DETERMINATION OF 5-LOG REDUCTION TIMES IN *ESCHERICHIA COLI* O157:H7 AND *LACTOBACILLUS* SPECIES IN CUCUMBER JUICE MEDIUM WITH VARIED SALT TREATMENTS

by

DOROTHY E. DUPREE

(Under the Direction of Elizabeth L. Andress)

ABSTRACT

Survival of pathogenic bacteria, such as *Escherichia coli* O157:H7, in fermented and acidified vegetables continues to be an area of concern in the processed vegetable industry. Survival of fermentative microorganisms, such as *Lactobacillus* species (spp.), is desired. This study determined 5-log reduction times in 5-strain cocktails of *E. coli* O157:H7 and *Lactobacillus* spp. independently in cucumber juice media with varied salt treatments of 2% NaCl, 6% NaCl, and 1.1% $CaCl_2$. 5-log reductions were reached for *E. coli* O157:H7 in 14 hours (6% NaCl) and 18 hours (1.1% $CaCl_2$), but not observed by 18 hours in 2% NaCl. For *Lactobacillus* spp., a 5-log reduction was reached in 40 hours (6% NaCl) and 46 hours (2% NaCl), but not observed by 46 hours for $CaCl_2$. There is some evidence from this study that $CaCl_2$ could be useful for reducing *E. coli* O157:H7 populations while allowing survival of desirable *Lactobacillus* spp.

INDEX WORDS: *Escherichia coli* O157:H7, *Lactobacillus*, Cucumber, Acidified vegetables, Fermented vegetables, Salt treatment

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DOROTHY E. DUPREE

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DOROTHY E. DUPREE

Major Professor:	Elizabeth L. Andress
Committee:	Fred Breidt
	Jamie A. Cooper
	Judy A. Harrison
	Barbara M. Grossman

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
August 2017

DEDICATION

This scientific masterpiece is dedicated to my Mom and Dad.

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USDA Agricultural Research Service

Food Science Research Unit

Dept. of Food, Bioprocessing and Nutrition Sciences

322 Schaub Hall, Box 7624, NC State University

Raleigh, NC 27695-7624

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TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Pickled and acidified foods are popular components of the typical U.S. diet, as well as in international cultures. Commonly consumed acidified condiments are pickled fruits and vegetables, including mixtures such as salsas and other relishes (USDA, 2015). Pickles of one type or another are eaten by more than 67% of American households; the per capita American consumption is at least 9 pounds annually (Pickle Packers International, 2017). More than 30 states grow pickling cucumbers in the U.S. Up to half of the $1.5 billion per year pickled vegetable market consists of products that are actually preserved without thermal processing. This includes fermented products, which are naturally acidified and defined as acid foods (Breidt and Caldwell, 2011). Fermented food production today constitutes one of the world's largest industries, with some estimates showing that as much as one-third of all food eaten by humans worldwide is fermented. Commonly regarded benefits of fermented foods, which may explain their widely estimated consumption, include: preservation, health, energy efficiency and flavor (Katz, S.E., 2012).

Cucumbers as well as other vegetables can be quick pickled through the addition of acid brines (such as vinegar-based seasoned solutions). Vegetables can also be fermented in salt brine solutions where the growth of primarily lactic acid bacteria

decrease the reducing sugar content and increases acidity of the vegetable substrate (USDA, 2015). The result also changes the flavor to that of a pickled food.

Producing acidified foods for meeting regulatory-defined requirements for commercial processing (21 CFR 114) as well as standards for safe thermal processing at home as an acid food requires achieving an equilibrium pH of 4.6 or below (USDA, 2015). This goal is to prevent germination of *Clostridium botulinum* spores that would then result in cell proliferation and toxin formation during room temperature anaerobic storage. Shelf stability of some acidified foods is usually achieved by a short thermal (canning) process in home preservation, followed by storage in a hermetically sealed jar. In commercial processing, regulations mandate killing of vegetative bacterial pathogens and preventing growth of spoilage microorganisms in the product. Heat processing of filled jars may be used to achieve pathogen destruction. However, other controls are usually employed for very acid products. These include pasteurization and a hot-fill only or use of acids and/or salts to achieve reductions in bacterial populations (Breidt et al., 2013; National Food Lab, 2015).

Survival of pathogenic bacteria in fermented and acidified vegetable products continues to be an area of concern in the processed vegetable industry as alternatives to thermal processing are sought. One documented approach is to use a hold time at a given temperature to achieve a 5-log reduction of targeted pathogens (National Food Lab, 2015). However, sufficient scientific literature is not available to allow commercial processors and regulators to meet the Food Safety Modernization Act requirements for demonstrating safety by this 5-log reduction standard; researchers are actively

conducting studies to build databases that processing authorities can utilize (Breidt et al., 2007; Breidt et al., 2013; Ingham et al., 2017).

Acid-resistant microorganisms are the primary pathogens of concern in fermented and acidified vegetables. The pathogenic bacteria, *Escherichia coli* O157:H7, particularly is considered to be the most acid resistant pathogen of concern for non-fermented acidified foods. However, this organism is also of concern for its ability to survive in fermentation procedures. Recent research has shown that *E. coli* O157:H7 can require between 3 and 25 days to achieve a 5-log reduction in cell numbers in cucumber fermentation brines, depending on specific brine conditions (Breidt and Caldwell, 2011). Standard pickling methods have historically used sodium chloride (NaCl) as the salt of choice to inhibit microbial growth during fermentation and pickling. More recent research has been investigating the use of calcium chloride ($CaCl_2$) as an alternative, due to waste water issues near commercial processing plants (USDA, 2016).

Several research projects are ongoing to determine bacterial survival and reduction rates under various acidic conditions using both NaCl and $CaCl_2$ (USDA, 2016). As a precursor to studying specific acidified food systems, data are still needed to characterize certain survival and reduction patterns of bacteria of interest in pickling and fermentation. Pathogen control is required and focuses on *E. coli* O157:H7 control, as the most acid resistant pathogen of interest. At the same time, behavior of lactic acid bacteria under the same conditions must be understood because of their critical role in fermentation.

The current study was undertaken to determine 5-log reduction times of *E. coli* O157:H7 and *Lactobacillus* species (spp.) in a 50% cucumber juice medium with varied salt type and concentration. The growth medium was adjusted to a pH of 3.2 and equilibrated at 30 °C for all samples. Samples were inoculated with a 5-strain cocktail for each microorganism. Log reductions for samples were determined were by: 1) spiral plating samples at specified time points, 2) storing plates at 30 °C for *Lactobacillus* spp. and 37 °C for *E. coli* O157:H7 to allow for colony growth, and 3) counting surviving colonies. The results will contribute to the scientific database needed to meet newer regulatory goals for 5-log reduction in processed acidified foods.

CHAPTER 2

LITERATURE REVIEW

The microbiology of foods

Food microbiology is the study of the biology of the microorganisms present in food including: their growth characteristics, identification, and pathogenesis. Food microbiologists study the relationship between microorganisms and food poisoning, food spoilage, food preservation, and food legislation. Bacteria are the most abundant microorganisms and include species that are both beneficial and harmful to human health, allowing an opportunity for conduction of extensive research to characterize their individual behavior within a food system (USDA 2011). There are several parameters that affect the growth of microorganisms including pH, moisture content, oxidation-reduction potential, nutrient content, antimicrobial constituents within the food system, and temperature. Within research, these parameters are either controlled for or manipulated to assess their effects on growth and death of specific microorganisms within a food system (USDA 2011).

The microflora of fresh vegetables

Vegetables are commonly eaten raw or fermented (non-heat treated) and, as a result, may inherently present a food safety risk. Fresh vegetables are capable of serving as a natural habitat for a wide variety of microorganisms. Representative microflora of vegetables includes aerobic bacteria (*Pseudomonas*, *Staphylococcus*, *Bacillus*), lactic acid

bacteria (*Lactobacillus*, *Streptococcus*, *Pediococcus*), enteric bacteria (*Enterococcus*, *Enterobacter*, *Escherichia*), and yeasts and molds (*Aspergillus*, *Penicillium*, *Fusarium*). In raw vegetable products, lactic acid bacteria are actually outnumbered by non-lactic competitors by a thousand times or more. Total populations of *Pseudomonas*, *Flavobacterium*, *Bacillus*, and *Escherichia* are capable of reaching levels as high as 10^7 cells per gram, while lactic acid bacteria are normally present at only about 10^3 cells per gram (Mehta, B.M. 2012). It is worth noting that, despite the disparity in population numbers, successful lactic acid fermentation of vegetables can be done with appropriate salt concentration, temperature control, and a proper anaerobic environment as long as a minimal lactic acid population is present (Hutkins, R.W. 2006).

Increased fresh produce demand within the U.S. is closely related to the agricultural development of the central coast region of California. This region is actually known as the "Salad Bowl of America." This region in California is capable of providing leafy greens and other various vegetables year-round to the rest of the nation because of the ideal climate for production. The increased demand requires shipping over long distances rapidly. Increased efficiency of production/harvest, new/improved cultivars, and new methods for treating plant disease have been areas of innovation to handle this increased demand. However, there remains an increase in illnesses and outbreaks, including multistate outbreaks, many of which have a source region of California documented. *Escherichia coli* O157:H7 has been linked to leafy greens in several documented outbreaks dating back to 1995. *Salmonella* has also been implicated in several outbreaks of cantaloupe and tomato commodities. In 2006, a multistate *E. coli*

O157:H7 outbreak was seen in bagged baby spinach that originated from California. Over 200 individuals fell ill as a result. *S.* Newport was implicated in a tomato outbreak originating in Virginia that resulted in over 500 ill individuals (Fan et al., 2001).

Removal of pathogenic and spoilage bacteria from fresh fruits and vegetables prior to any processing is difficult. Biofilms containing pathogenic bacteria on fresh produce may be more resistant to sanitizing agents and other organic acid treatments than free cells (Kumar and Anand 1998; Bower and Daeschel 1999; Riordan, et al., 2001; Sapers 2001). The USDA AMS Microbial Data Program assessed the incidence of *E. coli* on selected fresh produce items from 2002-2007. Over 65,000 samples were analyzed. Both domestic and imported commodities were tested (including cantaloupe, leaf and romaine lettuce, tomatoes, green onions, etc.) for generic *E.* coli, including *E. coli* O157:H7, and *Salmonella*. Results from the multi-year studies indicated low levels of *E. coli* on produce items. Samples were considered positive for *E. coli* if >0.03 MPN/ml rinse was determined. (MPN refers to the most probable number and is generated using a standardized tool based on sample dilutions showing positive coliform and/or fecal coliform). Only 1.5-2.7% of samples each year were positive for *E. coli* at concentrations >10 MPN/ml (USDA 2008). Although major outbreaks are of concern, it should be emphasized that relative to the consumption frequency of ready-to-eat produce, outbreaks are not frequent, resulting in a low number of total cases per total consumptions. Nevertheless, researchers seek to identify what is a rare event versus practices within food production and handling that may lead to increased risk of contamination and possible outbreaks (Fan et al., 2001).

As stated previously, both lactic acid bacteria, such as *Lactobacillus*, and enteric bacteria, such as *Escherichia*, are naturally present in the environment. Vegetables can serve as a natural habitat for these microorganisms. Population numbers can vary widely though, in general, non-lactic acid competitors outnumber lactic acid bacteria. To reiterate, with an appropriate starting population of lactic acid bacteria, fermentation has long been understood to proceed, with colonies of lactic acid bacteria in brines demonstrating a competitive advantage versus other microorganisms, including enteric bacteria. Several additional factors, such as pH, temperature, acid concentration, and salt create an environment for such a competitive advantage to exist (Hutkins, R.W. 2006). Despite a wealth of anecdotal evidence showing this to be true, the scientific realm of research has yet to fully catch up. Although there has been a long history of vegetable fermentation and non-thermal acidified vegetable processing, the USDA and Center for Home Food Preservation have limited information to disseminate to consumers for safe home-fermentation. Industry is also looking for guidance to handle non-thermal processing of these products to achieve appropriate reduction in foodborne pathogens, such as *Escherichia coli* O157:H7, as the Food Safety Modernization Act (FSMA) begins to fully go into effect (Ingham, et al., 2017).

To date, much of the industry guidance relates to the Code of Federal Regulations (21 CFR 114; 17) mandate that vegetative cells of microorganisms of public health significance be destroyed during the manufacture of acidified canned foods. The FDA's 2010 draft *Guidance for Industry: Acidified Foods* further specifies that "To be adequate… the scheduled process for acidified foods should be sufficient to destroy or

prevent the presence of vegetative cells of…pathogenic microorganisms (such as *Escherichia coli* O157:H7, *Listeria monocytogenes,* and *Salmonella* species) that are acid-tolerant. Destruction of pathogens is usually achieved via a thermal process, ensuring a 5-log pathogen reduction (FDA, 2010). With many acidified and fermented vegetable products, thermal processing for a 5-log reduction does not take place. It is therefore important to characterize pathogen survival, particularly acid-resistant pathogens such as *E. coli* O157:H7, in environments the microorganisms are likely to face in an acidified or fermented product. Since lactic acid bacteria, such as *Lactobacillus*, will also be naturally occurring in such an environment, it is important to characterize its survival as well. As mentioned previously, in successful fermentations, lactic acid bacteria demonstrate a competitive advantage versus other initially present microorganisms. Before quantifying such a competitive advantage scientifically, it is important to first characterize their survival patterns independently.

Characteristics of *Escherichia coli (E. coli)*

What is *Escherichia coli?*

Escherichia coli (E. coli) is a Gram-negative, rod-shaped facultative anaerobic bacterium. Most *E. coli* strains colonize the gastrointestinal tract of humans and animals as part of the normal flora and do no harm (Lim et al., 2010). Some *E. coli* strains have evolved into pathogenic bacteria, however, by acquiring virulence factors through plasmids, transposons, bacteriophages, and/or pathogenicity islands. Categorization of these pathogenic types is based on serogroups, pathogenicity mechanisms, clinical symptoms, or virulence factors (Kaper et al., 2004).

Among these pathogenic categories is enterohemorrhagic *E. coli* (EHEC). EHEC are pathogenic *E. coli* strains that produce Shiga toxins (Stxs) and cause hemorrhagic colitis (HC) and potentially life-threatening hemolytic uremic syndrome (HUS) in humans. Several serotypes in EHEC are frequently associated with human diseases including: O26:H11, O91:H21, O111:H8, O157:NM, and O157:H7. *E. coli* O157:H7 is the most frequently isolated serotype of EHEC from ill individuals in the United States, Japan, and the United Kingdom (Lim et al., 2010).

E. coli O157:H7 was first recognized in 1982 as a human pathogen associated with outbreaks of bloody diarrhea in Oregon and Michigan and was also linked to sporadic cases of HUS in 1983. Since then, several outbreaks associated with EHEC have been reported in the United States. As a result, *E. coli* O157:H7 has become one of the most important foodborne pathogens (Lim et al., 2010).

Prevalence and Economic Costs of *E. coli* O157:H7

The Centers for Disease Control and Prevention (CDC) has estimated that *E. coli* O157:H7 infections cause 73,000 illnesses, 2,200 hospitalizations, and 60 deaths annually in the United States (Mead et al., 1999). Outbreak surveillance data from CDC has reported, however, that *E. coli* O157:H7 infections are decreasing after the peak in 1999. Despite this, large outbreaks and sporadic cases continue to occur. The annual cost of illness due to *E. coli* O157:H7 infections in 2005 was 405 million dollars at its peak, including lost productivity, medical care, and premature deaths (Frenzen et al., 2005).

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

E. coli O157:H7 expresses the somatic (O) antigen 157, a lipopolysaccharide, and the flagella (H) antigen. *E. coli* O157:H7 has other unique features useful for isolation purposes. *E. coli* O157:H7 has delayed D-sorbitol fermentation (>24 h) and inability of producing β-glucuronidase, which hydrolyzes 4-methyl-umeebelliferyl-D-glucoronide (Thompson et al., 1990). Detection of *E. coli* O157:H7 can thus be done by supplementing Sorbitol MacConkey (SMAC) agar with MUG. To increase *E. coli* O157:H7 selectivity, cefixime, potassium tellurite, and vancomycin can be added to SMAC agar plates to inhibit other Gram-negative bacterium (Lim et al., 2010).

***E. coli* O157:H7 Transmission**

Cattle are a natural reservoir for *E. coli* O157:H7. Healthy cattle carry and shed *E. coli* O157:H7 in their feces at any given time (Dunn et al., 2004). Food has remained the predominant transmission route of *E. coli* O157:H7. Transmission by food accounts for 61% of 8,598 outbreak cases from 1982-2002 (Dunn et al., 2004). While ground beef continues to be a primary vehicle for transmission, fresh produce is increasingly being indicated as well. Produce-associated outbreaks of *E. coli* O157:H7 infection were first reported in 1991. The largest outbreak of *E. coli* O157:H7 was actually traced to radish sprout contamination in Osaka, Japan in 1996 which saw 7,966 individuals diagnosed with confirmed infection (Michino et al., 1999). Raw vegetables continue to be implicated in many outbreaks in North America, Europe, and Japan (Rangel et al., 2005). A model of transmission of *E. coli* O157:H7 is shown below (Lim et al., 2010). The varied

possible transmission routes seen can be attributed to the very low infectious dose (~50 CFU) of *E. coli* O157:H7.

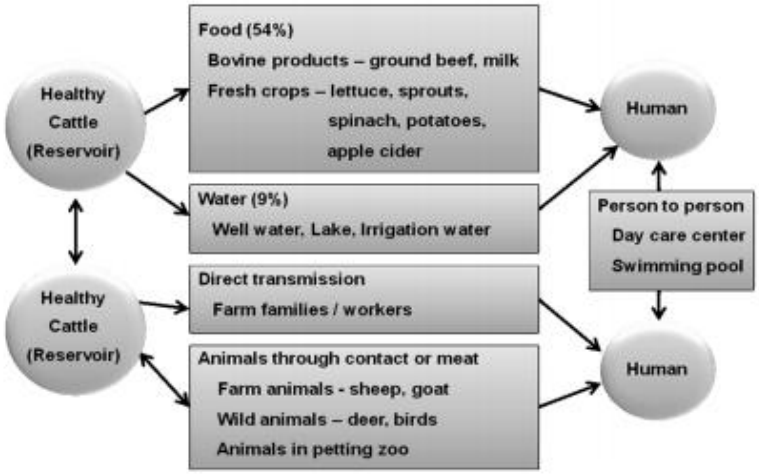


Figure 2.1. Schematic Depiction for *E. coli* O157:H7 Transmission.

Source: Lim, J.Y., Yoon, J.W., Hovde, C.J. 2010. A brief overview of *Escherichia coli* O157:H7 and its plasmid O157. Journal of Microbiology Biotechnology (20)1:14.

Acid Resistance of *E. coli* O157:H7

In general, acid resistance is the ability of bacteria to protect themselves from low pH (<pH 3.0). The low pH of the stomach is one of the first host defenses against foodborne enteric pathogens (Peterson et al., 1989). The ability to survive in the acidic environment of the stomach increases the chances of the bacteria to colonize the intestines and cause infection. An effect of acid resistance is that infectious dose of enteric pathogens is lowered, as seen with *E. coli* O157:H7 with an infectious dose seen to be as low as ~50 CFU. The low infection dose is one of the best known characteristics of *E. coli* O157:H7 and is what makes it a highly infectious pathogen (Lim et al., 2010).

Environmental Survival of *E. coli* O157:H7

E. coli O157:H7 can survive in varied environments including soil, water, animal reservoirs, and food. *E. coli* O157:H7 has been shown to survive for up to a year in manure-treated soil (Jiang et al., 2002). *E. coli* O157:H7 can also survive for long periods of time in water, especially if kept at cold temperatures (LeJeune et al., 2001). To survive in numerous environments, *E. coli* O157:H7 must adapt to variations or extreme changes in temperature, pH, and osmolarity. To combat acidic environments and temperature increases, *E. coli* O157:H7 produces various classes of exopolysaccharides (EPS) and alters its lipid composition in membranes (Yuk and Marshall, 2004).

Increased osmotic pressure has been used to control the growth of food spoilage and pathogenic bacteria specifically by desiccation or addition of high amounts of osmotically active ingredients, such as sodium chloride, which result in decreased water activity. When the osmotic pressure in the surrounding environment increases, cells activate osmoregulation systems to prevent shrinkage and plasmolysis. In *E. coli* specifically, osmotic stress conditions mimic physiological features of starvation-including the production of peroxisome assembly proteins and heat shock proteins. Ultimately, increased osmolarity in bacterial cells has been associated with the inhibition of DNA replication, cell growth, and nutrient uptake (Chung et al., 2006).

Environmental adaptations of *E. coli* O157:H7 help to explain the persistence and dissemination of this serotype on farms as well as the increasing transfer from cattle to cattle. The ability of it to survive outside the host reservoir also increases the risk that the pathogen can contaminate crops and produce via bovine manure contamination, irrigation using contaminated water and direct contact with infected animals (Maule,

2000). These sources of contamination help to demonstrate how fresh produce can become a source of a high enough dose of *E. coli* O157:H7 to cause infection.

Achievement of a 5-Log Reduction: How do various foodborne pathogens compare?

Breidt and others determined conditions needed for 5-log reductions in individual foodborne pathogen cocktails of *E. coli* O157:H7, *Salmonella enterica,* and *Listeria monocytogenes* (Breidt et al, 2013). Individual colonies of each pathogen strain were grown statically to induce acid resistance and were incubated at 37 °C for 16 hours. Individual cultures for each pathogen were combined resulting in three different cocktails for testing. Inoculation was done in cucumber juice medium. Inoculated cucumber juice had an approximate initial cell count of 10^8 CFU/ml for each pathogen. There were 15 different acid-pH combinations for cucumber juice medium with a pH of either 3.5 or 3.8. Acid conditions were more varied and ranged from 0.0%-2.5% depending on the acid used. Acids used included benzoic and acetic, which are commonly seen in acidified products processed commercially.

It was found that *E. coli* O157:H7 was more acid resistant than *Listeria monocytogenes* and *Salmonella enterica* under certain conditions. Among the different acid conditions tested, a 5-log reduction was seen in an average of less than 4 days for all pathogens at pH 3.5 with 2.5% acetic acid or at pH 3.8 with 2.5% acetic acid containing 0.1% benzoic acid. In addition, regardless of pH-acid combination, *Salmonella enterica* and *Listeria monocytogenes* samples specifically had mean 5-log reduction times of less than 2 days. In contrast, some *E. coli* samples were shown to have 5-log reduction times of longer than 10 days. For example, the pH 3.5 and 2.0% acetic acid

sample had a mean 5-log reduction time of 11.7 days. This was also seen when benzoic acid was used. The pH 3.8 and 1.5% acetic acid, 0.1% benzoic acid sample had a mean 5-log reduction time of 13.5 days.

This suggests that *E. coli* is more acid resistant, based on the extended time until a 5-log reduction was seen. In addition, this research demonstrated that acid killing was dependent on both the acid concentration and acid type. It was also concluded that, overall, benzoic acid significantly reduced the time needed for a 5-log reduction when it was the sole acid used or in combination with acetic acid. A pH value of 3.5, as expected, also resulted in overall quicker 5-log reduction (Breidt et al., 2013).

This research also supported earlier findings that found *E. coli* O157:H7 to have a longer predicted time for achievement of a 5-log reduction (5.7 days or 138 hours) compared to *Listeria monocytogenes* (0.5 days or 11.2 hours) and *Salmonella enterica* (2.1 days or 51 hours) respectively. Specific conditions in this particular study included holding temperatures of either 10 C or 25 C and addition of acetic acid to achieve a cucumber juice medium pH of 3.3 (Breidt et al., 2007). This helps demonstrate that *E. coli* O157:H7 is also more acid resistant across several pH measures when compared to *S. enterica* and *L. monocytogenes*.

Survival of *Escherichia coli* O157:H7 in Cucumber Brines

To answer the question of survival in a specific medium, Breidt and Caldwell have assessed *E. coli* O157:H7 survival in fermented cucumber brines specifically. Their methods for bacterial survival determination are widely used in cucumber fermentation research. Key points are outlined below (Breidt, F. and J.M. Caldwell 2011).

To obtain cucumber juice medium for inoculation, size 2B (3.5-3.8 cm in diameter) pickling cucumbers were processed to a cucumber slurry and then centrifuged. Sodium chloride was added to a final concentration of 2%. To create fermented brines, each sample medium was either inoculated with a single colony of *Lactobacillus plantarum* or *Leuconostoc mesenteroides* and incubated at 30°C for 96 hours followed by centrifugation and filtration. Brines were obtained at different stages of fermentation. Survival of *E. coli* O157:H7 in non-fermented cucumber juice medium was also studied. Samples of non-fermented cucumber juice medium (CJ), fermented cucumber juice medium (FCJ), and commercially obtained cucumber brines were each inoculated with a 5-strain *E. coli* O157:H7 cocktail. Strains were statically grown at 37 °C for 16 hours in LB broth supplemented with 1% glucose to induce acid resistance. *E. coli* cocktail was inoculated at 10^6, 10^8, and 10^8 CFU/ml for CJ, FCJ, and commercial brines. 0.5 ml samples were removed and diluted followed by plating with a spiral plater (Spiral Biotech Inc. Norwood, MA). Plates were stored at 37°C for 24 hours. Colonies were then counted with an automated spiral plate counter (Q-Count, Spiral Biotech Inc. Norwood, MA).

It was shown through death curves (utilizing polynomial functions) that *E. coli* O157:H7 strains required more than two weeks before a 5-log reduction could be achieved in brine fermented by *L. mesenteroides* and incubated at 10 °C. In brine fermented by *L. mesenteroides* but incubated at 30 °C, 5-log reduction was achieved more rapidly at less than two days. Interestingly, brine fermented by *L. plantarum* and incubated at 10 °C showed accelerated 5-log reduction time (about 4 days) compared to brine fermented by *L. mesenteroides* at the same incubation temperature. At an incubation temperature of 30 °C, 5-log reduction was seen in less than 1 day.

Analysis of commercially obtained brine samples showed a positive correlation between pH and 5-log reduction time specifically ($R^2 = 0.71$). It was also noted that protonated acid concentrations also correlated well; however, because pH is much easier to measure than protonated acid concentrations, only the correlation with pH was reported.

Overall conclusions from this body of research include: observation of a positive correlation between pH and *E. coli* O157:H7 5-log reduction time (in inoculated commercial brines) and prolonged survival of *E. coli* O157:H7 in cucumber juice fermented with *L. mesenteroides* and *L. plantarum* when incubation temperature was lower (10 °C). Higher incubation temperature (30 °C) resulted in accelerated 5-log reduction of *E. coli* O157:H7, with brine fermented by *L. plantarum* showing the quickest reduction at less than 1 day based on the death curve calculated utilizing a polynomial function (Breidt and Caldwell, 2011).

Treatment Determination for a 5-Log Reduction of *Escherichia coli* in Refrigerated Pickle Products

Lu et al. (2013) developed a method to assure a 5-log reduction of pathogenic *E. coli* in refrigerated pickle products. This research was conducted as a response to concerns of survival of *E. coli* at 4 °C in brines typical of commercial refrigerated products. Prior research demonstrated that *E. coli* strains were acid resistant and better survived in refrigerated acid solutions. A brine formulation of 25 mM fumaric acid, 5 mM benzoic acid, 70 mM acetic acid, and 342 mM (2%) NaCl was tested. This formulation is unique in that it contains fumaric acid and less acetic acid than what is typical of commercial brines. This formulation assured a 5-log reduction in cell numbers of *E. coli* O157:H7 without heat processing. Specifically, a 5-log reduction was seen for *E. coli* O157:H7 at 30 °C for 1.52 ± 0.15 d, at 20 °C for 3.12 ± 0.34 d, or at 10 °C for 8.83 ± 0.56 d (Lu et al., 2013).

Characteristics of Genus *Lactobacillus*

What are *Lactobacillus* species*?*

The lactic acid bacteria genus *Lactobacillus* includes a variety of gram-positive microaerophilic species capable of survival in a variety of environments, including nutrient-rich dairy environments, host habitats (such as the human gut), and natural ecological niches such as plants and soil. The genus *Lactobacillus* comprises over a hundred different species, including commercially relevant species such as *L. acidophilus, L. casei, L. bulgaricus*, *and L. plantarum*. For example, *L. plantarum* is a

naturally occurring species that has been reported to dominate on vegetable surfaces such as cabbage and lettuce (Yang et al., 2010a).

In addition to naturally occurring in produce, members of the genus *Lactobacillus* are widely used in food and feed manufacturing and commercially as health-promoting microbes in probiotic containing foods. Lactic acid bacteria, including genus *Lactobacillus*, are also known for their role in the process of carbohydrate fermentation. Fermentation may proceed relying solely on naturally occurring LABs or as a starter culture (addition of the individual LAB strain to already occurring natural LABs) to ensure a controlled, complete fermentation is accomplished. This is common commercially and is particularly important in the fermentation of vegetable products, such as sauerkraut, pickles, and kimchi, which have raw vegetables as a starting ingredient (Lahtinen et al., 2012).

Function of Lactic Acid Bacteria in Carbohydrate Fermentation

Lactic acid bacteria (LAB) obtain their energy through substrate-level phosphorylation. There are two basic fermentative pathways occurring in LAB. The homofermentative pathway (Embden-Meyerhof-Parnas pathway) produces only lactic acid. The heterofermentative pathway (pentose phosphoketolase pathway) produces lactic acid, ethanol, and significant amounts of carbon dioxide. *Lactobacillus* favors the homofermentative pathway. It nets 2 lactate molecules and 2 ATP molecules per 1 glucose molecule. The Embden-Meyerhof-Parnas pathway (glycolysis) oxidizes one glucose molecule to 2 pyruvates, 2 ATP, and 2 NADH. Both pyruvates are reduced to NADH, which is oxidized to NAD+. The lactate is then excreted into the surrounding

environment. Fermentation is generally regarded as a fast process. Although fermentation only generates 3% to 7% of the 38 ATPs that oxidative phosphorylation can potentially produce, fermentation actually produces ATP at about 100 times the rate of oxidative phosphorylation by comparison. Due to this accelerated rate, many cells ferment in the presence of glucose instead of respire, even if oxygen is present. This is known as the Crabtree effect, and it occurs in many bacteria in nature, including *Escherichia coli* when presented with high concentrations of glucose (Wright and Axelson, 2012).

Objective, Hypotheses and Specific Aims

The objective of this research was to determine 5-log reduction times in 50% cucumber juice media for two separate bacterial cocktails: *Escherichia coli* O157:H7 and *Lactobacillus.* Media differed by salt type and concentration: 2% NaCl, 6% NaCl, and 1.1% $CaCl_2$.

Hypotheses

1. The fastest 5-log reduction, regardless of bacterial cocktail, will be seen in media with a salt type of NaCl and a concentration of 6%.

2. The slowest 5-log reduction, regardless of bacterial cocktail, will be seen in media with a salt type of $CaCl_2$ and concentration of 1.1%.

Specific Aims

The specific aims of this project were to:

1. Determine 5-log reduction times of *Escherichia coli* O157:H7 in cucumber juice media that varied by salt type and concentration.

2. Determine 5-log reduction times of *Lactobacillus* in cucumber juice media that varied by salt type and concentration.

CHAPTER 3

METHODS

Pickling cucumbers were obtained and processed to a cucumber juice medium to be used in studying characteristics of *E. coli* O157:H7 and *Lactobacillus* species (spp.) survival in the presence of certain salt concentrations. Samples of cucumber juice had the addition of either 2% NaCl, 6% NaCl or 1.1% $CaCl_2$. Lactic acid was also added to samples. Samples of these cucumber juice media were then inoculated with one of two 5-strain cocktails: *Lactobacillus* spp. or *Escherichia coli* O157:H7. Three replications of each treatment were performed. Samples were serially diluted, auto-plated, and counted at specific time points to determine log reduction times.

Cucumber Juice Preparation

Size 2B (3.5-3.8 cm in diameter) fresh cucumbers were obtained from Mt. Olive Pickle Company in Mount Olive, NC. Cucumbers were covered in aluminum foil and stored in a walk-in refrigerator unit at 4 °C for up to 1 week. Cucumbers were scrubbed with warm water to remove any dirt and debris. Visual inspection of cucumbers for mold was also done; those with signs of mold were removed. Cucumbers were chopped and blended in an industrial blender to make a slurry. The cucumber slurry was then poured into clean, empty gallon jars and frozen in a walk-in freezer maintained at 0 °C for 2-3 weeks.

To prepare cucumber juice, frozen slurry was pulled from walk-in freezer and thawed at room temperature overnight. Thawed slurry was strained through a cheesecloth to separate the pulp from the juice. Equal amounts of cucumber juice were separated into 250 ml centrifuge bottles. Bottles were only filled 2/3 full to prevent accidental spillover. Bottles were placed into the centrifuge in a balanced configuration. The Sorvall GSA rotor head was closed and the centrifuge (Sorvall RC-5B refrigerated superspeed centrifuge, DuPont Instruments, Wilmington, DE) was set to 5x1000 rpm for 45 minutes to further separate the pulp and cucumber juice.

After centrifugation, the cucumber juice was carefully removed and pelleted pulp and debris were discarded. Cucumber juice was passed through a vacuum filtration unit with a 40 µm filter (Millipore SteriFlip vacuum-driven filtration system 50 ml, Millipore Corporation, Billerica, MA) to remove final remaining particulates. Bottles were labeled and stored in a refrigerator at 4 °C until ready for use. Storage can be from 2-3 months. Visual inspection of juice should be performed before use. Juice should remain clear of any particulates and should not have a clouded appearance.

Bacterial Strains and Growth Conditions

Five strains each of *Escherichia coli* O157:H7 and *Lactobacillus* spp. were obtained from stock cultures in a -80 °C freezer. *E. coli* strains included: B0200 (human feces), B0201 (apple cider outbreak), B0202 (salami outbreak), B0203 (ground beef), and B0204 (pork). *Lactobacillus* spp. strains included: LA0471, LA0023, LA0513, LA0516, and LA0445 (all pickle isolates from cucumber fermentation). *E. coli* strains were streaked on Luria Bertani (LB) plates (Becton, Dickinson and Company, Franklin Lakes, NJ) and

incubated at 37 °C for 17 hours. *Lactobacillus* strains were streaked on Lactobacilli deMan Rogosa and Sharpe (MRS) plates (Becton, Dickinson and Company, Franklin Lakes, NJ) and stored at 30 °C for 36 hours.

Once colony growth was seen on plates, a colony for each individual strain was removed with a disposable wire loop and deposited in 15 ml screwcap tubes (Corning CentriStar that contained 5 ml of MRS broth (if a *Lactobacillus* spp. strain) or LB broth (if an *E. coli* strain) (Becton, Dickinson and Company, Franklin Lakes, NJ). *E. coli* strains were grown statically at 37 °C for 18 hours to induce acid resistance. *Lactobacillus* was also grown statically at 30 °C for 36 hours.

Making Cucumber Juice Treatments

Three different test treatments and one control were made for both *E. coli* and *Lactobacillus* spp. cocktails. All treatments were 50 milliliters total volume, with 25 milliliters being cucumber juice and 25 milliliters being filtered deionized water. See Tables 3.1 and 3.2 for test conditions for each treatment. All conditions consisted of a controlled mM acid content and differed by salt amount and/or type only. All treatments were stored in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI) at 30 °C for 48 hours to equilibrate before inoculation with bacterial strains.

Table 3.1. Cucumber Juice Treatments for *Escherichia coli* O157:H7

Code	Treatment	Acid[c]	pH[d]
D	2% NaCl[a]	50 mM	3.2
E	6% NaCl	50 mM	3.2
F	1.1% $CaCl_2$[b]	50 mM	3.2
control	--	50 mM	3.2

[a] Sodium chloride (Fischer Scientific, Pittsburgh, PA)
[b] Calcium chloride dehydrate (Aldrich Chemical Company, St. Louis, MO)
[c] Sodium L-lactate (Aldrich Chemical Company, St. Louis, MO)
[d] pH was tested in all treatments (Accumet AB150, Fischer Scientific, Pittsburgh, PA) and adjusted as needed to target of 3.2 with hydrochloric acid (Aldrich Chemical Company, St. Louis, MO)

Table 3.2. Cucumber Juice Treatments for *Lactobacillus* spp.

Code	Treatment	Acid[c]	pH[d]
A	2% NaCl[a]	350 mM	3.2
B	6% NaCl	350 mM	3.2
C	1.1% $CaCl_2$[b]	350 mM	3.2
control	--	350 mM	3.2

[a] Sodium chloride (Fischer Scientific, Pittsburgh, PA)
[b] Calcium chloride dehydrate (Aldrich Chemical Company, St. Louis, MO)
[c] Sodium L-lactate (Aldrich Chemical Company, St. Louis, MO)
[d] pH was tested in all treatments (Accumet AB150, Fischer Scientific, Pittsburgh, PA) and adjusted as needed to target of 3.2 with hydrochloric acid (Aldrich Chemical Company, St. Louis, MO)

Making 5-Strain Cocktails

E. coli O157:H7 and *Lactobacillus* spp. cocktails were made by combining individual strains. All strains were obtained from the culture collection of the Food Safety and Foodborne Disease Prevention laboratory at North Carolina State University. 15 ml screwcap tubes with strains were placed into the centrifuge rotor balanced. Individual strains were centrifuged (Sorvall RC-5B, SS-34 rotor, refrigerated superspeed centrifuge, DuPont Instruments, Wilmington, DE) for 10 minutes at 5x1000 rpm. Cells were then resuspended in 1 ml of sterile saline (0.85 NaCl) using pipette (SL1000, Rainin Instruments, Oakland, CA) and disposable pipette tips. Cell cultures of the same species were then combined in a 50 ml screwcap tube. All screwcap tubes were vortexed (Daigger Vortex Genie 2, Scientific Industries Inc., Bohemia, NY) before and after cell culture combination to ensure adequate mixing. All work was performed in a class II, type A2 laminar flow biological safety cabinet (NU425-500, NuAire Inc., Plymouth, Minnesota).

Treatment Inoculation Procedures

Inoculation of all test treatments was performed in an anaerobic chamber (Coy Laboratory Products Inc., Grass Lake, MI). 10 ml Vacutainer tubes containing prepared cucumber juice + salt media were inoculated with bacterial cocktails. Vacutainer tubes were used because sampling can be done without introducing oxygen to the treatments, sustaining an anaerobic environment within the tubes. Use of Vacutainer tubes acts as a control, affirming that cell death is likely occurring due to the effects of salt and acid, not the combined effects of dissolved oxygen and organic acid/pH. 9 ml of

each equilibrated treatment were inserted into separate Vacutainer tubes using a 10 ml syringe with a 21G x 2 needle (Becton, Dickinson and Company, Franklin Lakes, NJ). 1 ml of cocktail was then added to Vacutainer tubes with treatments and vortexed. Control Vacutainer tubes were not inoculated with either cocktail.

Sampling of Treatments

Original inoculation cell counts were determined by withdrawing 1 ml of each bacterial cocktail with 1 ml TB syringe with intradermal bevel needle (Becton, Dickinson and Company, Franklin Lakes, NJ). Original cell inoculation counts were determined to be approximately 10^9 CFU/ml for all treatments.

Serial dilutions were done for all inoculated cucumber juice treatments and are specified in Tables 3.3 and 3.4. Dilutions were consistent across reps. Dilutions were made into vials containing 900 µl 1% MOPS-Saline (0.85%) for each subsequent sample. 0.3 ml for each treatment was withdrawn with 1 ml TB syringe with intradermal bevel needle (Becton, Dickinson and Company, Franklin Lakes, NJ) into a blank vial. 100 µl was then pipetted (P200, Rainin Instruments, Oakland, CA) for each dilution with disposable pipette tips.

Table 3.3. Serial Dilutions for *Escherichia coli* O157:H7 Samples.

Time Period[a]	Sample[b]	Dilution (1/10)
T0	D	5.00
T0	E	5.00
T0	F	5.00
T1	D	2.00
T1	E	2.00
T1	F	2.00
T2	D	2.00
T2	E	2.00
T2	F	2.00
T3	D	0.00
T3	E	0.00
T3	F	0.00
T4	D	0.00
T4	E	0.00
T4	F	0.00
T5	D	0.00
T5	E	0.00
T5	F	0.00

[a] Time periods: T0= 1 hour, T1= 4 hours, T2= 7 hours, T3= 10 hours, T4= 14 hours, T5= 18 hours

[b] Samples: D= 2% NaCl sample, E= 6% NaCl sample, F= 1.1% $CaCl_2$ sample

Table 3.4. Serial Dilutions for *Lactobacillus* spp. Samples.

Time Period	Sample	Dilution (1/10)
T0	A	5.00
T0	B	5.00
T0	C	5.00
T1	A	3.00
T1	B	2.00
T1	C	3.00
T2	A	3.00
T2	B	2.00
T2	C	3.00
T3	A	2.00
T3	B	1.00
T3	C	2.00
T4	A	1.00
T4	B	0.00
T4	C	2.00
T5	A	0.00
T5	B	0.00
T5	C	2.00

[a] Time periods: T0= 1 hour, T1= 6 hours, T2= 20 hours, T3= 26 hours, T4= 40 hours, T5= 46 hours

[b] Samples: D= 2% NaCl sample, E= 6% NaCl sample, F= 1.1% $CaCl_2$ sample

Plating Treatments

Plates used for experiments were sterile, polystrene 100 mm x 15 mm (Fischer Scientific, Pittsburgh, PA). MRS and LB agar (Becton, Dickinson and Company, Franklin Lakes, NJ) were mixed separately, autoclaved, poured into petri dishes, and allowed to cool and set overnight at room temperature. Culture populations in inoculated media were determined by plating serial dilutions using a spiral plater (Spiral Biotech Inc., Norwood, MA) and either MRS or LB pre-made plates. *E. coli* plates were incubated at 37 °C for 18 hours. *Lactobacillus* spp. plates were incubated at 30 °C for 36 hours.

Serial dilutions were plated at specific time intervals. Samples of *E. coli* were plated at 1, 4, 7, 10, 14, and 18 hours. Samples of *Lactobacillus* spp. were plated at 1, 6, 20, 26, 40, and 46 hours. Hours of plating were based on prior unpublished pilot research that showed when adequate log reduction and colony growth would occur.

Colony Counting

After storing plates to allow for colony growth, colony counting was performed using a Q-Count 510 colony counter (Spiral Biotech, Norwood, MA). A shutter speed of 1/60 was used for all plates. Data generated from Q-Count is cfu/ml, which was then transformed to logarithmic values for statistical analysis.

CHAPTER 4

RESULTS

Raw data collected for microbial counts were expressed as CFU/ml which were then converted to common logarithmic units for analysis. The main independent variable was salt concentration. Log reduction was considered the dependent variable. This is true for both data sets: log reductions in *E. coli* O157:H7 and *Lactobacillus* species (spp.). Results for *E. coli* O157:H7 and *Lactobacillus* data will be presented separately.

Repeated-measures analysis of variance (ANOVA) was used to test statistical significance among salt treatments (SAS Version 9.3, Cary, NC) for both sets of data, with each set of data representative of 3 repetitions (reps) per salt level. This approach was selected based on factors including: type of observations, number of samples, and nature of variables for the study. The following table (Figure 1) from Ilstrup (1990) was used to confirm the approach. Repeated-measures ANOVA was shown to be appropriate based on the study's design which included: dependent observations, 3 samples (treatments), and continuous variables.

TABLE 2. Significance tests

Type of observations	No. of samples	Nature of variable (reference)		
		Nominal	Continuous Gaussian	Ordinal or continuous non-Gaussian
I. Dependent	2	Sign test or McNemar's test (20)	Paired *t* test (18)	Wilcoxon signed-ranks test (20)
II. Independent	2	Relative deviate test (19) Chi-square (20)	Two-sample *t* test (4)	Wilcoxon rank sum test (4, 13)
III. Dependent	3 or more	Cochran Q test (20)	Repeated-measures analysis of variance (12, 21)	Friedman's procedure (20)
IV. Independent	3 or more	Chi-square test (20)	One-way analysis of variance (4)	Kruskal-Wallis one-way analysis of variance (20)

Figure 4.1. Univariate Methods for Statistical Analysis in Microbiology.

Source: Ilstrup, D.M. 1990. Statistical methods in microbiology. Clinical Microbiology Reviews 3:219-226.

Two different models were generated: one model for *E. coli* O157:H7 cocktails and one for *Lactobacillus* spp. cocktails. It is worth noting that this is not the only approach for statistical analysis with this type of microbial data. Additional approaches have been generated by statisticians in the field. For example, the Weibull model, a parametric survival model, was utilized in selected studies referenced in chapter 2 (Breidt et al., 2013; Breidt et al., 2011). Repeated-measures ANOVA was ultimately chosen for analysis based on the number of dependent samples in this study and the allowance for reproducibility of results.

E. coli O157:H7 Results

SAS generated the following General Linear Model (GLM) for comparing significance in *E. coli* O157:H7 log reductions:

$$\begin{aligned}
&\boldsymbol{LogCFUijt}=\boldsymbol{\alpha}+\boldsymbol{\beta 1 Isaltlevel}(\boldsymbol{i}=\mathbf{1})+\boldsymbol{\beta 2 Isaltlevel}(\boldsymbol{i}=\mathbf{2})+\boldsymbol{\gamma 1 ITimeHour}(\boldsymbol{t}=\mathbf{4})\\
&+\boldsymbol{\gamma 2 ITimeHour}(\boldsymbol{t}=\mathbf{7})+\boldsymbol{\gamma 3 ITimeHour}(\boldsymbol{t}=\mathbf{10})+\boldsymbol{\gamma 4 ITimeHour}(\boldsymbol{t}=\mathbf{14})+\boldsymbol{\gamma 5 ITi}\\
&\boldsymbol{meHour}(\boldsymbol{t}=\mathbf{18})+\boldsymbol{\delta 1 Isaltlevel}(\boldsymbol{i}=\mathbf{1})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{4})\\
&+\boldsymbol{\delta 2 Isaltlevel}(\boldsymbol{i}=\mathbf{1})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{7})\\
&+\boldsymbol{\delta 3 Isaltlevel}(\boldsymbol{i}=\mathbf{1})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{10})\\
&+\boldsymbol{\delta 4 Isaltlevel}(\boldsymbol{i}=\mathbf{1})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{14})\\
&+\boldsymbol{\delta 5 Isaltlevel}(\boldsymbol{i}=\mathbf{1})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{18})\\
&+\boldsymbol{\delta 6 Isaltlevel}(\boldsymbol{i}=\mathbf{2})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{4})\\
&+\boldsymbol{\delta 7 Isaltlevel}(\boldsymbol{i}=\mathbf{2})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{7})\\
&+\boldsymbol{\delta 8 Isaltlevel}(\boldsymbol{i}=\mathbf{2})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{10})\\
&+\boldsymbol{\delta 9 Isaltlevel}(\boldsymbol{i}=\mathbf{2})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{14})\\
&+\boldsymbol{\delta 10 Isaltlevel}(\boldsymbol{i}=\mathbf{2})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{18})\\
&+\boldsymbol{\varepsilon ijt},\ \boldsymbol{for\ i}=\mathbf{1,2};\boldsymbol{t}=\mathbf{4,7,10,14,18};\boldsymbol{j}=\mathbf{1,2,3}
\end{aligned}$$

Where α indicates the baseline, which corresponds to an observation under the condition that the salt level to be (NaCl, CaCl)=(0, 1.1) and time of observation to be 0 hours; i=1 and i=2 indicate the salt level to be (NaCl, CaCl)=(2, 0.0) and (NaCl, CaCl)=(6, 0.0); t=4,t=7,t=10,t=14 and t=18 indicate the time of observation to be 4, 7, 10, 14 hours. $Isal(i)\times ITimeHour(t)$ represents the interaction effect between salt level of i and time t.

When looking at overall log reductions in *E. coli* O157:H7 samples, a 5-log reduction was reached in 6% NaCl by 14 hours and in 1.1% $CaCl_2$ by 18 hours (Table 4.1). The log reductions in 2% NaCl proceeded at the slowest rate overall. At 18 hours only a 4.43 log reduction was reached.

Table 4.1. Surviving Populations of *E. coli* O157:H7 in log CFU/ml[a] in Varied Salt Types and Concentrations over Time and Time Required to Achieve a 5-Log Reduction.

Time (Hours)	**Salt Type and Concentration**		
	2% NaCl	**6% NaCl**	**1.1% $CaCl_2$**
1	8.34	8.30	8.42
4	7.07	7.03	7.04
7	6.65	5.08	5.89
10	5.78	4.13	4.99
14	4.66	3.26[b]	4.04
18	3.91	2.95	3.21[b]
Total log reduction by 18 hours	4.43	5.35	5.21

[a] Values in a column represent the average of 3 reps
[b] Represents the time point a 5-log reduction is first observed

A Type III Test of Fixed Effects was run (Table 4.2). All effects including salt level $F(2, 34) = 655.50$, Pr > F= <.001, time of observation $F(5,34) = 5249.00$, Pr > F= <.001, and interaction (salt level x time of observation) $F(10,34)= 69.38$, Pr > F= <.001 are significant at significance level $\alpha = .01$. Since the interaction effects were significant, these will be the focus of interpretation rather than the main effects of salt level and time of observation. This significance demonstrates that the interaction of these two variables is an important predictor for the dependent variable (log reduction).

Table 4.2. Type III Test: Results of Fixed Effects Analysis in Log Reductions of *E. coli* O157:H7 in Media with Varying Salt Types and Concentrations.

Effect	Numerator DF	Denominator DF	F Value	Pr > F
Salt level	2	34	655.50	<.001
Time	5	34	5249.00	<.001
Salt level x time	10	34	69.38	<.001

To interpret if there were significant differences across the interaction effects specifically, differences of least square means was calculated at the significance level $\alpha = .01$ (Table 4.3). A few trends were revealed through the statistical model: 1) no significant difference exists between log reductions for all salt concentrations at a time point of zero hours, 2) no significant difference exists between log reductions for all salt concentrations at a time point of four hours, and 3) a significant difference in log reductions exists between all salt concentrations at all other time points.

Table 4.3. Equality Test: Differences of Least Square Means of Log Reductions in *E. coli* O157:H7 Cocktail Population When Comparing Salt Levels at Various Time Points.

Time Point (Hours)	Salt Levels Compared[a]	Estimate	Standard Error (SE)	DF	t value	Pr > | t |[b]
1	1, 2	0.04000	0.06378	34	0.63	0.5347
1	1, 3	-0.07667	0.06378	34	-1.20	0.2377
1	2, 3	-0.1167	0.06378	34	-1.83	0.0762
4	1, 2	0.03667	0.06378	34	0.57	0.5691
4	1, 3	0.03333	0.06378	34	0.52	0.6046
4	2, 3	-0.00333	0.06378	34	-0.05	0.9586
7	1, 2	1.5667	0.06378	34	24.56	<.0001*
7	1, 3	0.7600	0.06378	34	11.92	<.0001*
7	2, 3	-0.8067	0.06378	34	-12.65	<.0001*
10	1, 2	1.6467	0.06378	34	25.82	<.0001*
10	1, 3	0.7867	0.06378	34	12.33	<.0001*
10	2, 3	-0.8600	0.06378	34	-13.48	<.0001*
14	1, 2	1.4033	0.06378	34	22.00	<.0001*
14	1, 3	0.6233	0.06378	34	9.77	<.0001*
14	2, 3	-0.7800	0.06378	34	-12.23	<.0001*
18	1, 2	0.9633	0.06378	34	15.10	<.0001*
18	1, 3	0.7033	0.06378	34	11.03	<.0001*
18	2, 3	-0.2600	0.06378	34	-4.08	<.0001*

[a] Where salt level is denoted as 1= 2% NaCl, 2= 6% NaCl, and 3= 1.1% $CaCl_2$

[b] Results with * indicate significant difference at significance level $\alpha = .01$

***Lactobacillus* spp. Results**

SAS generated the following General Linear Model (GLM) for *Lactobacillus* spp. log reductions:

$$\boldsymbol{LogCFUijt}=\boldsymbol{\alpha}+\boldsymbol{\beta 1 Isaltlevel}(\boldsymbol{i}=\mathbf{1})+\boldsymbol{\beta 2 Isaltlevel}(\boldsymbol{i}=\mathbf{2})+\boldsymbol{\gamma 1 ITimeHour}(\boldsymbol{t}=\mathbf{6})$$
$$+\boldsymbol{\gamma 2 ITimeHour}(\boldsymbol{t}=\mathbf{20})+\boldsymbol{\gamma 3 ITimeHour}(\boldsymbol{t}=\mathbf{26})+\boldsymbol{\gamma 4 ITimeHour}(\boldsymbol{t}=\mathbf{40})+\boldsymbol{\gamma 5 ITime\ Hour}(\boldsymbol{t}=\mathbf{46})+\boldsymbol{\delta 1 Isaltlevel}(\boldsymbol{i}=\mathbf{1})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{6})$$
$$+\boldsymbol{\delta 2 Isaltlevel}(\boldsymbol{i}=\mathbf{1})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{20})$$
$$+\boldsymbol{\delta 3 Isaltlevel}(\boldsymbol{i}=\mathbf{1})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{26})$$
$$+\boldsymbol{\delta 4 Isaltlevel}(\boldsymbol{i}=\mathbf{1})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{40})$$
$$+\boldsymbol{\delta 5 Isaltlevel}(\boldsymbol{i}=\mathbf{1})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{46})$$
$$+\boldsymbol{\delta 6 Isaltlevel}(\boldsymbol{i}=\mathbf{2})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{6})$$
$$+\boldsymbol{\delta 7 Isaltlevel}(\boldsymbol{i}=\mathbf{2})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{20})$$
$$+\boldsymbol{\delta 8 Isaltlevel}(\boldsymbol{i}=\mathbf{2})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{26})$$
$$+\boldsymbol{\delta 9 Isaltlevel}(\boldsymbol{i}=\mathbf{2})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{40})$$
$$+\boldsymbol{\delta 10 Isaltlevel}(\boldsymbol{i}=\mathbf{2})\times\boldsymbol{ITimeHour}(\boldsymbol{t}=\mathbf{46})$$
$$+\boldsymbol{\varepsilon ijt},\ \boldsymbol{for\ i}=\mathbf{1,2};\boldsymbol{t}=\mathbf{6,20,26,40,46};\boldsymbol{j}=\mathbf{1,2,3}$$

Where α indicates the baseline, which corresponds to an observation under the condition that the salt level to be (NaCl, CaCl)=(0, 1.1) and time of observation to be 0 hour; i=1 and i=2 indicate the salt level to be (NaCl, CaCl)=(2, 0.0) and (NaCl, CaCl)=(6, 0.0); t=6,t=20,t=26,t=40 and t=46 indicate the time of observation to be 6, 20, 26, 40 and 46 hours. $Isal(i)\times ITimeHour(t)$ represents the interaction effect between salt level of i and time t.

When looking at overall log reductions in *Lactobacillus* spp. samples, a 5-log reduction was reached in 2% NaCl by 46 hours and 6% NaCl by 40 hours (Table 4.4). The log reductions in 1.1% $CaCl_2$ proceeded at the slowest rate overall. At 46 hours only a 4.34 log reduction was reached.

Table 4.4. Surviving Populations of *Lactobacillus* in log CFU/ml[a] in Varied Salt Types and Concentrations over Time and Time Required to Achieve a 5-log Reduction.

Time (Hours)	Salt Type and Concentration		
	2% NaCl	**6% NaCl**	**1.1% $CaCl_2$**
1	8.53	8.49	8.54
6	6.83	6.71	6.97
20	4.78	4.45	5.55
26	3.87	3.60	4.70
40	3.62	3.31[b]	4.37
46	2.57[b]	2.71	4.20
Total log reduction by 46 hours	5.96	5.78	4.34

[a] Values in a column represent the average of 3 reps

[b] Represents the time point a 5-log reduction is first observed

A Type III Test of Fixed Effects was run (Table 4.5). All effects including salt level $F(2, 34) = 223.87$, Pr > F= <.001, time of observation $F(5,34) = 2354.71$, Pr > F= <.001, and interaction (salt level x time of observation) $F(10,34)= 21.06$, Pr > F= <.001 are significant at significance level $\alpha = .01$. Since the interaction effects were significant, these will be the focus of interpretation rather than the main effects of salt level and time of observation. This significance demonstrates that the interaction of these two variables is an important predictor for the dependent variable (log reduction).

Table 4.5. Type III Test: Results of Fixed Effects Analysis in Log Reduction of *Lactobacillus* spp. in Media with Varying Salt Types and Concentrations.

Effect	Numerator DF	Denominator DF	F Value	Pr > F
Salt level	2	34	223.87	<.001
Time	5	34	2354.71	<.001
Salt level x time	10	34	21.06	<.001

To interpret if there were significant differences across the interaction effects specifically, differences of least square means was calculated at the significance level $\alpha = .01$ (Table 4.6). A few trends were revealed through the statistical model: 1) no significant difference exists between log reductions for all salt concentrations at a time point of zero hours, 2) no significant difference exists between log reductions for salt concentrations of 2% NaCl and 6% NaCl and 2% NaCl and 1.1% CaCl at a time point of six hours, 3) no significant difference exists between log reductions for salt concentrations of 2% NaCl and 6% NaCl at 46 hours, and 4) a significant difference in log reductions exists between all salt concentrations at all other time points.

Table 4.6. Equality Test: Differences of Least Square Means of Log Reductions in a *Lactobacillus* spp. Population When Comparing Salt Levels at Various Time Points.

Time Point (Hours)	Salt Levels Compared[a]	Estimate	Standard Error (SE)	DF	t value	Pr > | t |[b]
1	1, 2	0.04667	0.1042	34	0.45	0.6572
1	1, 3	-0.01000	0.1042	34	-0.10	0.9241
1	2, 3	-0.05667	0.1042	34	-0.54	0.5902
6	1, 2	0.1233	0.1042	34	1.18	0.2449
6	1, 3	-0.1333	0.1042	34	-1.28	0.2094
6	2, 3	-0.2567	0.1042	34	-2.46	0.0190*
20	1, 2	0.3367	0.1042	34	3.23	<.0001*
20	1, 3	-0.7733	0.1042	34	-7.42	<.0001*
20	2, 3	-1.1100	0.1042	34	-10.65	<.0001*
26	1, 2	0.2767	0.1042	34	2.65	<.0001*
26	1, 3	-0.8300	0.1042	34	-7.96	<.0001*
26	2, 3	-1.1067	0.1042	34	-10.62	<.0001*
40	1, 2	0.3067	0.1042	34	2.94	0.0058*
40	1, 3	-0.7567	0.1042	34	-7.26	<.0001*
40	2, 3	-1.0633	0.1042	34	-10.20	<.0001*
46	1, 2	-0.1500	0.1042	34	-1.44	0.1592
46	1, 3	-1.6333	0.1042	34	-15.67	<.0001*
46	2, 3	-1.4833	0.1042	34	-14.23	<.0001*

[a] Where salt level is denoted as 1= 2% NaCl, 2= 6% NaCl, and 3= 1.1% $CaCl_2$

[b] Results with * indicate significant difference at significance level $\alpha = .01$

CHAPTER 5

DISCUSSION

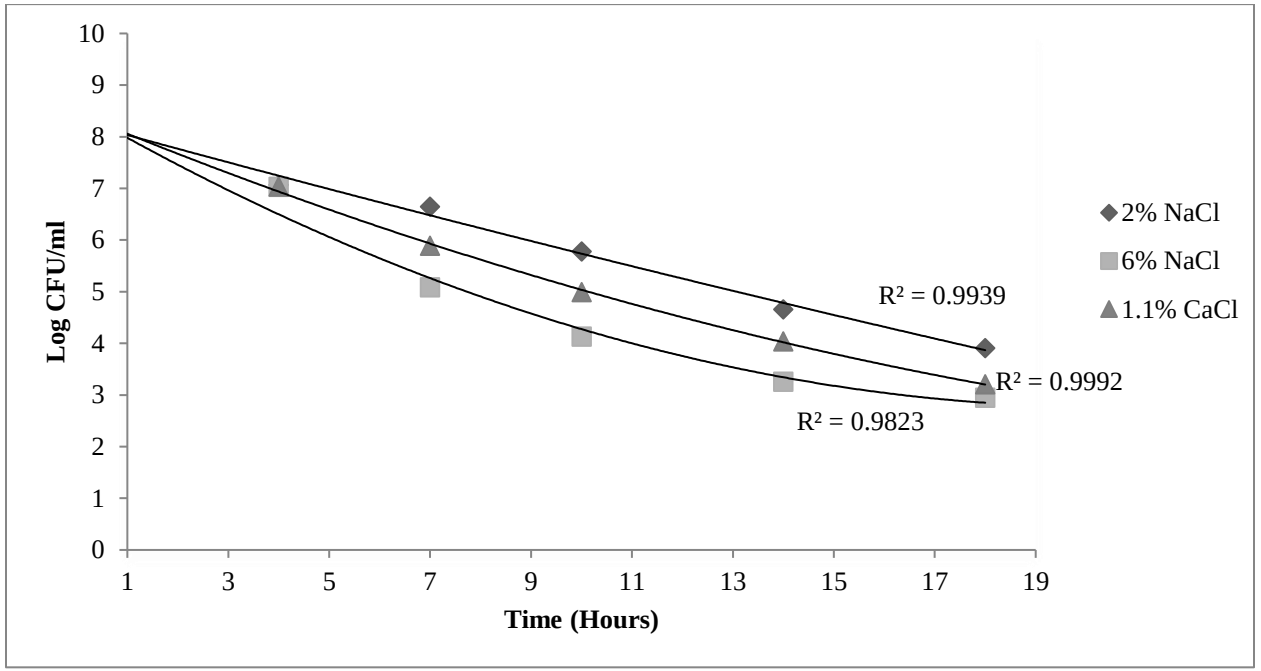
The objective of this research was to determine 5-log reduction times in 50% cucumber juice medium for two separate bacterial cocktails: *Escherichia coli* O157:H7 and *Lactobacillus* species (spp.). Media differed by salt type and concentration: 2% NaCl, 6% NaCl, and 1.1% $CaCl_2$. The results of this study showed a significant interaction effect for time and salt level for both *E. coli* O157:H7 and *Lactobacillus* spp. cocktails. This indicates that the interaction between these two variables is an important predictor for the outcome variable of log reduction.

Escherichia coli O157:H7 Log Reductions

Overall, *E. coli* O157:H7 log reduction was fastest in the cucumber juice medium with 6% NaCl compared to the other two media. This was expected based on prior literature and the known osmotic stress of NaCl salt in bacteria (Breidt et al., 2013). This confirmed one of the key hypotheses generated prior to the study. In contrast, *E. coli* O157:H7 log reduction was slowest in the cucumber juice medium with 2% NaCl. This was an unexpected finding. It was hypothesized that log reduction would be slowest in the cucumber juice medium with 1.1% $CaCl_2$. This appears to be evidence that lower concentrations of $CaCl_2$ could be effective in promoting log reductions. Average log reductions of >5 CFU/ml were achieved within 18 hours in both 6% NaCl and 1.1% $CaCl_2$. At 18 hours, the log reduction reached in 2% NaCl was only an average of 4.43 CFU/ml

(Figure 5.1). Figure 5.1 depicts the reduction of *E. coli* in the three salt media used. The equations used to generate these lines were: $y = 0.0013x^2 - 0.2691x + 8.3005$ (2% NaCl), $y = 0.0063x^2 - 0.4043x + 8.4543$ (1.1% $CaCl_2$), and $y = 0.0137x^2 - 0.5626x + 8.5282$ (6% NaCl).

Differences of Least Square (LS) means of log reductions were also determined through a repeated measures analysis of variance (ANOVA) general linear model (GLM). Across all salt levels at a time point of 1 and 4 hours, no significant difference was seen in log reduction. At all other time points sampled, significant differences were seen across all salt levels. With a significant difference noted at time points of 7, 10, 14, and 18 hours, comparisons can be made on pace of log reduction based on salt level.



Where R^2 is the coefficient of determination. Correlations between salt concentrations and log reductions are all strong, positive. The equations used to generate these lines were: $y = 0.0013x^2 - 0.2691x + 8.3005$ (2% NaCl), $y = 0.0063x^2 - 0.4043x + 8.4543$ (1.1% $CaCl_2$), and $y = 0.0137x^2 - 0.5626x + 8.5282$ (6% NaCl).

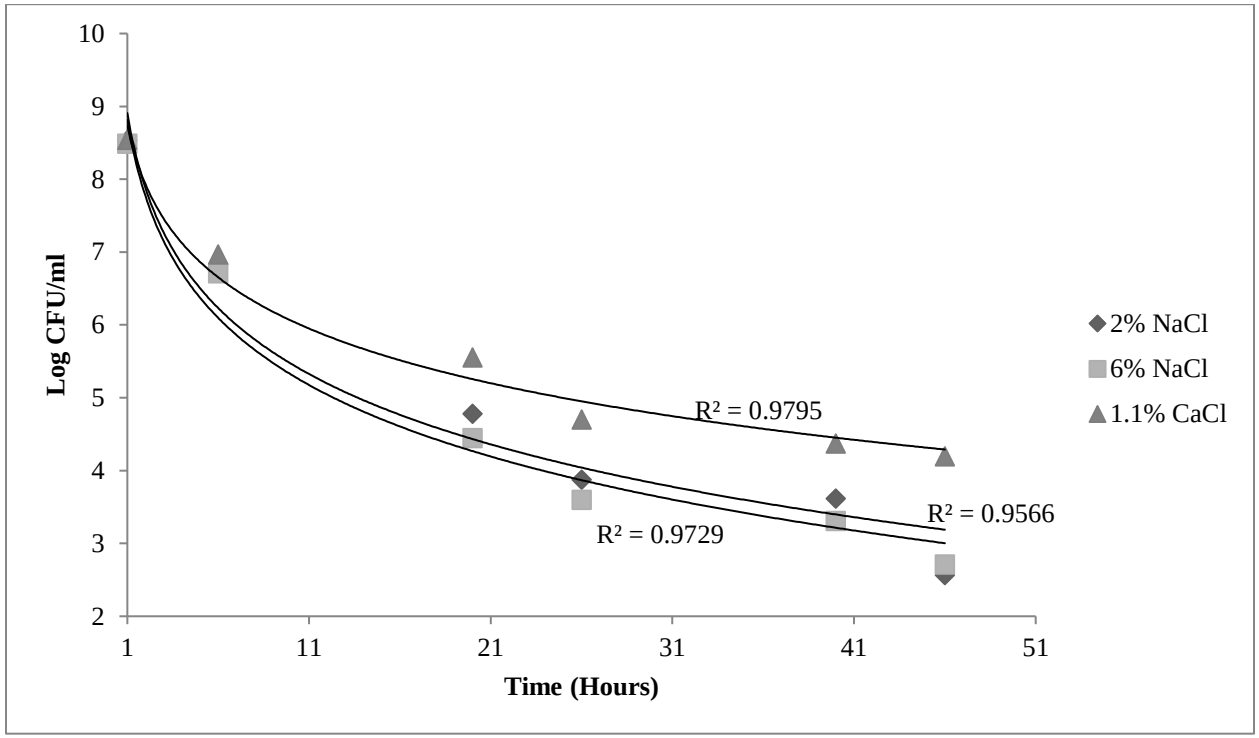
Figure 5.1. Mean Log Numbers of *E. coli* O157:H7 in a Cucumber Juice Medium over Time in Varied Salt Treatments Stored at 30 °C.

***Lactobacillus* spp. Log Reductions**

Overall, in the medium with 1.1% $CaCl_2$, log reduction proceeded at a slower rate when compared to medium with 2% and 6% NaCl. This confirmed one of the key hypotheses generated at the study. In contrast, log reduction in the medium with 6% NaCl proceeded at a faster rate, although it is important to note there was no significant difference between it and 2% NaCl at the final sampling time period of 46 hours. Again, this finding supported the original hypothesis generated at the start of the study that log reduction would proceed fastest in the medium with 6% NaCl. Average log reductions of >5 CFU/ml were achieved within 46 hours in both 6% NaCl and 2% NaCl. At 46 hours, the log reduction reached in 1.1% $CaCl_2$ was only an average of 4.34 CFU/ml (Figure 5.2). As with *E. coli* O157:H7, the effective concentration of salt will vary based on salt type. Figure 5.2 depicts the reduction of *Lactobacillus* spp. in the three salt media used. The equations used to generate these lines were: $y = -1.161\ln(x) + 8.7333$ (1.1% $CaCl_2$), $y = -1.494\ln(x) + 8.9082$ (2% NaCl), and $y = -1.518\ln(x) + 8.8164$ (6% NaCl).

Differences of Least Square (LS) means of log reductions were determined through a repeated measures analysis of variance (ANOVA) general linear model (GLM). Across all salt levels at a time point of 1 hour, no significant difference was seen in log reductions. At a time point of 6 hours, no significant difference was seen between log reduction in this cucumber juice medium when comparing 2% NaCl and 6% NaCl or when comparing 2% NaCl and 1.1% $CaCl_2$. A significant difference was seen at a time point of 6 hours when comparing 6% NaCl and 1.1% $CaCl_2$, however. Log reduction began to be significantly less in the cucumber juice medium with 1.1% $CaCl_2$ compared

to the medium with 6% NaCl. At time points of 20, 26, and 40 hours, there is a significant difference across all salt levels. At these time points, log reduction proceeded faster in the cucumber juice medium with 6% NaCl, while log reduction proceeded slower in the medium with 1.1% $CaCl_2$. Log reduction times for the medium with 2% NaCl is between these two. Interestingly, at the final time point of 46 hours, no significant difference was seen in log reduction between 2% NaCl and 6% NaCl. The medium with 1.1% $CaCl_2$ remained significantly different from both media with 2% and 6% NaCl, consistent with the findings in previous time points sampled.



Where R^2 is the coefficient of determination. Correlations between salt concentrations and log reductions are all strong, positive. The equations used to generate these lines were: y = -1.161ln(x) + 8.7333 (1.1% $CaCl_2$), y = -1.494ln(x) + 8.9082 (2% NaCl), and y = -1.518ln(x) + 8.8164 (6% NaCl).

Figure 5.2. Mean Log Numbers of *Lactobacillus* spp. in a Cucumber Juice Medium over Time in Varied Salt Treatments Stored at 30 °C.

Limitations

Several key limitations exist in regards to the experiments conducted. Most notably, results apply only to the specific salt concentrations studied: 2% NaCl, 6% NaCl, and 1.1% $CaCl_2$. Results cannot be generalized to other concentrations, though general trends can be predicted. This is important to note particularly when it comes to newer technologies involving $CaCl_2$.

It is seen that *E. coli* reduction is potentially achieved fastest based in this model with 1.1% $CaCl_2$ compared to 2% NaCl which is a standard salt amount in some acidified food processing. *Lactobacillus* log reduction seems to proceed slowest in the context of this model with 1.1% $CaCl_2$ when compared to both 2% and 6% NaCl. *Lactobacillus* spp. itself are not foodborne pathogens like *E. coli* O157:H7, however. They are considered probiotic bacteria with potential health effects. This research provides evidence to use $CaCl_2$ as a salt for preservation when *E. coli* populations are desired to be reduced significantly, while *Lactobacillus* spp. are not. This is a primary goal in both home and commercial fermentations.

Results were also obtained for these two microorganisms separately. Competition studies with both microorganisms in a cucumber juice medium could yield different results for both times in log reduction and for differences in reduction based on salt type/level in the medium. Lactic acid was also considered a control in both parts of this research. Amount and type of acid did not vary as salt did in these experiments. If a different acid were to be used, e.g. acetic or benzoic, log reduction times could also be different as a result.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Survival of pathogenic bacteria in acidified and fermented vegetable products continues to be an area of concern in the processed vegetable industry. The pathogenic bacteria *Escherichia coli* O157:H7 particularly is considered to be the most acid resistant pathogen of concern for non-fermented acidified foods. While determining methods to eliminate *E. coli* O157:H7, it is also useful to characterize *Lactobacillus* species (spp.) survival independently. *Lactobacillus* spp. are naturally occurring on the vegetable surface as well and are important for successful fermentations of vegetables. Therefore, methods used to reduce or eliminate pathogens need to, at the same time, preserve necessary *Lactobacillus* spp. for fermentations.

It also possible that competition among these microorganisms could influence survival. Before competition studies can be considered between these two microorganisms, however, it is fundamental to understand and characterize their behavior in media independently. With this consideration in mind, this research sought to determine log reduction times for *E. coli* O157:H7 and *Lactobacillus* spp. cocktails separately. A repeated measures analysis of variance (ANOVA) showed that, overall, both *E. coli* O157:H7 and *Lactobacillus* spp. log reduction proceeded fastest in a cucumber juice medium with 6% NaCl compared to either a 2% NaCl or 1.1% $CaCl_2$ concentration. *E. coli* O157:H7 in cucumber juice medium with 2% NaCl showed the

slowest log reduction. This differed from the *Lactobacillus* spp. cocktail where log reduction progressed slowest in 1.1% $CaCl_2$.

Differences of Least Squares (LS) means of log reductions showed if there were significant differences across salt treatments for *E. coli* O157:H7 and *Lactobacillus* spp. cocktails. Across all salt levels at a time point of 1 and 4 hours, no significant difference was seen in log reduction for *E. coli* O157:H7. At all other time points sampled, significant differences were seen across all salt levels for *E. coli* O157:H7.

Across all salt levels at a time point of 1 hour, no significant difference was seen in log reduction for *Lactobacillus* spp. At a time point of 6 hours, no significant difference was seen between log reduction in cucumber juice medium when comparing 2% NaCl and 6% NaCl nor 2% NaCl and 1.1% $CaCl_2$ for *Lactobacillus* spp. A significant difference was seen at a time point of 6 hours when comparing 6% NaCl and 1.1% $CaCl_2$, however in *Lactobacillus* spp., Log reduction began to be significantly less in cucumber juice medium with 1.1% $CaCl_2$ compared to medium with 6% NaCl. At time points of 20, 26, and 40 hours, there is a significant difference across all salt levels for *Lactobacillus* spp. Additional research is needed, but there is some evidence from this study that calcium chloride could be useful for both reducing *E. coli* O157:H7 populations while allowing prolonged survival of desirable *Lactobacillus* spp.

An additional concept that still demands exploration is the “hurdle effect” in minimally processed foods. This refers to the combination of two or more inhibitory agents (such as acid and salt) in processing to reduce the risk of foodborne illness. Researchers have observed that a combination of effects is not always more inhibitory

to foodborne pathogens, however. Some work has demonstrated that *E. coli* can use 4% NaCl to counteract acidification of its cytoplasm by organic acids, demonstrating that the combination of salt and acid cannot always achieve an additive antimicrobial effect (Casey, P.G. and S. Condon, 2002). Other work showed that the addition of varied concentrations of NaCl significantly reduced the inhibitory effect of acetic acid on *E. coli* O157:H7 in laboratory media and pickled cucumbers. This suggests that the addition of salt at a certain concentration may actually increase the acid resistance response of *E. coli* O157:H7 (Bae, Y. and S. Lee, 2017). Not all researchers exploring this idea have utilized the same salt and acid concentrations and types though.

Suggestions for Future Research

There are several opportunities for future research related to determination of log reduction times in acidified vegetable products. For example, a fixed temperature of 30 °C was used for the cucumber juice media in these experiments. This served as an important control variable throughout. Repeat of experiments utilizing other temperatures as controls could provide useful information for processors. 30 °C was initially chosen because most acidified vegetable processing (including commercial fermentations) occurs at ambient temperatures. Testing with temperatures such as 20 °C would provide for a lower end to an ambient temperature range. In addition, it is important to consider testing extremes of 10 °C and 40 °C. Temperature extremes are known to occur during commercial fermentations, for example.

As mentioned in Chapter 5, log reduction times will vary when *E. coli* O157:H7 and *Lactobacillus* spp. are studied in competition. Determination of these log reduction

times in competition will be particularly impactful for formulation of industry recommendations, which rely on determinations of 5-log reductions as an industry performance standard (FDA, 2003).

An additional consideration is that this study explored log reductions of a population. Future research could also address growth patterns of these microorganisms in certain acid conditions. This study did not explore bacterial growth and death during a time of fermentation. Characterization of growth and death in fermentative conditions (including before lactic acid production) is warranted.

pH was also used as a control variable throughout these experiments. A pH of 3.2 was used for all media. Processing of acidified vegetables, as well as commercial fermentations, can be carried out across a range of pH values (such as 3.0-3.8), however (Breidt et al., 2013). Future research can be performed using similar protocols, but altering the control pH based on values in this range. In addition, different acid types and concentrations can also be compared in future studies.

While *E. coli* O157:H7 has been shown to be an acid resistant pathogen of concern, other pathogens such as *Salmonella* and *Listeria* are worth studying in terms of competition with *Lactobacillus* spp. These pathogenic microorganisms can occur on the surface of vegetables as well. Although they are widely considered to be less acid resistant (compared to *E. coli* O157:H7), there is a need for evaluating *Salmonella* and *Listeria* behavior in media supplemented with $CaCl_2$, considering this is still an innovative preservation technology (Kim et al., 2015).

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