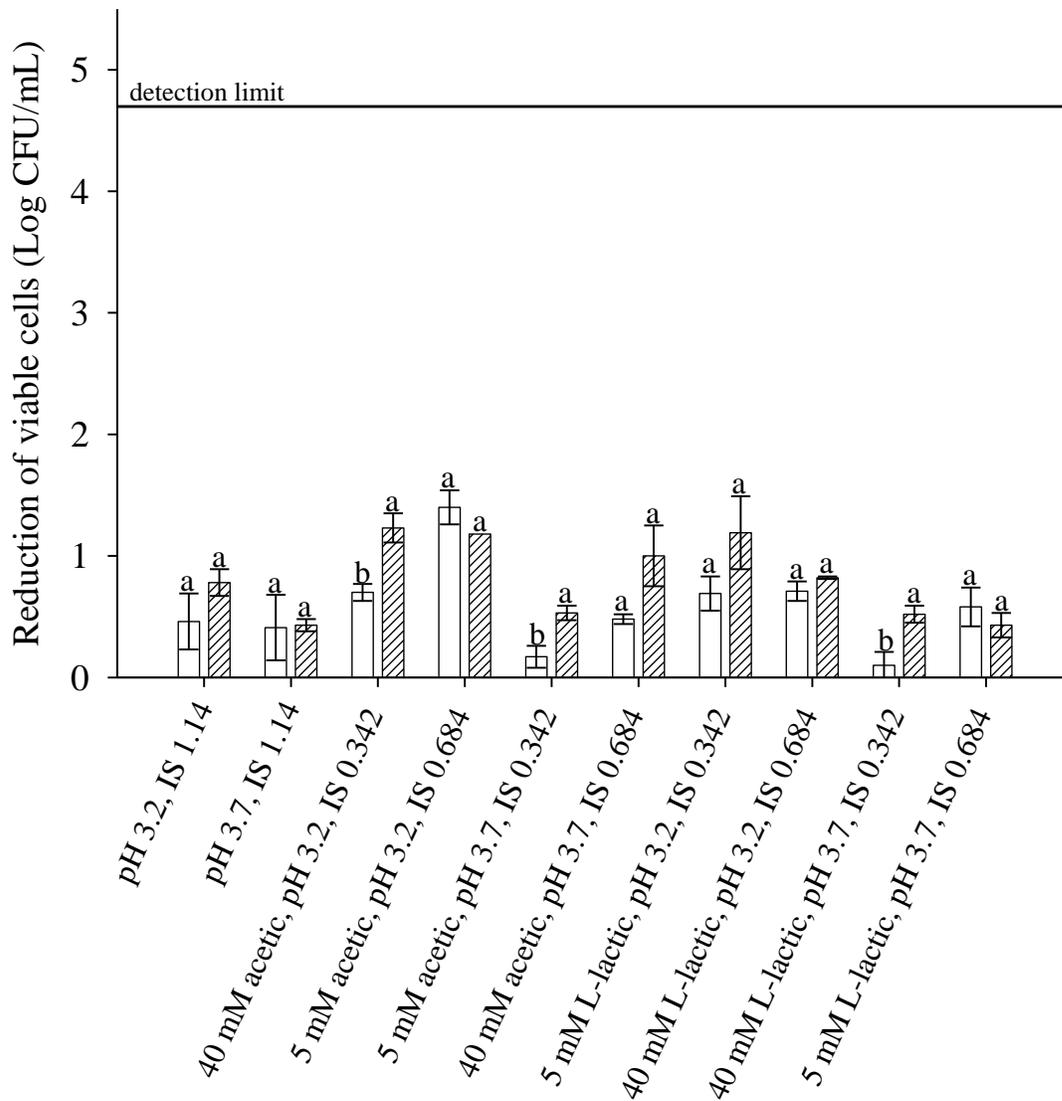
Figure 13. Comparison of log reduction of *E. coli* O157:H7 five-strain cocktail to *E. coli* O157:H7 B202 challenged in various low pH solutions under anaerobic conditions
E. coli O157:H7 B202 (clear bars) *E. coli* O157:H7 five-strain cocktail (striped bars) were incubated under anaerobic conditions in solutions with μ of 0.342 at pH 3.2 for 6 h at 30ºC. Error bars indicate the standard deviation for 3 trials. Within the test solution, mean values without the same lower case letter are significantly different ($P \leq 0.05$).
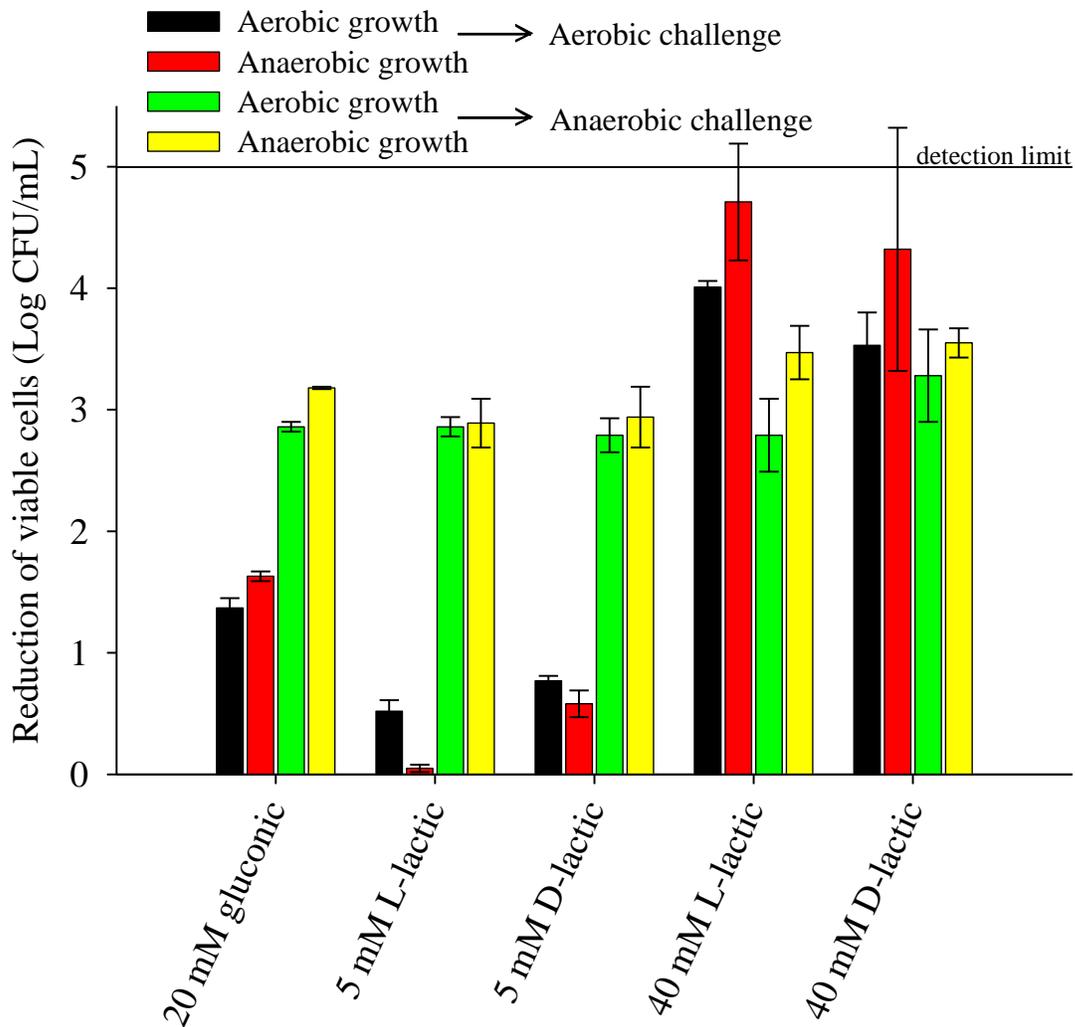
Figure 14. Comparison of growth atmosphere and challenge atmosphere in the reduction of *E. coli* O157:H7 in protonated D- and L-lactic solutions at pH 3.2
Cells grown under aerobic (black bars) and anaerobic (red bars) conditions and challenged under aerobic conditions in solutions with μ of 0.342 at pH 3.2 for 6 h at 30ºC. Cells grown under aerobic (green bars) and anaerobic (yellow bars) conditions and challenged under anaerobic conditions in the same test solutions. Error bars indicate the standard deviation for 3 trials.
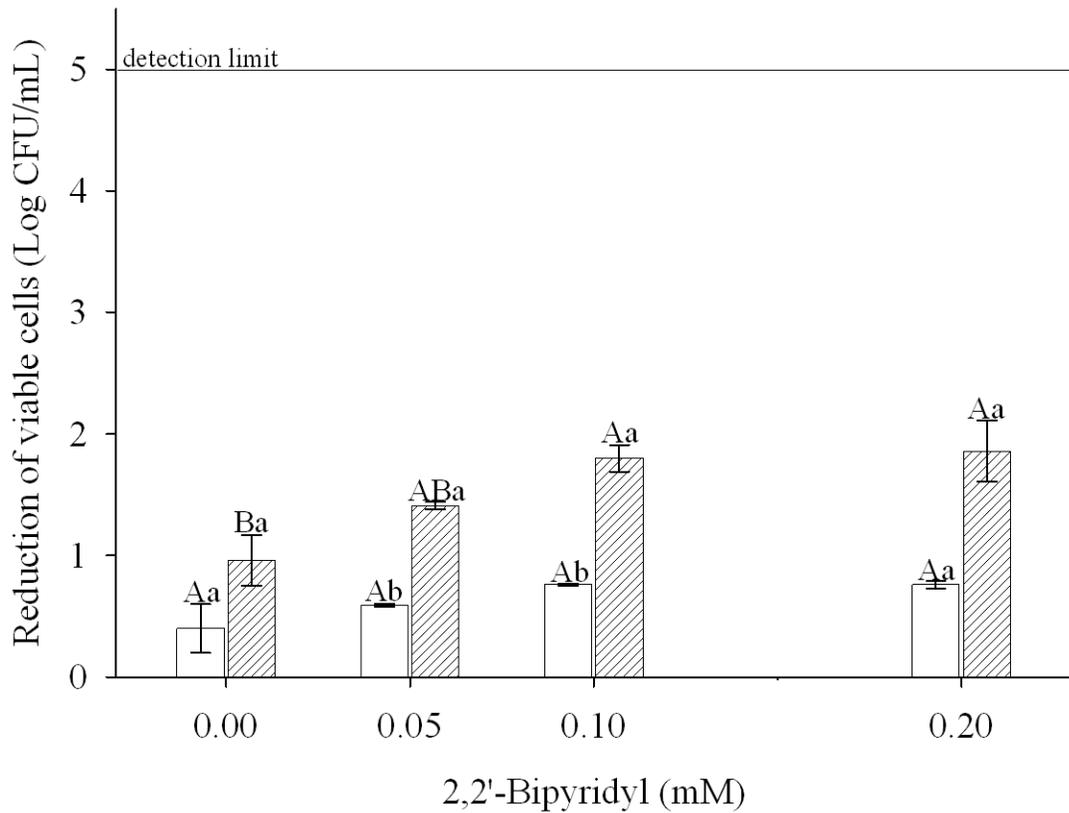
Figure 15. Log reduction of *E. coli* O157:H7 in various 2,2'-bipyridyl concentrations in 20 mM gluconic acid at pH 3.2
Cells were incubated under aerobic (empty bars) and anaerobic conditions (striped bars) in solutions with μ of 0.342 at pH 3.2 for 6 h at 30ºC. Error bars indicate the standard deviation for 3 trials. Within the same atmosphere, mean values without the same capitalized letter are significantly different ($P \leq 0.05$). Within the same concentration, mean values without the same lower case letter are significantly different ($P \leq 0.05$).

Figure 16. Log reduction of *E. coli* O157:H7 in acid solutions with 0.2 mM 2,2'-bipyridyl at pH 3.2

Cells were incubated under aerobic (empty bars) and anaerobic conditions (striped bars) in solutions with μ of 0.342 at pH 3.2 for 6 h at 30ºC. Error bars indicate the standard deviation for 3 trials. Within the same atmosphere, mean values without the same capitalized letter are significantly different ($P \leq 0.05$). Within the same concentration, mean values without the same lower case letter are significantly different ($P \leq 0.05$).

Figure 17. Log reduction of *E. coli* K-12 and OxyR mutant in various acid solutions at pH 3.2 under aerobic conditions
Wild type (clear bars) and OxyR mutant (striped bars) cells were incubated under aerobic conditions in solutions with μ of 0.342 at pH 3.2 for 6 h at 30°C. Error bars indicate the standard deviation for 3 trials. Within the same strain, mean values without the same capitalized letter are significantly different ($P \leq 0.05$). Within the same concentration, mean values without the same lower case letter are significantly different ($P \leq 0.05$).

CHAPTER III

Effects of pH, Dissolved Oxygen, and Ionic Strength on the Survival of *Escherichia coli*

O157:H7 in Acid Solutions

3.1 Abstract

The ability of *Escherichia coli* O157:H7 to survive in acidified vegetable products is of concern because of previously documented outbreaks associated with fruit juices. A study was conducted to determine the survival of *E. coli* O157:H7 in organic acids at pH values typical of acidified vegetable products (pH 3.2 and 3.7) under different dissolved oxygen conditions ($\leq$ 0.05 and 5 mg/L) and a range of ionic strengths (μ, 0.086 to 1.14). All solutions contained 20 mM gluconic acid, which was used as a non-inhibitory low pH buffer to compare the individual acid effect to that of pH alone on the survival of *E. coli* O157:H7. *E. coli* O157:H7 cells challenged in buffered solution with ca. 5 mg/L dissolved oxygen (present in tap water) over a range of ionic strengths at pH 3.2 exhibited a decrease in survival as the ionic strength was increased over 6 h at 30ºC. Cells challenged in 40 mM protonated L-lactic and acetic acid solutions with μ of 0.684 achieved a > 4.7 log CFU/mL reduction at pH 3.2. However, under oxygen limiting conditions in an anaerobic chamber, with less than or equal to 0.05 mg/L oxygen, *E. coli* O157:H7 cells showed $\leq$ 1.55 log CFU/mL reduction regardless of pH, acid type, concentration, or ionic strength. Many acid and acidified foods are sold in hermetically sealed containers with oxygen limiting conditions. Our results demonstrate that *E. coli* O157:H7 may survive better than previously expected from studies with acid solutions containing little to no dissolved oxygen.

3.2 Introduction

Outbreaks of disease from the consumption of acid foods, such as apple cider and apple juice, have raised concerns about the safety of foods that use low pH or organic acids as the main barrier to pathogenic bacteria (9, 10). The U.S. Code of Federal Regulations (21 CFR part 114) defines acid and acidified foods as having a pH of 4.6 or lower. Acid foods are foods that naturally have a pH below 4.6. Acidified foods reach their equilibrated pH of 4.6 or lower through the addition of acid or acid food ingredients, such as organic acids.

Organic acids and their salts have been used for many years as food preservatives in sauces, mayonnaises, dressings, drinks, and fruit juices (24). These organic acids are thought to enter the bacterial cell in their undissociated form by diffusion across the non-polar cell membrane (7). Studies by Eklund (18) demonstrated that the undissociated sorbic acid was 15 to 600 times more effective in inhibiting the growth of *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* than the dissociated form. The acid anion cannot readily diffuse through the cell membrane and may accumulate to molar amounts in the cell cytoplasm, depending on the difference between the internal and external pH (15). Organic acids may act on the cell by inhibiting metabolic reactions and lowering intracellular pH. The intracellular accumulation of acid anions may also have deleterious effects (7). The antimicrobial activity of an organic acid may be affected by several factors such as the pK_a, temperature, specific type of acid, acid concentration, pH, and ionic strength.

Escherichia coli O157:H7 is a gram negative, facultative anaerobic bacterium commonly found in the gut of ruminants, specifically cattle (17). The bacterium can cause mild diarrhea, bloody diarrhea, life-threatening hemolytic uremic syndrome (HUS) in children, and thrombotic thrombocytopenic purpura (TTP) in adults (17, 27). In the United States, an estimated 62,458 illnesses, 1,843 hospitalizations, and 52 deaths are caused annually by foodborne infections of *E. coli* O157:H7 (22). Studies by Leyer *et al.* (21) demonstrated the ability of *E. coli* O157:H7 to survive in apple cider (pH 3.42) held for 3 days at 6ºC. The low infectious dose of *E. coli* O157:H7 estimated at ca. 100 cells (11) coupled with its ability to survive passage through the human stomach (pH 2; anaerobiosis) (23) demonstrates the need to better investigate the survival of this pathogen under conditions typical of acidified vegetable products (2,5).

The objectives of this study were to examine the effects of pH, acid type, ionic strength, and dissolved oxygen on the survival of *E. coli* O157:H7 cells. Many acidified vegetable products have brine pH values between 3.0 and 3.3 (condiments) or around pH 3.7 (acidified cucumber pickles). Salt concentrations for acidified vegetables typically range from 2% to 4% NaCl, equivalent to ionic strength values of 0.342 or 0.684, respectively. These products are prepared in hermetically sealed jars, resulting in an anaerobic environment. Our results indicate that *E. coli* O157:H7 may survive better than previously expected from studies where anaerobic conditions are not used.

3.3 Materials and Methods

3.3.1 Bacterial strains

E. coli B200 (strain O157:H7, ATCC 43888, human feces isolate), *E. coli* B201 (strain O157:H7, SRCC 1675, apple cider outbreak isolate), *E. coli* B202 (strain O157:H7, SRCC 1486, salami outbreak isolate), *E. coli* B203 (strain O157:H7, SRCC 206, ground beef isolate), *E. coli* B204 (strain O157:H7, SRCC 1941, pork isolate) were used.

3.3.2 Preparation of inocula for acid challenge

E. coli B202 was used in these experiments, because it has been shown to be the most acid resistance of the five listed above (1). Stock cultures of *E. coli* B202 stored in tryptic soy broth (TSBG, BBL/Difco, Sparks, MD) supplemented with 1% glucose (Sigma Chemical Co., St. Louis, MO) and 16% glycerol at -80°C were streaked by loop inoculum (ca. 10 µl) onto tryptic soy agar (TSAG, BBL/Difco) supplemented with 1% glucose and incubated for 24 h at 30ºC (to be consistent with the temperature of acid treatments). Three colonies of strain B202 were then transferred by loop inoculum into 10 mL of TSBG and incubated statically for ca. 18 h at 30ºC to induce acid resistance as previously described (8). The overnight cultures typically attained 10^9 CFU/mL and a pH of ca. 4.9. Cultures were centrifuged, washed, and resuspended in 0.85% saline solution. To determine initial populations, cells were serially diluted, plated in duplicate (Model 4000, Spiral Biotech, Inc., Norwood, MA) on TSAG, and incubated at 30ºC for 24 h.

3.3.3 Preparation of inocula for acidified cucumber brine

Five strains of *E. coli* O157:H7 were used: *E. coli* B200, *E. coli* B201, *E. coli* B202, *E. coli* B203, and *E. coli* B204. Cultures were prepared as described (2). Briefly, each strain was grown statically in TSBG for 18 h as described above. The cell suspensions, containing ca. 10^9 CFU/mL, were centrifuged, and the cell pellet was resuspended in 0.1 fraction of the original culture volume using sterile saline (0.85% NaCl). Equal amounts of each suspension were combined into a cocktail. The initial inoculum in the cucumber brine was ca. 1×10^8 CFU/mL for the strain mixture. The initial cell concentration was determined by plating as described above.

3.3.4 Preparations of acid solutions

Calculations of pH, ionic strength, and protonated acid concentrations for the buffered acid solutions were made using custom Matlab routines (pH$_{tools}$, available at: http://www.mathworks.com/matlabcentral) as previously described (16). This program also allows for the adjustment of pK$_a$ values based on ionic strength and temperature. L-lactic and acetic acid (Sigma) were tested at concentrations ranging from 0.1 to 40 mM. We used L-lactic acid for our experiments because we have found that D-lactic acid can have a protective effect on the survival of *E. coli* O157:H7 at pH 3.2, compared to the inhibition caused by buffered control solution at the same pH (2). All solutions contained gluconic acid (Sigma) which was used as a non-inhibitory low pH buffer (20 mM) to determine the inhibitory effects of pH 3.2 and 3.7 in the absence of added organic acids as previously

described (6). Solutions containing only 20 mM gluconic acid (buffered solution, 0 mM) were used as the controls. The acid solutions were adjusted using HCl/NaOH to reach the desired pH and NaCl was added, as needed, to produce the indicated ionic strength. Solutions incubated in the anaerobic chamber (COY, Grass lake, MI) were allowed to equilibrate for at least 48 h prior to use. The mixed anaerobic gas atmosphere consisted of 5% CO_2, 10% H_2, and 85% N_2. The dissolved oxygen content of solutions was measured using the CellOx 325 dissolved oxygen sensor (WTW, Weilheim, Germany). The lower limit of detection with this instrument was ca. 0.05 mg/L.

3.3.5 Acid challenge

The acid solutions (180 μl) were dispensed into wells of a 96 well microtiter plate (Microtest flat bottom plate, BD, Franklin Lakes, NJ) and allowed to equilibrate at 30ºC for 1 h before the inoculum was added. The inoculum (20 μl) resulted in an initial cell suspension of ca. 2×10^8 CFU/mL. The solutions were mixed thoroughly and incubated statically for 6 h at 30ºC. After incubation, 100 μl of the test suspension was diluted in 900 μl of 50 mM MOPS buffer (Sigma) and vortexed to facilitate neutralization. Suspensions were serially diluted in 450 μl saline solutions and plated in duplicate on TSAG. The lower limit for detection of bacterial cells by this method was ca. 4×10^3 CFU/mL. The plates were incubated at 30ºC for 24 h to determine the reduction of viable cells.

3.3.6 Acid challenge in acidified cucumber brine

Acidified pickles were prepared as previously described (4). Briefly, size 2B cucumbers (ca. 4 cm diameter) were obtained from a local supplier. Cucumbers (789 ± 2 g) were packed in 571 ± 2 mL of cover solution in 1.36 L (46 oz) glass jars to equilibrate at 2% sodium chloride, 0.1% calcium chloride, and pH 3.3. Food grade vinegar (20% acetic acid) was used to achieve the target pH (ca. 400 mM acetic acid). After filling and sealing, the jars were heat processed so that the cold point of each jar was held at 74.4^{o}C for 15 min. The jars were stored at 25°C for at least 10 days to allow the equilibration of water-soluble components (e.g., acids, salt, and sugars) between the cover solution and the cucumbers. Brine was removed from the jars and added to double walled fermentation flasks as previously described (5). Remaining jars and flasks were inoculated with a cocktail of five *E. coli* O157:H7 strains prepared as described above to give an initial inoculum of 10^{8} CFU/mL. Jars were sampled through rubber septa affixed to the lids as previously described (4). Both the jars and flasks were incubated at 30°C, brine samples (0.5 mL) were taken at the indicated time intervals, serially diluted and plated as described above.

3.3.7 Statistical analysis

Experiments with buffered acid solutions were replicated three times with two test solutions per replicate, and brine experiments were carried out in triplicate. Log reduction was calculated as the difference between the initial count and the final count ($\log_{10}$ [N_0/N]). Log numbers were analyzed using the general linear models with Tukey adjustment of the

Statistical Analysis Systems version 8.0 (Statistical Analysis System, SAS Institute, Cary,

N.C.). For modeling the reduction in CFU/mL for brine experiments, a Weibull model was

used as previously described (5). Results were considered significant when the P-value was

$P \leq 0.05$.

3.4 Results

3.4.1 Dissolved oxygen

Solutions prepared for use in experiments conducted on the bench-top were found to

contain 5.3 ± 0.2 mg/L dissolved oxygen at $25 \pm 2°C$, and were designated as 'aerobic'. The

maximum dissolved oxygen concentration of distilled water at sea level (37°C) is ca. 6.7

mg/L (0.21 mM). The solutions in the anaerobic chamber equilibrated for up to one week

had oxygen limiting conditions, and retained 0.05 mg/L dissolved oxygen or less, designated

as 'anaerobic'.

3.4.2 Ionic strength

The log reduction of *E. coli* O157:H7 cells recovered from solutions with various

ionic strengths (μ) buffered at pH 3.2 and 3.7 (adjusted with HCl) are shown in Figures 18A

and 18B. There were no significant differences ($P > 0.05$) in cell survival between μ of

0.086 and μ of 0.342 under aerobic conditions, regardless of pH. However, there was a

gradual decrease in the survival as the ionic strength increased up to 1.14 at pH 3.2 and 3.7

(Figure 18A). Under anaerobic conditions ($\leq$ 0.05 mg/L), cells treated with μ of 0.342 were

reduced by 1.19 log CFU/mL compared to only a 0.45 log CFU/mL reduction with μ of

1.140 at pH 3.2 (Figure 18B).

3.4.3 Lactic acid

The log reduction of *E. coli* O157:H7 cells recovered from solutions containing 0 to

40 mM protonated lactic acid at pH 3.2 are shown in Figures 19A and 19B. There was no

significant difference ($P > 0.05$) in the survival of *E. coli* O157:H7 cells recovered from the

control solution and solutions containing up to 5 mM protonated lactic acid (μ, 0.342) under

aerobic conditions (Figure 19A). There was a 1.67 log CFU/mL reduction in cells treated

with 5 mM protonated lactic acid (μ, 0.684) compared to a 2.96 log CFU/mL reduction in

cells treated with the control solution. An increase in survival at 5 mM lactic acid (pH 3.2, μ,

0.60-0.68, 25ºC) was also reported by Bjornsdottir *et al.* (2). Under oxygen limiting

conditions, there was no significant difference ($P > 0.05$) in the reduction of *E. coli* O157:H7

cells treated with the control solution (0.85 log CFU/mL) compared to 40 mM lactic acid

(0.71 log CFU/mL; μ, 0.684) (Figure 19B). Survival of cells treated with solutions of μ of

0.342 or μ of 0.684 were not significantly different ($P > 0.05$) under oxygen limiting

conditions.

The log reduction of *E. coli* O157:H7 cells recovered from solutions of protonated

lactic acid (0 to 40 mM) at pH 3.7 are shown in Figures 20A and 20B. *E. coli* O157:H7 cells

treated with 40 mM protonated lactic acid (μ, 0.342) were reduced in number by 1.27 log

CFU/mL, while cells in 40 mM protonated lactic acid (μ, 0.684) were reduced by > 4.7 log

CFU/mL. There was no significant difference ($P > 0.05$) in the survival of *E. coli* O157:H7

cells recovered from any test solutions containing μ of 0.342 under oxygen limiting

conditions (Figure 20B), and all anaerobic test solutions resulted in a reduction of $\leq$ 0.58 log

CFU/mL regardless of ionic strength.

3.4.4 Acetic acid

The log reduction in *E. coli* O157:H7 cells recovered from solutions of protonated

acetic acid (0 to 40 mM) at pH 3.2 are shown in Figures 21A and 21B. There was no

significant difference ($P > 0.05$) in the reduction of *E. coli* O157:H7 cells treated with acid

solutions ranging from 0 to 20 mM protonated acetic acid at μ of 0.342. For all

concentrations of protonated acetic acid tested, we observed a significant decrease ($P \leq 0.05$)

in survival of *E. coli* O157:H7 cells treated with solutions of μ of 0.684 compared to μ of

0.342. *E. coli* O157:H7 cells treated with solutions containing μ of 0.684 decreased in

survival as the concentration of acetic acid was increased up to 40 mM. Cells treated with

solutions containing 40 mM protonated acetic acid were reduced by 2.20 log CFU/mL at μ of

0.342 and > 4.7 log CFU/mL at μ of 0.684. Under oxygen limiting conditions, there was no

significant difference ($P > 0.05$) in the reduction of *E. coli* O157:H7 cells treated with

solutions containing up to 40 mM acetic acid, regardless of ionic strength.

Cells treated with 40 mM protonated acetic acid (pH 3.7) at μ of 0.342 and 0.684,

were reduced by 0.62 log CFU/mL and 1.26 log CFU/mL, respectively (data not shown).

Solutions containing protonated acetic acid (up to 40 mM) were not significantly different (P > 0.05) in reducing *E. coli* O157:H7 cells under oxygen limiting conditions ($\leq$ 0.80 log CFU/mL), regardless of ionic strength (data not shown).

3.4.5 Cucumber brine

Survival of *E. coli* O157:H7 in acidified cucumber brine was measured in sealed jars, as well as with the equilibrated brine removed from the jars. Immediately upon opening, the dissolved oxygen measured in the jars was 0.5 mg/L, however, similar measurements were obtained with solutions having 0.05 mg/L oxygen or less that were measured immediately upon removal from the anaerobic chamber (data not shown). It was not possible to directly measure the dissolved oxygen in the sealed jars. The bacterial cell counts from the brine removed from the jars were below the limit of detection within 12 h. More than 60 h was required for the detection limit to be reached with cells inoculated and sampled through the septa in the jars (Figure 22). Because the brine composition, pH, and ionic strength was the same for all samples, the data indicate that reduced oxygen content of the brine in the jars was responsible for the 48 h difference in the time needed to achieve a 5 log reduction in *E. coli* O157:H7 cell numbers.

3.5 Discussion

We investigated the ability of *E. coli* O157:H7 to survive in acid solutions under anaerobic conditions. Acidified vegetable products such as pickles, banana peppers, and

olives are typically prepared in hermetically sealed jars (anaerobic conditions). These types

of products are protected through a hurdle strategy using a combination of pasteurization, salt

(2 to 4%) and low pH (typically pH 3 to 4) via addition of organic acids. However, some

acidified vegetable products can not be pasteurized and maintain acceptable sensory

properties. Although there have not been any known outbreaks of disease from acid-resistant

vegetative pathogens in acidified vegetables, recent research has shown that *E. coli* O157:H7

can survive in these products and potentially cause disease.

Salt has long been used to preserve and flavor foods. Studies by Entani *et al.* (20)

showed that NaCl worked synergistically with acetic acid to increase its bactericidal action

against *E. coli* O157:H7. Clavero and Beuchat (12) demonstrated that the combination of

decreased pH (pH 4.8) and a_w (0.90, adjusted by NaCl) increased the inactivation or

inhibition of *E. coli* O157:H7 in TSB at 30°C over 48 h. Our results show that the addition

of NaCl to the organic acid solutions under aerobic, but not anaerobic, conditions correlated

with a decrease in the survival of *E. coli* O157:H7 for the conditions tested. Additional

studies with greater acid concentrations will be carried out to further investigate this effect.

Lactic acid is a common fermentation product and is used to lower the pH of foods

(19, 26). Acetic acid is used in the food industry as an acidulant and flavorant in food items.

Organic acid toxicity for bacterial cells is attributed to lowering cytoplasmic pH and

intracellular accumulation of acid anion (15). A > 4.7 log CFU/mL reduction was achieved

after 6 h when cells were treated with 40 mM protonated lactic or acetic acid at pH 3.2,

regardless of ionic strength. Under oxygen limiting conditions, there was $\leq$ 1.55 log

CFU/mL reduction in the populations of *E. coli* O157:H7 incubated for these solutions. Cleary and McFeeters (13) found the dissolved oxygen content of commercial acidified cucumber pickles to be ca. 0.45 ± 0.9 mg/L, when measurements were taken upon opening the jars. Due to rapid equilibration, it is likely that the dissolved oxygen content was less than 0.45 mg/L. As shown in Figure 22, once brine is exposed to dissolved oxygen, the survival of *E. coli* O157:H7 is significantly reduced. Further research is being carried out to investigate and exploit this phenomenon.

Previous experiments conducted on the inhibition of acid-resistant pathogenic bacteria by organic acids have been confounded by differences in experimental design, and methods for calculating acid effects. For example, data from Young and Foegeding (28) can be used to show that citric acid was more inhibitory to *Listeria monocytogenes* than lactic and acetic acids, based on protonated acid concentrations, but for total acid concentrations (acid anion plus the protonated acid), the order is reversed. Ryu *et al.* (25) and Deng *et al.* (14) found that acetic acid was the most inhibitory to *E. coli* O157:H7 when added to media to reach the desired pH. By controlling pH, ionic strength, and temperature, Bjornsditter *et al.* (2) found that protonated lactic acid was the most inhibitory to *E. coli* O157:H7. However, these reports on the inhibitory effects of organic acids on *E. coli* O157:H7 do not take into consideration the effects of dissolved oxygen in the acid solutions.

We have demonstrated that an *E. coli* O157:H7 strain can survive significantly better in acid solutions under oxygen limiting conditions compared to aerobic conditions. For the acid solutions tested in the absence of oxygen *E. coli* O157:H7 was only reduced by ≤ 1.55

log CFU/mL for all conditions tested. We found that increasing the incubation time from 6 h up to 24 h did not enhance the reduction of *E. coli* O157:H7 cells under anaerobic conditions (1). Under aerobic conditions, a > 4.7 log CFU/mL or greater reduction was observed. Results from previous research carried out with oxygen containing bench-top solutions may not be representative of acid or acidified food products in hermetically sealed containers or anaerobic digestive organs of animals. Further characterization of the effect of oxygen limitation on the survival of *E. coli* and other acid-resistant pathogens in acid solutions may help assure the safety of acid and acidified foods.

3.6 Acknowledgements

3.7 References

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18A

18B
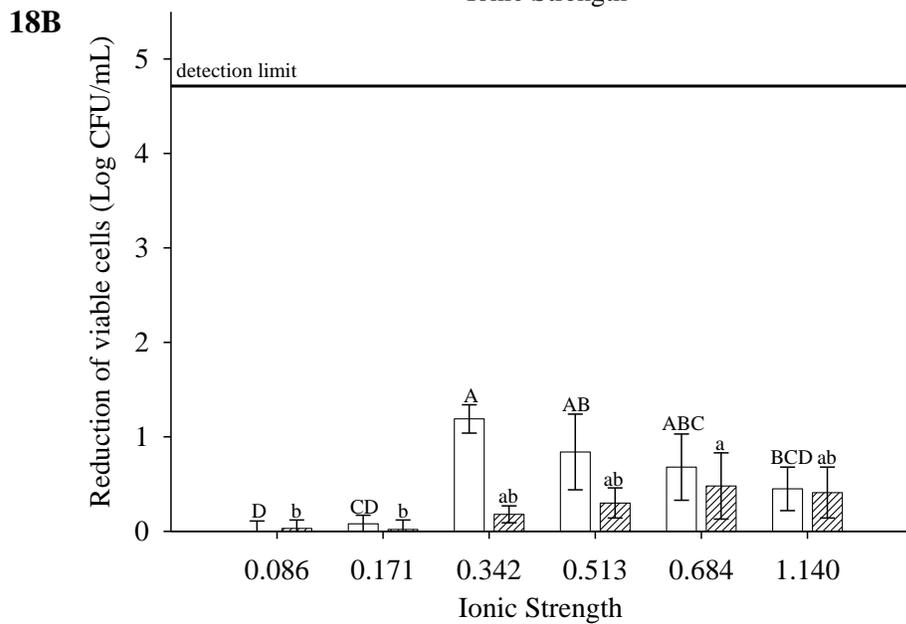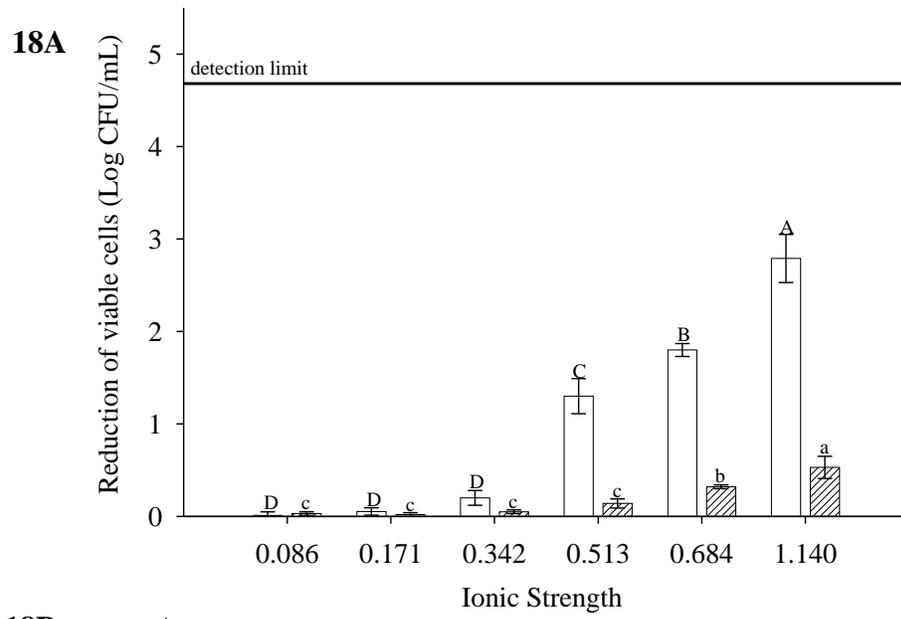
Figure 18. The log reduction of *E. coli* O157:H7 in a range of ionic strengths.
Cells were incubated under (A) aerobic and (B) oxygen limiting conditions in solutions at pH 3.2 (empty bars) and 3.7 (stripped bars) for 6 h at 30ºC. Error bars indicate the standard deviation for 3 trials. Identical letters over error bars indicate there is no significant difference between treatments ($P > 0.05$) (Capital letters for pH 3.2, lower case letter for pH 3.7).
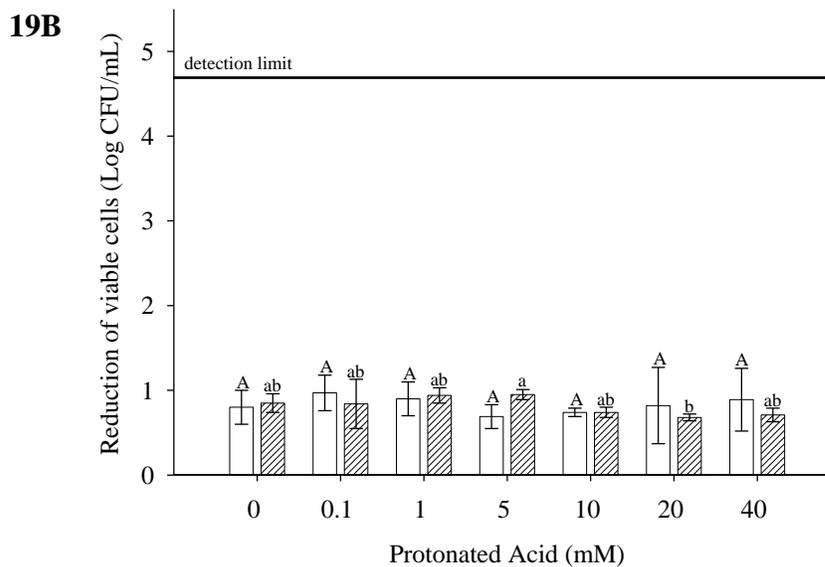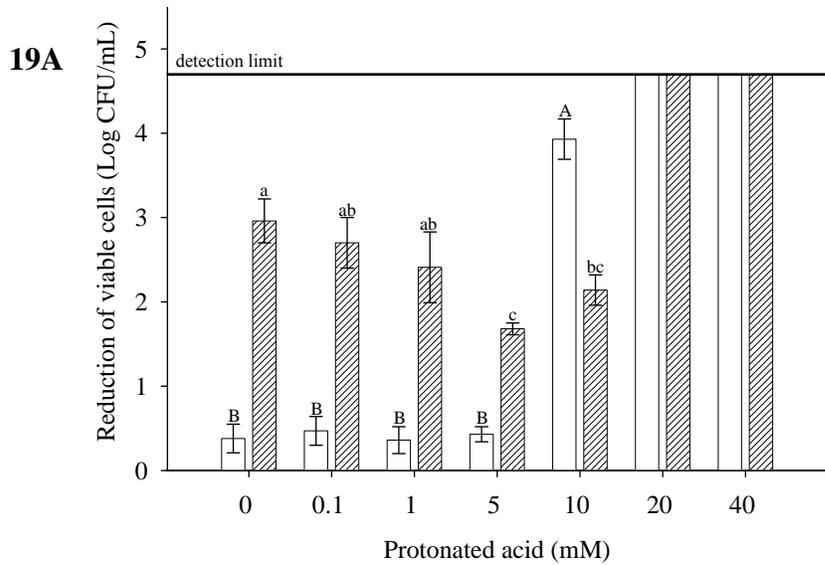
Figure 19. The log reduction of *E. coli* O157:H7 in concentrations of L-lactic acid at pH 3.2.

Cells were incubated under (A) aerobic and (B) oxygen limiting conditions in solutions with ionic strength (μ) of 0.342 (empty bars) and 0.684 (striped bars) at pH 3.2 for 6 h at 30ºC. Error bars indicate the standard deviation for 3 trials. Identical letters over error bars indicate there is no significant difference between treatments ($P > 0.05$); capital letters, $\mu = 0.342$; lowercase, $\mu = 0.684$.

20A
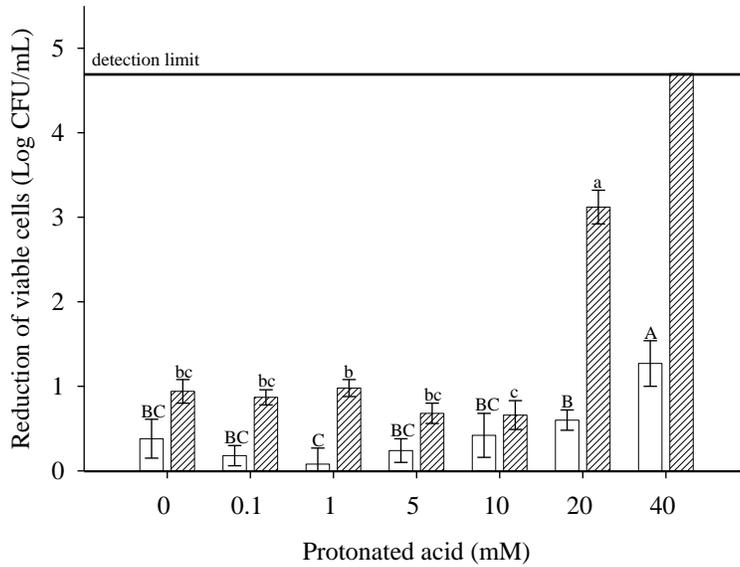
20B
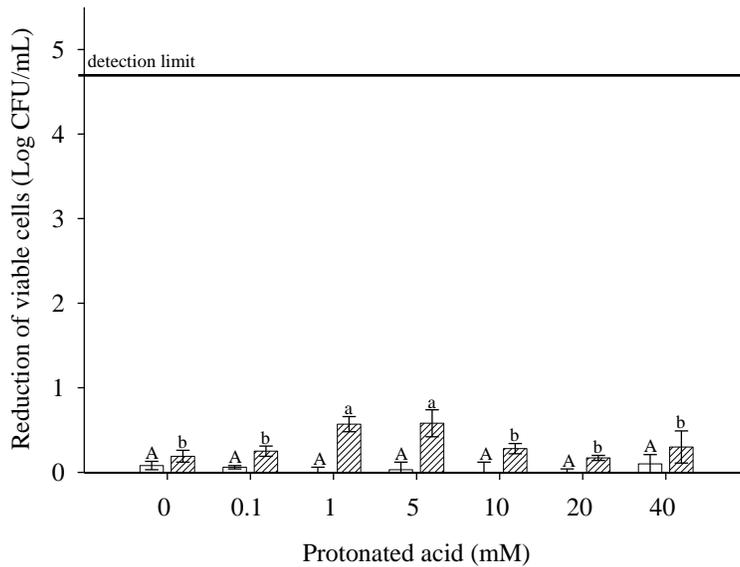
Figure 20. The log reduction of *E. coli* O157:H7 in concentrations of L-lactic acid at pH 3.7

Cells were incubated under (A) aerobic and (B) oxygen limiting conditions in solutions with μ of 0.342 (empty bars) and 0.684 (striped bars) at pH 3.7 for 6 h at 30ºC. Error bars indicate the standard deviation for 3 trials. Identical letters over error bars indicate there is no significant difference between treatments ($P > 0.05$); capital letters, μ = 0.342; lowercase, μ = 0.684.

21A

21B

Figure 21. The log reduction of *E. coli* O157:H7 in concentrations of acetic acid at pH 3.2

Cells were incubated under (A) aerobic and (B) oxygen limiting conditions in solutions with μ of 0.342 (empty bars) and 0.684 (striped bars) at pH 3.2 for 6 h at 30ºC. Error bars indicate the standard deviation for 3 trials. Identical letters over error bars indicate there is no significant difference between treatments ($P > 0.05$); capital letters, μ = 0.342; lowercase, μ = 0.684.

**Figure 22. The survival of *E. coli* O157:H7 in acidified pickle brines.**
Bacteria cell counts were obtained from sealed jars through rubber septa (triangles) or in brine removed from jars and exposed to air (circles). The lines represent the Weibull models of each data with three replications. Obtained from Breidt (3)

CHAPTER IV

Determination of Internal pH and Catalase Activity of *Escherichia coli* O157:H7 in

Lactic Acid Solutions

4.1 Abstract

Previous research has shown the lack of oxygen or the presence of D-lactic acid under aerobic conditions can increase the survival of *Escherichia coli* O157:H7 in low pH (pH 3.2) organic acid solutions at ionic strength (μ) of 0.684. Cells held at an μ of 0.342 survived better aerobically than anaerobically, however. This study was conducted to determine if changes in internal pH and catalase activity could explain how dissolved oxygen or D- versus L-lactic acid affected the survival of *E. coli* O157:H7 when the organism was exposed to low pH (3.2) values typical of fermented and acidified foods (μ, 0.342). For lactate concentrations of up to 40 mM, there was no significant difference in internal pH values when cells were incubated in D- versus L-lactic acid solutions. Unexpectedly, cells incubated in 5 mM lactic acid under aerobic conditions maintained a significantly higher internal pH (ca. 5.8) than cells incubated under anaerobic conditions (ca. 5.4), and cells under aerobic conditions survived better compared to anaerobic conditions (1.5 log reduction vs. 3 log reduction over 6 h, respectively) at pH 3.2, ionic strength of 0.342, at 30°C. Catalase activity significantly decreased in cell extracts when the pH dropped ≤ 6.0 in the phosphate buffer, regardless of the dissolved oxygen content. Catalase activity was reduced by the high ionic strength (μ, 0.5) representative of internal cell conditions for cells exposed to D-lactic acid at pH 3.2 (external). However, further work will be needed to explain the changes in survival between aerobic and anaerobic incubation.

4.2 Introduction

Previous research by Kreske *et al.* (26) has shown that the presence of oxygen can reduce the survival of *Escherichia coli* O157:H7 in low pH organic acid (OA) solutions. Maurer *et al.* (28) demonstrated that *Escherichia coli* K-12 induces genes in response to oxidative stress in an acidic pH environment and repressed these genes in a basic environment. They deduced that low pH may amplify the toxicity of oxygen radicals. Outbreaks of disease (7-11) in the 1990s demonstrated the capability of bacterial pathogens to survive in juices, and other low pH (pH 3 to 4) products, previously thought to be safe because of the presence of organic acids. The acid resistance of *E. coli* O157:H7 is of concern because of its acid resistance systems, its low infectious dose of ca. 100 cells or less (12), the ability of the organism to cause life-threatening hemolytic uremic syndrome (HUS) in children, and thrombotic thrombocytopenic purpura (TTP) in adults (21, 42). Bearson *et al.* (1) demonstrated that both AR2 and AR3 protected *E. coli* O157:H7 from oxidative stress caused by the addition of hydrogen peroxide when challenged at pH 2.5 for 4 h.

Most of the research on the effects of low pH (pH 3 to 4) and OA on survival of pathogens (15, 17, 37, 45) has been conducted on the bench top and does not evaluate survival under anaerobic conditions, where there is little or no dissolved oxygen. This is a problem because pickles, banana peppers, olives and most other acidified products that are typically prepared in hermetically sealed jars result in an anaerobic environment. The antimicrobial activity of an organic acid may be affected by several factors, such as the dissolved oxygen, pK_a, temperature, specific type of acid, acid concentration, pH, ionic

strength (μ), and growth phase or physiological state of the target organism. Kreske *et al.* (26) reported that there was a decrease in survival of *E. coli* O157:H7 as ionic strength (adjusted by NaCl) increased from 0.086 to 1.14, when cells were challenged in 20 mM gluconic acid at pH 3.2 for 6 h at 30˚C.

Organic acids are inhibitory to bacterial cells because of the accumulation of acid anions within the cell cytoplasm, reduced intracellular pH, and the inhibition of metabolic reactions (5). Lactic acid is a carboxylic acid that is colorless, nonvolatile, and is soluble in water. The acid has a pK_a of 3.86 and has two optical isomers, D- and L-lactic acid (39). Bjornsdottir *et al.* (3) showed a significant increase in survival of *E. coli* O157:H7 cells when incubated in D-lactic acid versus L-lactic acid at low concentration (1 to 20 mM), pH 3.2 and μ of 0.6, at 25°C. *E. coli* O157 and non-O157 isolates were more susceptible to L-lactic acid (50-200 mM) than D-lactic acid at pH 3.8 (30).

E. coli K-12 and other bacteria maintain an internal pH around neutral for optimum growth (32, 33) which allows cellular processes to occur. Diez-Gonzalez and Russell (18) demonstrated the ability of *E. coli* O157:H7 to decrease intracellular pH and thus prevent the accumulation of acetate anion (160 mM sodium acetate) when growing anaerobically.

Oxygen can serve as a terminal electron acceptor, but the presence of oxygen may result in the generation of reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide, and the hydroxyl radical which are detrimental to cell components (22). The hydroxyl radical can react with all cellular macromolecules and cause cell death (24, 40). The objectives of this study were to examine the effects of low pH acid solutions on the

survival of *E. coli* O157:H7 cells under aerobic and anaerobic conditions. Understanding

how dissolved oxygen and oxygen radicals affect the survival of *E. coli* O157:H7 in the

presence of D- and L-lactic acid may aid in the development of safer acidified foods.

4.3 Materials and Methods

4.3.1 Preparation of inocula

E. coli O157:H7 (USDA/ARS Food Fermentation Culture Collection, Raleigh, NC

strain B202, previous strain designation SRCC1486, salami outbreak isolate) was used in

these experiments, because it was more acid-resistant than other food isolates used in Chapter

II. Stock cultures of *E. coli* B202 were inoculated into 25 mL of tryptic soy broth (TSBG,

BBL/Difco) supplemented with 1% glucose and incubated statically for ca. 18 h at 30ºC to

induce acid resistance as previously described (6). The overnight cultures typically attained

10^{8-9} CFU/mL and a pH of ca. 4.9. Cultures were centrifuged (3,000 X *g*, 10 min, 20 $\pm$ 2°C),

washed, and resuspended in 0.85% saline solution. To determine initial populations, cells

were serially diluted, plated in duplicate (Model 4000, Spiral Biotech, Inc., Norwood, MA)

on tryptic soy agar supplemented with 1% glucose (TSAG, BBL/Difco), followed by

incubation at 30ºC for 24 h. Cell numbers were determined using an automated plate reader

(Q-count, Spiral Biotech, Inc., Norwood, MA). Cultures referred to as GS (grown statically)

were cells grown in TSBG statically for 18 h at 30°C; cultures referred to as GSA (grown

statically in the anaerobic chamber) were cells grown in TSBG statically in the anaerobic chamber for 18 h at 30°C.

4.3.2 Preparations of acid solutions

Calculations of pH, ionic strength, and protonated acid concentrations for the buffered acid solutions were made using custom Matlab routines (pH$_{tools}$, available at: http://www.mathworks.com/matlabcentral) as previously described (20). This program also allows for the adjustment of pK$_a$ values based on ionic strength (μ) and temperature. The acid solutions for internal pH experiments were adjusted using HCl or NaOH to reach the desired pH of 3.2 and NaCl was added to produce an ionic strength of 0.342 (equivalent to 2% salt, common in acidified food products). Unless otherwise noted, acid concentrations are reported as the protonated acid concentration, because the protonated form of the acid can freely diffuse through cell membranes (13, 19). Solutions were incubated aerobically (no additional treatment), or anaerobically by placing in an anaerobic chamber (COY Laboratory Products, Grass Lake, MI) and allowed to equilibrate with the anaerobic gas (5% CO_2, 10% H_2, and 85% N_2) for $\geq$ 48 h at room temperature (24 $\pm$ 2°C) prior to use.

4.3.3 Internal pH measurements

Internal pH was determined using a dual radiolabel acid distribution method as described by Riebeling *et al*. (34). Acid solutions were prepared with D- or L-lactic acid (Sigma) at concentrations 5 and 40 mM protonated acid at pH 3.2 and an ionic strength (μ) of

0.342. All solutions contained gluconic acid (20 mM, Sigma) which was used as a non-inhibitory low pH buffer to determine the inhibitory effects of pH 3.2 in the absence of other organic acids as previously described (4). Cells from overnight cultures (cell concentration of 10^{9-10} CFU/mL) were added to 800 μl of acid solutions under aerobic or anaerobic conditions along with triated water ^3H_2O (American Radiolabeled Chemicals, Inc, St. Louis, MO) to determine cell pellet volumes. The internal/external cell partitioning of benzoate [^{14}C] (American Radiolabeled Chemicals, Inc) was used to determine internal pH, and propylene glycol [^{14}C] (PEG, American Radiolabeled Chemicals, Inc) was used to determine external cell pellet water space. Cell suspensions in acid solutions were incubated at 30°C for 30 min (750 μl), and then centrifuged through silicone oil (750 μl, 65:35 of oil types 550 and 556, Dow Chemical Co., Midland, MI) in a microcentrifuge (16,000 X g, 15 min, 4 $\pm$ 2°C) to separate supernatant fractions and cell pellets. Supernatant fractions (200 μl) were removed immediately following centrifugation from the top (aqueous) layer in the microcentrifuge tube. The cell pellets were obtained by clipping off the bottom of tubes using dog toenail clippers (Millers Forge, Inc., Plano, TX) after freezing at -80°C for $\geq$ 40 min. Aliquots of the supernatant (100 μl) were combined with 500 μl of detergent solution (0.5% Tween 80, 50 mM Tris, Fisher scientific, Fair Lawn, NJ) and 5 mL of Scintisafe solution (Fisher chemicals, Fair Lawn, NJ) into 7 scintillation vials (Perkin Elmer, Waltham, MA) and mixed by inverting until clear. Similarly, 600 μl of detergent solution (0.5% Tween 80, 50 mM Tris) was added to the cell pellet within the microcentrifuge tube tip in a

scintillation vial (Perkin Elmer) and vortexed. Once the cells were resuspended, 5 mL of Scintisafe solution (Fisher chemicals) was added to the vial and inverted until clear.

Radioactive counts from the supernatant or pellet fractions were determined with a Beckmann LS 6500 scintillation counter (Beckmann Coulter, Inc, Fullerton, CA) using dual label ^{14}C, ^{3}H radionuclide counting with quenching, to determine disintegrations per minute (DPM). The DPM values from the supernatant and pellet fractions ([^{14}C] PEG and ^{3}H$_2$O) were used to determine the external water volumes in the cell pellet as described by Rottenberg (35). DPM values for [^{14}C] benzoate from the pellet fractions were then used to determine internal pH as described (37). The total internal concentration of lactic acid for each treatment was calculated based on the calculated internal pH, with the assumption that the internal cell and external protonated acid concentrations were equal.

4.3.4 Preparation of cell-free extracts

Cultures of *E. coli* O157:H7 (2-25 mL) were grown for 18 h in TSBG under static conditions as described above. The cells were collected by centrifugation (3,000 X *g*, 10 min, 20 $\pm$ 2°C) and resuspended in cold 0.05 M phosphate buffer at pH 7. Cell suspensions were added to 1.5 mL O-ring screw cap microcentrifuge tubes (T55-129, Lab source, Inc., Willowbrook, IL) containing 0.5 g of glass beads (acid washed, <106 μm, Sigma) and were disrupted in a mini-beadbeater (BioSpec products, Bartlesville, OK) at max speed for 1 min interval(s), with 1 min intervals on ice. The extracts were clarified by centrifugation (16,000

X *g,* 15 min, 4 ± 2°C) and were dialyzed (8000 MW) overnight at 4°C against the 0.05 M

phosphate buffer before assayed for protein and enzymatic activity.

4.3.5 Catalase assay

Cell extracts were assayed for catalase activity by the method of Beers and Sizer (2)

with minor modification. All cell extracts were assayed for protein content using the Lowry

method (27) based on a standard curve with bovine serum albumin (Sigma). To determine

the pH profile of catalase from acid-adapted *E. coli* O157:H7 cell-free extracts, assays were

carried at pH 5.0, and 5.5, in 0.05 M potassium acetate buffer and at pH 6.0, 6.5, 7.0 and 7.5,

in 0.05 M potassium phosphate buffer.

To determine the catalase activity in conditions (pH and total acid concentration) that

mimic the cell's environment, solutions were prepared at the previously calculated pH values

and total acid concentrations inside the cell. Assay solutions prepared in 0.05 M potassium

phosphate buffer at pH 5.8 (internal pH measurement from treatment with 5 mM protonated

lactic acid under aerobic conditions) contained 0.5 M total D- or L-lactic acid (Sigma), 0.5 M

total D- or L-lactic acid with 20 mM gluconic acid, and 0.48 M KCl with 20 mM gluconic

acid (as an ionic strength control). Assay solutions prepared in 0.05 M potassium acetate

buffer at pH 5.4 (internal pH measurement from treatment with 5 mM protonated lactic acid

under anaerobic conditions) contained 0.2 M total D- or L-lactic acid (Sigma), 0.2 M total D-

or L-lactic acid with 20 mM gluconic acid, and 0.18 M KCl with 20 mM gluconic acid.

Assays were carried out at room temperature (24 ± 2°C) using a Kontron Spectrophotometer (Research Instruments International, San Diego, CA).

4.3.6 Statistical analysis

Experiments were independently replicated three times. Data were analyzed using the general linear models with Tukey adjustment of the Statistical Analysis Systems version 9.1 (Statistical Analysis System, SAS Institute, Cary, N.C.). Results were considered significant when the P-value was $P \leq 0.05$.

4.4 Results

4.4.1 Internal pH

Internal pH measurements were taken after 30 min of incubation in 20 mM gluconic acid with protonated D- or L-lactic acid (5, 40 mM) at 30º C as shown in Figure 23. *E. coli* O157:H7 incubated in 20 mM total gluconic acid alone at pH 3.2 maintained an internal pH $\geq$ 6.64, regardless of atmosphere (aerobic or anaerobic) during incubation, resulting in an internal to external pH gradient of $\geq$ 3.44 units. There was no significant difference ($P >$ 0.05) in internal pH values when cells were incubated in D- versus L-lactic acid solutions for concentrations up to 40 mM protonated acid (ca. 55 mM total external acid). Cells incubated in 5 mM protonated D- or L-lactic acid under aerobic conditions maintained a significantly higher ($P \leq 0.05$) internal pH (ca. 5.8) than cells incubated under anaerobic conditions (ca.

5.4, Figure 23). A significant decline ($P \leq 0.05$) in internal pH (ca. pH 5.2) was measured

when *E. coli* O157:H7 cells were incubated in 40 mM protonated L-lactic acid solutions

compared to 20 mM gluconic acid (pH 6.64) (Figure 23). There was no significant

difference ($P > 0.05$) found in the internal pH of cells incubated in 40 mM protonated lactic

acid, regardless of isomer or atmosphere.

4.4.2 Catalase activity

Catalase activity was measured in phosphate buffer at pH 5.0 to 7.5 to determine a pH

profile. All results are reported as percentages in relation to the optimum pH 7 which was

28.66 ± 3.79 units/mg protein for GS cell extracts and 19.92 ± 2.79 units/mg protein for GSA

cell extracts. Catalase activity of GSA cell extracts ($82 \pm 5\%$) at pH 7.5 was significantly

higher ($P \leq 0.05$) than from GS cell extracts ($73 \pm 3\%$, Figure 24). The catalase activity of

GS cell extracts measured at pH 5.5 and 5.0 were $51 \pm 3\%$ and $24 \pm 3\%$ the activity of the

optimum, respectively (Figure 24).

Using calculated internal pH values (Figure 23) from incubation in 5 and 40 mM

protonated D- or L-lactic acid, the internal acid anion concentrations were determined using

the Henderson-Hasselbalch equation: $pH_i = pK_a + \log_{10}([A\text{-}]/[HA])$, where HA is 5 or 40

mM and the pK_a of lactic acid is 3.8. The internal anion concentration of lactic acid (Table

2) was calculated based on the measured internal pH assuming that the protonated acid was

equal inside and outside the cell. The internal pH value and internal anion concentration

were used to determine how the catalase enzyme would behave inside the cell when exposed

to the test solutions. Gluconic acid, 20 mM, had no significant effect ($P > 0.05$) on catalase activity in cell-free extracts (data not shown) when measured at pH 6.6 (internal pH measurement from treatment with 20 mM gluconic acid). At pH 5.8 (internal pH measurement from treatment with 5 mM protonated lactic acid under aerobic conditions), GSA catalase activity ($72 \pm 4\%$) was significantly higher ($P \leq 0.05$) than activity from GS cell extracts ($54 \pm 2\%$, Figure 25). There was a significant increase ($P \leq 0.05$) in catalase activity when cell extracts were measured in 0.5 M total L-lactic acid (see Table 2) compared to D-lactic acid at pH 5.8. At pH 5.4 (internal pH measurement from treatment with 5 mM protonated lactic acid under anaerobic conditions), catalase activity from GS cell extracts was significantly higher ($P \leq 0.05$) than catalase activity from GSA cell extracts when measured in 0.2 M total L-lactic acid alone or with 20 mM gluconic acid (Figure 26). In previous experiments, gluconic acid was used as a non-inhibitory low pH buffer (4), to determine how the presence of gluconic acid (20 mM, Sigma) effects catalase activity it was added to lactic acid solutions. Catalase activity of cell extracts at pH 5.4 with L-lactic with 20 mM gluconic acid was significantly higher ($P \leq 0.05$) than other lactic acid containing treatments. At pH 5.2 (internal pH measurement from treatment with 40 mM protonated lactic acid) there was a significant decline ($P \leq 0.05$) in catalase activity when cell extracts were measured in 1 M total L-lactic acid (See Table 2) or with 20 mM gluconic acid compared to 20 mM gluconic acid solution (data not shown).

Because ionic strength can affect enzyme activity, potassium chloride was substituted for lactic acid to confirm that changes in catalase activity where a result of the presence of

lactic acid (0.48 M potassium chloride plus 20 mM gluconic acid at pH 5.8 and 0.18 M

potassium chloride plus 20 mM gluconic acid at pH 5.4). At pH 5.8, the catalase activity in

0.48 M potassium chloride plus 20 mM gluconic acid was not significantly different ($P >$

0.05) than that from 0.5 M total D-lactic or with 20 mM gluconic acid (Figure 25).

4.5 Discussion

The ability of *E. coli* O157:H7 to survive and cause infection in products that use low

pH and organic acids as their main hurdles is of concern because of previously documented

disease outbreaks associated with fruit juices (9-11). Previous research with low pH acid

solutions by Kreske *et al.* (26) has shown a significant decrease in survival of *E. coli*

O157:H7 under aerobic conditions compared to anaerobic conditions, determining the

intracellular pH and activity of oxygen detoxifying enzymes may help explain these results.

A study was conducted to determine 1) the internal pH of *E. coli* O157:H7 in the presence of

D- or L-lactic acid at pH 3.2 (µ, 0.342) under different dissolved oxygen conditions at 30°C

and; 2) the catalase activity of *E. coli* O157:H7 cell extracts in phosphate buffers with pH

values corresponding to the internal pH measurements with the addition of lactic acid.

Understanding how dissolved oxygen and oxygen radicals affect the survival of *E. coli*

O157:H7 in the presence of organic acids may aid in the development of safer acidified

foods.

Lactic acid is used commonly in food products for flavor or to lower pH. Research

has shown that the two isomers of lactic acid affect cells differently, a decrease in survival is

observed when cells are exposed to L-lactic acid compared to D-lactic acid (3, 16, 30). For

concentrations up to 40 mM, there was no significant difference ($P > 0.05$) in internal pH

values when cells were incubated with protonated D- versus L-lactic acid solutions (Figure

23). This is in agreement with previously published papers (23, 30). After 30 min of

incubation, the pH gradient when *E. coli* O157:H7 cells were incubated in 40 mM lactic acid

was ca. 2 pH units above the external pH (3.2), regardless of dissolved oxygen (Figure 23).

It was hypothesized that there would be a considerable drop in pH after 30 min because of

the low external pH and presence of organic acids, however, cells were able to maintain an

internal pH around 5. Shechter *et al.* (38) demonstrated with fluorescence dyes that 3 strains

of *E. coli* K-12 maintained an internal pH ca. 7.5 for external pH 5.5-7.5. Cells incubated in

5 mM D- or L-lactic acid under aerobic conditions maintained a significantly higher ($P \leq$

0.05) internal pH (ca. 5.8) than cells incubated under anaerobic conditions (ca. 5.4, Figure

23) which creates a lower pH gradient. Kreske *et al.* (26) determined that survival of *E. coli*

O157:H7 was significantly better when cells were incubated in lactic acid solutions under

anaerobic conditions. Studies by Russell (36) demonstrated that acid tolerant bacteria

allowed their intracellular pH to drop along with the extracellular pH to maintain a small pH

gradient which prevented an accumulation of volatile fatty acid anions within the cell (until

the pH < 5.2). The internal pH of *Lactobacillus plantarum* decreased to ca. 4.5 as the

external pH dropped to 3.0 in modified HHD media (29). The pH gradient of *L. plantarum*

(modified HHD, pH 5.0) decreased with the addition of sodium acetate or lactic acid up to

160 mM. The ability of *L. plantarum* to lower internal pH as the pH decreases allows the

organism to predominate vegetable fermentations. Diez-Gonzalez and Russell (18) demonstrated the ability of *E. coli* O157:H7 to decrease intracellular pH and thus prevent the accumulation of acetate anion (up to 160 mM sodium acetate) when growing anaerobically at pH 5.9. Jordan *et al.* (25) reported that *E. coli* O157:H7 achieved a final cytoplasmic pH of ca. 5.2 when 50 mM protonated lactic acid was added to the incubation media at pH 4.0 (25). The ability of microorganisms to lower internal pH aids in survival by reducing the accumulation of organic acids.

Under anaerobic conditions, there was no significant difference ($P > 0.05$) in the reduction of *E. coli* O157:H7 cells regardless of lactic acid concentration (as shown in Figure 14). For cells challenged in 20 mM gluconic acid there is no significant difference ($P > 0.05$) in internal pH values under aerobic and anaerobic conditions but the reduction of viable *E. coli* cells was different. Under aerobic conditions, there was a significant decline ($P \leq 0.05$) in internal pH when *E. coli* O157:H7 cells were incubated in 40 mM protonated L-lactic acid solutions (From 6.6 to 5.13-5.18, Figure 23) and a corresponding reduction in cell survival (1.5 log CFU/mL reduction to 4-4.7 log CFU/mL reduction, Figure 14). However, under anaerobic conditions, as the pH declines along with catalase activity, there is no difference in the reduction of viable cells. Our results show that catalase activity plays a role in *E. coli* O157:H7 survival in low pH acid solutions under aerobic conditions and further research needs to be performed.

A decline in *E. coli* O157:H7 catalase activity under acid and oxygen stress may contribute to the inability of the organism to detoxify ROS which may lead to decreases in

survival. The optimum pH for catalase activity of *E. coli* O157:H7 was 7.0. Moore *et al.*

(31) determined the optimum activity of *E. coli* catalase (*katG*) to be at pH 6.5. Singh *et al.*

(41) demonstrated the pH optimum for catalase activity (*katG*) of *Archaeoglobus fulgidus,*

Bacillus stearothermophilus, *Burkholderia pseudomallei*, *Escherichia coli*, *Mycobacterium*

tuberculosis, *Rhodobacter capsulatus* and *Synechocystis* PCC 6803 was between 6 and 6.5.

Varnado *et al*. (43) determined the optimum of catalase activity (*katP*, perisplamic) of *E. coli*

O157:H7 to pH 7.2 and cited a sharp pH dependence. Results from the pH profile

demonstrated that catalase activity is dependent on pH and of the pH tested activity ranked

6.5, 6.0, 7.5, 5.5, and 5.0 (Figure 24). A significant decline ($P \leq 0.05$) in catalase activity

was observed when the pH was > 7 and ≤ 6 (Figure 24). Claiborne and Fridovich (14)

reported a similar decline in *katG* activity when the pH was < 6 for *E. coli* B. Catalase

activity measured at pH 5.4 and 5.2 was $36 \pm 6\%$ and $29 \pm 4\%$ from GSA cell extracts

correlates with internal pH measurements when *E. coli* O157:H7 cells were incubated in 5

and 40 mM D- or L-lactic acid under anaerobic conditions. Internal pH of *E. coli* O157:H7

cells incubated in 5 mM lactic acid under aerobic conditions was ca. 5.8 and the

corresponding catalase activity was $54 \pm 2\%$. This significant difference observed in internal

pH between aerobic (pH 5.8) versus anaerobic (pH 5.4) cells treated with 5 mM D- or L-

lactic acid shows a increase in catalase activity under aerobic conditions as a result of the

higher internal pH. Bearson (1) reported that acid resistance system 2 and 3 protect *E. coli*

O157:H7 from oxidative stress at low pH by increasing internal pH. As shown in our results

the higher the internal pH the higher the catalase activity thus aiding in cell survival. This

increase in catalase activity under aerobic conditions helps the cell deal with ROS possibly explaining the protective effect seen when *E. coli* O157:H7 is treated with 5 mM lactic acid.

The activity of enzymes under pH stress is dependent upon several factors, such as temperature, buffer (nature and concentration), ionic strength, etc. (44). At pH 5.8, catalase activity in potassium chloride and D-lactic acid solutions were not significantly different ($P > 0.05$). However there was a significant increase ($P \leq 0.05$) in activity when cells extracts were tested in the presence of L-lactic. These results were unexpected since previous literature has shown that *E. coli* O157:H7 survives better in D-lactic solutions (3). The increase in catalase activity in the presence of L-lactic acid is most likely a result of the production of pyruvate produced by lactate dehydrogenase. The pyruvate nonenzymatically reacts with hydrogen peroxide to produce acetic acid and water thus there would be an increase in the disappearance of hydrogen peroxide. Further experiments are needed to determine if pyruvate production was responsible for the apparent increase in catalase activity observed with L-lactic acid.

Previous work by Kreske *et al.* (26) has shown a significant difference in survival of *E. coli* O157:H7 in organic acid solutions to be dependent on the presence of oxygen during exposure to the acid. Overall under aerobic conditions, as the external acid concentration at pH 3.2 (µ, 0.342) increased from 0 to 40 mM lactic acid, internal pH declined, catalase activity was reduced and *E. coli* O157:H7 cell populations were reduced. Internal pH of cells incubated aerobically in 5 mM lactic acid was significantly higher ($P \leq 0.05$) than cells incubated anaerobically. Previous data on the survival of *E. coli* O157:H7 in acid solutions

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was carried out for 6 h (3, 26), however, to maintain viable cell populations for internal pH experiments, cells were incubated for only 30 min. Under anaerobic conditions (5 mM lactic acid), the pH gradient was lower than under aerobic conditions thus decreasing the amount of acid anion from entering the cell, however, the relationship between internal pH, ROS and cell survival may be more complex than previous work indicates.

4.6 Acknowledgements

4.7 References

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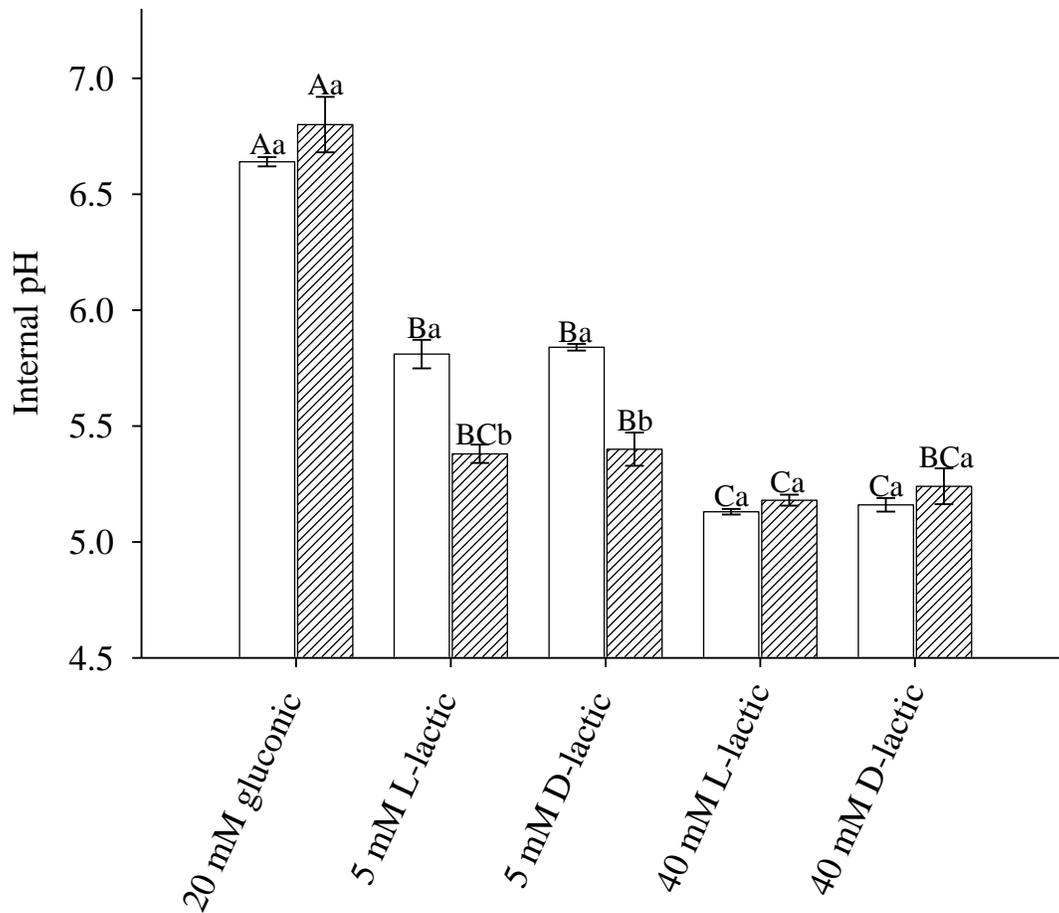
Figure 23. The internal pH of *E. coli* O157:H7 in lactic acid solutions at pH 3.2.
Cells were incubated under aerobic (empty bars) and anaerobic conditions (striped bars) in solutions with an ionic strength (μ) of 0.342 at pH 3.2 for 30 min at 30ºC. Error bars indicate the standard deviation for 3 trials. Within the same atmosphere, mean values without the same capitalized letter are significantly different ($P \leq 0.05$). Within the same concentration, mean values without the same lower case letter are significantly different ($P \leq 0.05$).

Conc. (mM)	Lactic acid isomer	Aerobic			Anaerobic		
		pH_i	Conc. (mM)[a]	SD	pH_i	Conc. (mM)[a]	SD
5	L	5.81	B 511.60 a	71.6	5.38	B 190.10 b	17.4
	D	5.84	B 548.24 a	18.6	5.40	B 199.10 b	51.4
40	L	5.13	A 855.18 a	23.7	5.18	A 959.50 a	33.4
	D	5.16	A 916.36 a	62.4	5.24	A 1101.60 a	202.7

[a]Mean values in the same column that are not preceded by the same capitalized letter are significantly different ($P \leq 0.05$). Within the same row, mean values that are not followed by the same lower case letter are significantly different ($P \leq 0.05$).

Table 2. Anion accumulation of *E. coli* O157:H7 incubated in lactic acid solutions at pH 3.2 at 30°C
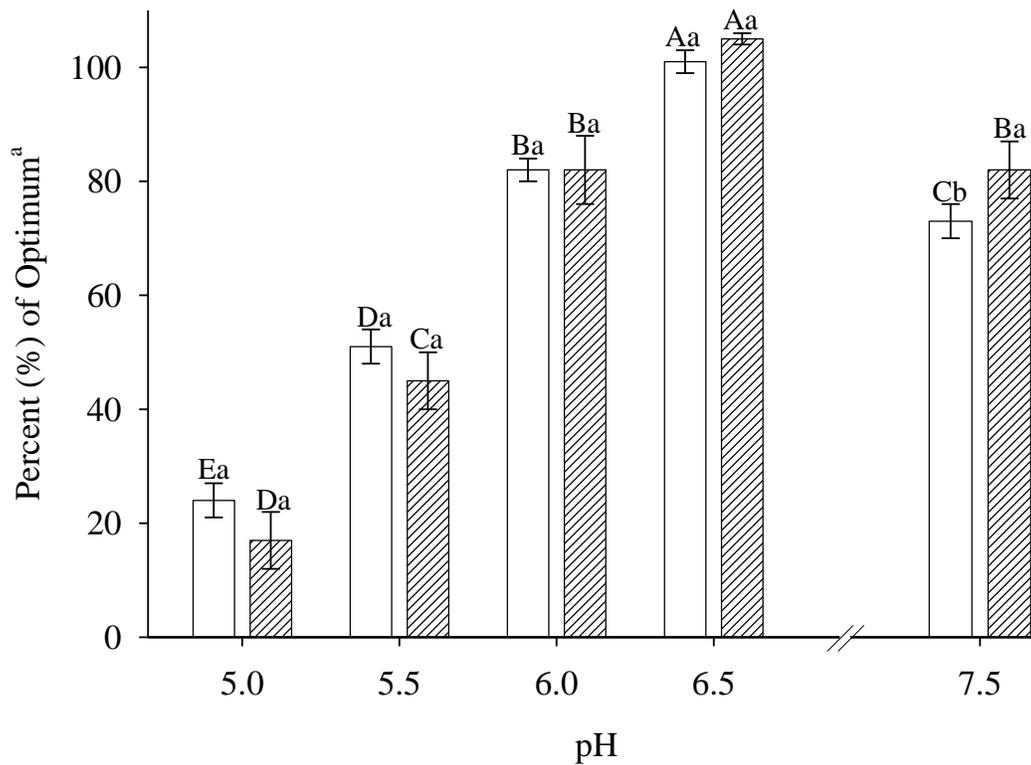
Figure 24. Effect of pH on catalase activity of _E. coli_ O157:H7 cell-free extracts
Cells were grown statically under aerobic conditions (empty bars) and anaerobic conditions (striped bars). In the range 5.0 to 5.5, buffering was achieved with potassium acetate; 6.0 to 7.5, potassium phosphate. Error bars indicate the standard deviation for 3 trials. [a] All results were reported as percentages in relation to the optimum pH (7). Within the same atmosphere, mean values without the same capitalized letter are significantly different ($P \leq$ 0.05). Within the same pH, mean values without the same lower case letter are significantly different ($P \leq 0.05$).
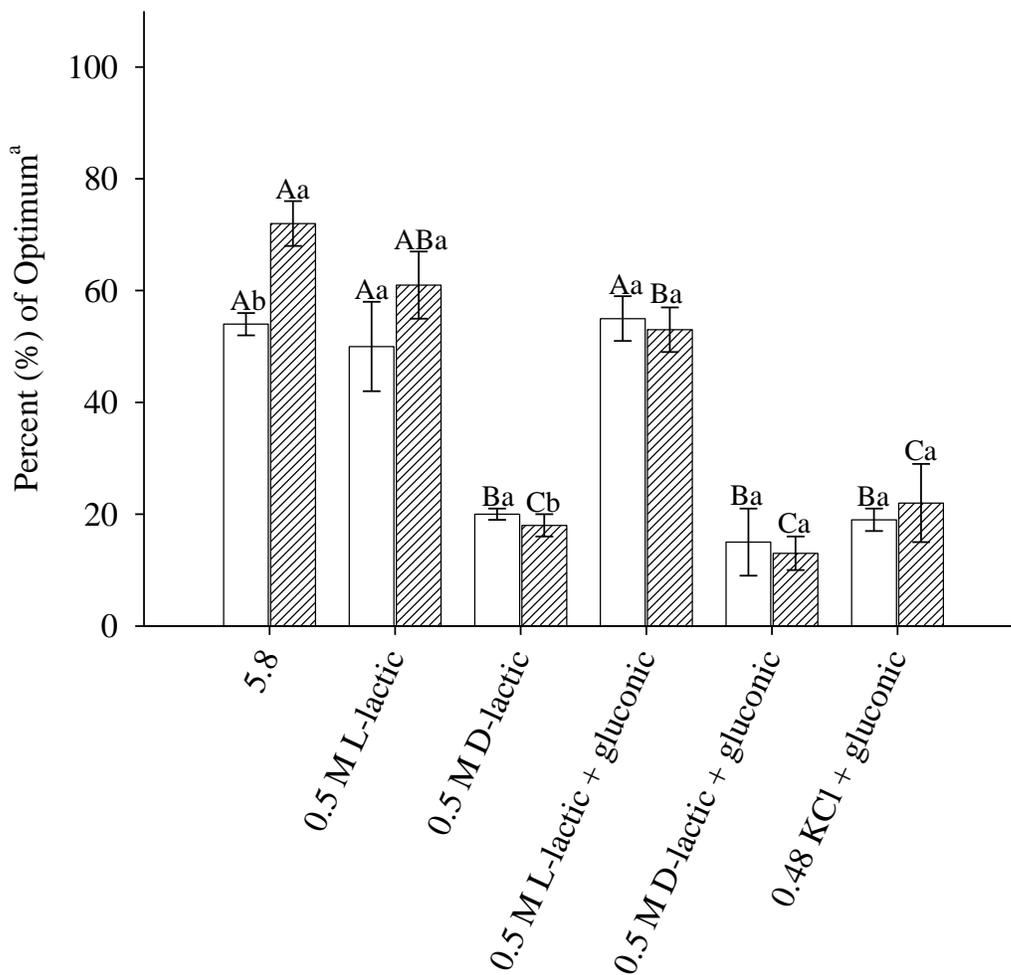
Figure 25. Catalase activity of *E. coli* O157:H7 cell-free extracts exposed to lactic acid at pH 5.8.
Cells were grown statically under aerobic conditions (blank bars) and anaerobic conditions (striped bars). The potassium phosphate buffer was adjusted to pH 5.8 and lactic acid, gluconic acid and potassium chloride were added as indicated. The ionic strength of solutions containing lactic acid and potassium chloride was 0.5 and solutions containing lactic acid and gluconic acid was 0.52. Error bars indicate the standard deviation for 3 trials. Within the same atmosphere, mean values without the same capitalized letter are significantly different ($P \leq 0.05$). Within the same test solution, mean values without the same lower case letter are significantly different ($P \leq 0.05$). [a] All results were reported as percentages in relation to the optimum pH (7).
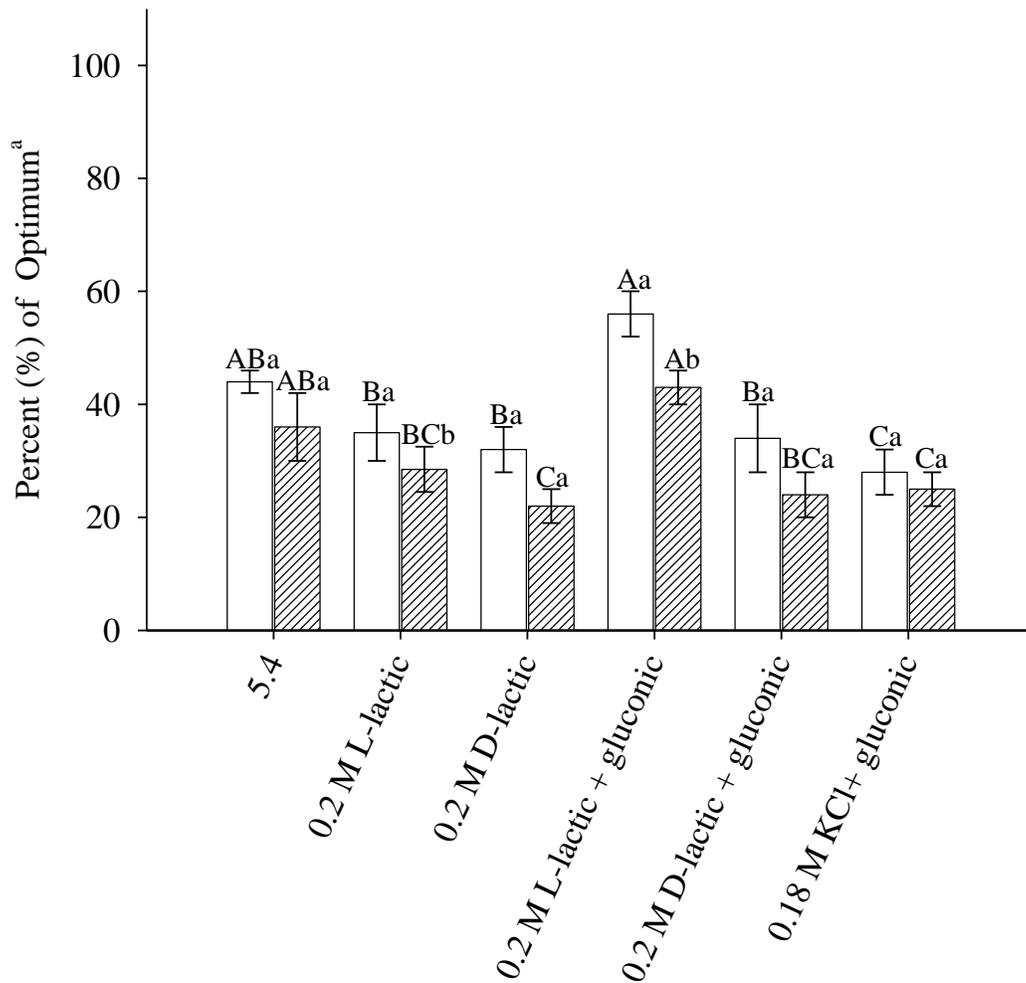
Figure 26. Catalase activity of *E. coli* O157:H7 cell-free extracts exposed to lactic acid at pH 5.4

Cells were grown statically under aerobic conditions (blank bars) and anaerobic conditions (striped bars). The potassium acetate buffer was adjusted to pH 5.4 and lactic acid, gluconic acid and potassium chloride were added as indicated. The ionic strength of solutions containing lactic acid and potassium chloride was 0.2 and solutions containing lactic acid and gluconic acid was 0.22. Error bars indicate the standard deviation for 3 trials. Within the same atmosphere, mean values without the same capitalized letter are significantly different ($P \leq 0.05$). Within the same test solution, mean values without the same lower case letter are significantly different ($P \leq 0.05$). [a] All results were reported as percentages in relation to the optimum pH (7).