ABSTRACT

FRAGEDAKIS, NICHOLAS. A Challenge Study: Inhibition of *Listeria monocytogenes* in Ready-to-eat Pork BBQ. (Under the direction of Arritt, F.M. PhD, Hanson, D.J. PhD., Smith, D. PhD.)

North Carolina eastern-style pork BBQ is a ready-to-eat (RTE) food product that is cooked, treated with a vinegar sauce, cooled, and packaged. There is a concern of *Listeria monocytogenes* (*Lm*) contamination due to post-lethality exposure primarily from food contact surfaces and packaging. Processors who produce RTE meat and poultry products must use one of three Alternatives (Alt) in order to meet requirements stated under 9 CFR Part 430, the *Listeria* guideline to ensure a safe product: Alt 1- post-lethality treatment (PLT) and an antimicrobial agent or process (AMAP), Alt 2 – PLT or an AMAP, Alt 3 – sanitation only. PLT’s can include steam pasteurization, radiant heating, and high pressure processing. Examples of AMAP’s can include any of the following; vinegar, lactates, diacetates, fermentation, drying, and freezing. Many North Carolina BBQ processors operate under Alt 3, depending on sanitation alone for preventing adulterated product. Operating under Alt 3 places the processor under increased end product and environmental testing. However, operating under Alt 2 per 9 CFR part 430 will save processors both time and money with fewer inspections as well as reduced environmental and product sampling. The *Listeria* guideline updated and released in January 2014 states that vinegar can be considered an antimicrobial agent if the final product pH is below 4.39 and even be considered a post-lethality treatment if there is at least 5-log *Lm* death before the product leaves the establishment. This challenge study explored 4 different concentrations of acetic acid (1.0%, 0.75%, 0.5%, 0.0%) which correlate to the following approximate final pH for the BBQ samples respectively ~4.63, 5.01, 5.27, and 6.11 using apple cider vinegar (5.0% acetic acid)
Pork shoulders were purchased at a local grocery store and prepared in Schaub Hall of the North Carolina State Food Science department. Inoculated meat samples (ca. 5.0 log CFU/g) were stored at 4°C (optimal) and 10°C (abuse) temperatures over 80 days and 60 days respectively. *Lm* counts were taken at 4 separate intervals for samples stored in both temperatures over the expected shelf life of the product. Results indicate that at 1.0% acetic acid (pH ~4.63), *Lm* was inhibited over the shelf life (p<0.05) at both optimal and abuse temperatures with respect to other treatments. Processors may use the findings in this study to meet Alt 2 requirements.
A Challenge Study: Inhibition of *Listeria monocytogenes* in Ready-to-eat Pork BBQ

by

Nicholas Fragedakis

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

Food Science

Raleigh, North Carolina

2014

APPROVED BY:

______________________________________________  ______________________________________
Fletcher Arritt, PhD                                      Dana Hanson, PhD
Committee Chair                                          

__________________________________________

Doug Smith, PhD
DEDICATION

To my family and friends
BIOGRAPHY

Nicholas Fragedakis came back to school after years in other industries to pursue his passion for helping others. The Food Science Department at North Carolina State University has allowed Nick to develop his interest in food science, while finding new ways that he can have a positive impact on society.
ACKNOWLEDGMENTS

First, I would like to thank God for directing my path and blessing me with the many opportunities that have come my way. I pray that I always make the most of the blessings in my life.

I would like to thank my wife for encouraging me to go back to school and finish what I started years ago. Without her love, prayers, and support I’m not sure I would have pursued these goals. I am so thankful for God bringing her into my life and blessing me with such a wonderful family.

I would also like to thank my academic advisor, Dr. Fletcher Arritt for the opportunity to work with him both in the entrepreneurial program and as a graduate student. I am appreciative of my committee for their guidance throughout this entire process. I also want to thank the people in my lab and in the Food Science Department that were an encouragement through these past two years.
# TABLE OF CONTENTS

LIST OF TABLES ........................................................................................................ vii

LIST OF FIGURES ...................................................................................................... viii

CHAPTER 1 Introduction.............................................................................................. 1

CHAPTER 2 Review Literature Review ....................................................................... 6

2.1 CHARACTERISTICS OF *LISTERIA* SPECIES .............................................. 6

2.2 History of *Listeria monocytogenes* ................................................................. 7

2.3 Recognition of *Listeria monocytogenes* as a Foodborne Pathogen ............. 8

2.4 Listeriosis .......................................................................................................... 9

2.5 Virulence and Pathogenicity ............................................................................ 10

2.6 Reservoirs ......................................................................................................... 10

2.7 *Listeria monocytogenes*: a robust organism ............................................... 11

2.8 Antimicrobial effect of organic acids .............................................................. 13

2.9 Ready-to-Eat (RTE) Meats ............................................................................ 15

2.10 Post-Processing Contamination ..................................................................... 16

2.11 Rational and Significance .............................................................................. 16

References .............................................................................................................. 18

CHAPTER 3 ................................................................................................................. 23

Abstract .................................................................................................................. 23

3.1 Practical Applications ...................................................................................... 23

3.2 Introduction ...................................................................................................... 24

Materials and Methods.......................................................................................... 26
LIST OF TABLES

Table 2.1  Growth limits of *Listeria monocytogenes* ........................................41
Table 2.2  Fecal Carriage Rates of *Listeria monocytogenes* .................................42
Table 2.3  *Listeria* species Serotypes ..................................................................43
Table 2.4  Food Contact and Non-Food Contact Contamination Sites in
Food Processing Plants ......................................................................................44
Table 2.5  Provisional cases of infrequently reported notifiable diseases
United States, week ending August 31, 2013 (35th week) ..............................45
Table 4.1  Analysis of RTE Pork BBQ product ......................................................46
Table 4.2  Recovery of *Listeria monocytogenes* at 4°C for 80 days in
RTE Pork BBQ with various concentrations of Acetic Acid .........................47
Table 4.3  Recovery of *Listeria monocytogenes* at 10°C for 60 days in
RTE Pork BBQ with various concentrations of Acetic Acid .........................48
LIST OF FIGURES

Figure 2.1  2012 Recalls Reported by the Food and Drug Administration (FDA) due to *Listeria monocytogenes* ............................................................... 49

Figure 2.2  2012 Recalls Reported by the United States Department of Agriculture Food Safety Inspection Service (USDA-FSIS) due to *Listeria monocytogenes* ............................................................... 50

Figure 2.3  Categorization of Listeriosis as invasive and noninvasive ............... 51

Figure 2.4  *Listeria monocytogenes* biofilm formation ....................................................... 52

Figure 4.1  *Listeria monocytogenes* survival at 4°C during shelf life of 80 days ........................................................................................................ 53

Figure 4.2  *Listeria monocytogenes* survival at 10°C during shelf life of 60 days ........................................................................................................ 54

Figure 4.3  Total aerobic plate count at 4°C during shelf life of 80 days ................. 55

Figure 4.4  Total aerobic plate count at 10°C during shelf life of 60 days ............ 56
CHAPTER 1

Introduction

Data from the CDC released in 2013 estimate that 1 in 6 Americans acquire a foodborne illness annually. Listeriosis is an important public health problem in the United States as *Listeria monocytogenes* can cause serious health issues and even death, usually with the elderly, immunocompromised, infants, and pregnant women (CDC, 2013). Symptoms of Listeriosis vary from person to person, but common signs may be fever, fatigue, nausea, vomiting, muscle aches, diarrhea and more severe illnesses such as meningitis and septicemia (Awaisheh, 2009). The most recent data (June, 2013) from the Centers for Disease Control and Prevention (CDC) show that there are approximately 1600 reported illnesses each year in the U.S., and of these 260 deaths. Humans generally acquire Listeriosis by eating foods which have been contaminated by animal sources and in 1981, contaminated coleslaw led to 34 perinatal and 7 adult cases of Listeriosis. It is thought that the source of contamination came from manure of two sheep which died of Listeriosis and was used as fertilizer for cabbage for the implicated coleslaw (Schlech et al., 1983). Two more recent Listeriosis outbreaks involved cantaloupes from a farm in Colorado and imported Frescolina Marte Brand ricotta cheese. Both outbreaks occurred in multiple states which led to 147 and 22 illnesses respectively. According to the CDC, the outbreak from the ricotta cheese led to four deaths and one fetal loss, while the outbreak involving the cantaloupes led to thirty-three deaths and one miscarriage. Current data reported by the CDC (2013) in the Morbidity and Mortality Weekly Report (MMWR) shows Listeriosis cases leveling off for the past 5 years.
(Table 2.5) (CDC, 2013). More specifically, between the years 2009-2011 there were 1651 cases of Listeriosis reported. Of these cases there was a 21% fatality rate of which the highest among the elderly (≥65 years of age). During these two years there were 12 reported outbreaks with 224 affected people in 38 states. MMWR also indicated of the 12 reported outbreaks five of these were due to soft cheeses made from pasteurized milk and two from raw produce. *L. monocytogenes* is listed by the CDC as causing the third highest number of deaths among foodborne pathogens (CDC, 2012). One of the largest reported Listeriosis outbreaks associated with death occurred in 1985 involving Mexican-Style cheese causing 142 illnesses and 48 deaths including 20 fetuses, 10 neonates, and 18 adults (Linnan et al., 1988).

Ready-to-eat (RTE) meat products such as deli meats, hotdogs, and BBQ pose potential health issues because of the types of pathogenic growth that can be supported including *L. monocytogenes*. The genus *Listeria* is found ubiquitous in nature and has unique growth characteristics that give the pathogen *L. monocytogenes* the ability to survive and grow in processing facilities. For example, *L. monocytogenes* has the ability to grow at refrigerated temperatures, form biofilms which protect from sanitizing agents (Pan et al., 2006), and is naturally found on meats and raw produce (Lado & Yousef, 2007). Biofilm formation on various surfaces suggests that there is potential for *L. monocytogenes* contamination on food contact surfaces as well as packaging. Biofilm formation occurs in the following sequence; cell deposition on surface, cell adhesion to surface, surface colonization, biofilm formation, and finally biofilm development (Figure 2.4) (Lado & Yousef, 2007). This
is especially important for RTE meat processing facilities since the product is handled after the lethality step.

Companies producing RTE meat products that may be exposed to contamination after a thermal treatment must adhere to sanitation guidelines put forth under 9 CFR 430.4, the *Listeria* Guideline. This guideline states that a processor must have a Hazard Analysis and Critical Control Point (HACCP) plan in place or prevent contamination by Sanitation Standard Operating Procedures (SSOPs) or other prerequisite program. Processors of RTE meats are given the choice of following one of three alternative plans under 9 CFR 430. Processors following Alternative 1 use a post-lethality treatment (i.e. antimicrobial agent) to reduce or eliminate microorganisms on the product and an antimicrobial agent or process that inhibits the growth of *L. monocytogenes*. The antimicrobial agent or process used to limit microbial growth must be included in the processors HACCP plan or sanitation plan and must be validated. Alternative 2 allows the processor to use either a post-lethality treatment (i.e. antimicrobial agent) that reduces or eliminates microorganisms on the product or an antimicrobial agent that inhibits growth of *L. monocytogenes*. Since this alternative allows the processor the choice of either the post-lethality treatment or an antimicrobial to inhibit *L. monocytogenes* the processor must provide for testing of food contact surfaces in the post-lethality processing environment, conduct hold-and-test procedures, state the frequency of testing, include the size and location of testing sites, and explain why this will be sufficient to control *L. monocytogenes* or indicator organisms. Choosing Alternative 2 the processor is more likely to be subjected to a higher frequency of verification by the FSIS in comparison to Alternative 1. Alternative 3 is the use of sanitation only to control *L. monocytogenes*. 
Alternative 3 follows the same requirements as Alternative 2 and includes additional requirements for establishments producing deli meats or hotdog products. Additional testing and verification must be done on food contact surfaces after an initial positive test for *L. monocytogenes* or an indicator organism as well as conducting follow up tests to ensure the effectiveness of the sanitation. If further testing reveals positives for *L. monocytogenes* or an indicator organism the processor must hold the lots of product for microbial sampling. After corrective actions have been successful the processor must ensure that the product is not contaminated and document results. The processor may rework the product in a way that is destructive to *L. monocytogenes*.

The Food Safety Inspection Service (FSIS) released a *Listeria* Guideline in 2014 for controlling this bacterium in facilities that make RTE meat and poultry products. RTE meat processors can choose from three alternatives to control *L. monocytogenes*, however those that choose alternative 3 (sanitation only) will have increased environmental and end product sampling by the USDA-FSIS (Miller & Dickson, 2009). Under the FSIS *Listeria* guideline, processors who are using Alternative 1 or 2 and show that the antimicrobial agent or process used inhibits *L. monocytogenes* growth to 2-log or less over the estimated shelf life, will be subject to reduced product sampling by FSIS (FSIS *Listeria* Guideline, 2014).

Food preservation techniques such as the addition of organic acids, reducing the water activity, adding salts, and drying have been used for many years. It has also been shown that reducing the pH of a food along with thermal treatment destroys *L. monocytogenes* (Beuchat et al., 1986). Another study done by the Center for Red Meat Safety in Fort Collins, Colorado showed that both pH and water activity independently and
combined can inhibit growth of *listeria monocytogenes* and that inoculum levels played an
important role (Koutsoumanis et al., 2005). Limits for growth of *L. monocytogenes* as
published by the International Commission on Microbiological Specifications for Foods
states that *Listeria* can grow at temperatures ranging from -0.4°C to 45°C, pH ranging from
4.1-9.4, and can grow at a water activity as low as 0.92.
CHAPTER 2

Review of Literature

It is estimated that foodborne bacterial pathogens acquired in the United States cause 9.4 million episodes of foodborne illness annually (Scallan et al., 2011). Of these 9.4 million foodborne illnesses that were reported, roughly 55,961 led to hospitalizations and approximately 1,351 deaths. There were 31 major pathogens that were reported in this study and only 4 that were implicated as the cause of death. Of the 4 pathogens that caused death, \textit{Listeria monocytogenes} attributed to 19\% of the total. It is estimated that 14 of the 31 major foodborne pathogens that were reported by Scallan et al., account for $14.0$ billion (ranging from $4.4$-$33.0$ billion) in cost of illness and a loss of 61,000 quality-adjusted life years (QALY) (Hoffman et al., 2012). Five of the fourteen foodborne pathogens account for 90\% of these costs; \textit{Salmonella enterica}, \textit{Toxoplasma gondii}, \textit{Campylobacter spp}, Norovirus, and \textit{Listeria monocytogenes}. Of the top five \textit{Listeria monocytogenes} is estimated to account for $2.6$ billion in health care costs and the loss of 9,400 QALYs (Hoffman et al., 2012).

2.1 Characteristics of \textit{Listeria} species

\textit{Listeria} are Gram-positive, non-spore-forming, rod shaped, facultative anaerobes. Individual cells measure 1-2\(\mu\)m in length and are motile at 25\(^\circ\)C although at 37\(^\circ\)C these bacteria do not appear motile (Kamp, H. 2011). The genus \textit{Listeria} has six species which include \textit{L. monocytogenes}, \textit{L. ivanovii}, \textit{L. seeligeri}, \textit{L. welshimeri}, and \textit{L. grayi} (Rocourt, J. 1996). Of the six species of \textit{Listeria} only two appear to be pathogenic, \textit{L. monocytogenes} and
L. ivanovii (Schmid et al., 2005). Of the two pathogenic species, L. monocytogenes is of greatest concern to human health where L. ivanovii is primarily an animal pathogen (Swaminathan et al., 2007). L. monocytogenes when motile produce five to six peritrichous flagella which may be beneficial for outcompeting non-motile organisms for the colonization of the gastrointestinal tract (O’Neil & Marquis, 2006). Table 2.1 displays various limits for growth of L. monocytogenes.

2.2 History of Listeria monocytogenes

In the early 1900’s a rod-shaped, Gram-positive, non-spore forming bacterium causing disease in rabbits and guinea-pigs was isolated. This bacterium was first named Bacterium monocytogenes because it infected the monocytes in the blood (Microorganisms in Foods 5, 1996). J.H. Harvey Pirie isolated a similar organism and proposed the name Listerella hepatolytica as the name for this genus and species in 1927 in the honor of Joseph Lister, the famous surgeon (Murray et al., 1926). In 1939 J.H. Harvey Pirie was informed at the Third International Congress for Microbiologists that this name was already in use by Jahn in 1906. Since the genus had become important to both human and animal sciences and the disease had become referred to as “Listerellosis”, Pirie had proposed the name Listeria (Pirie, 1940).

Reports have been made of this bacterium causing illnesses not only in humans but in a variety of animals including cattle, sheep, birds, rodents, and fish (Gray & Killinger, 1966). Contaminated silage (animal feed) had been implicated as the cause of animal Listeriosis and even with the knowledge of a large outbreak in Germany in the late 1940s from the
consumption of raw milk (Seeliger, 1961) it was not widely accepted that this may be the cause of human Listeriosis until the early 1980s (Linnan et al., 1988).

2.3 Recognition of *Listeria monocytogenes* as a Foodborne Pathogen

It was not until the early 1980s that a series of outbreaks led to *L. monocytogenes* being recognized as a dangerous foodborne pathogen. A paper published in 1983 by Schlech et al. examined an outbreak in Canada that led to 7 adult cases and 34 perinatal cases of meningitis. It was discovered that cabbage was the common factor with the illnesses. This provided the medical community reason to examine the risk factors for the acquisition of this uncommon infection (Schlech et al., 1983). A *L. monocytogenes* infection in 1979 led to 23 patients being admitted to Boston hospitals where raw vegetables were found to be the common factor (Ho et al., 1986). The summer of 1983 in Massachusetts, 49 patients acquired Listeriosis from pasteurized milk (Fleming et al., 1985). Of the 49 illnesses, 42 were adults and 7 were immunocompromised (i.e. fetuses, infants, elderly) with a mortality rate of 29%. *L. monocytogenes* serotype 4b was isolated from 32 of the 49 patients. The milk came from a farm in which cows were found to have Listeriosis during the time of the outbreak. This particular case raised the question of pasteurization being effective in destroying *L. monocytogenes*. The outbreak that probably had the biggest impact of alerting the world that animals and food can spread Listeriosis was in California (1985) that led to 142 illnesses and 48 deaths (Linnan et al., 1988). The food implicated with this outbreak was Mexican-style soft cheese and studies revealed *L. monocytogenes* serotype 4b as the cause of Listeriosis.
This study revealed that soft cheeses were often contaminated with unpasteurized milk (Linnan et al., 1988).

### 2.4 Listeriosis

Listeriosis impacts high risk groups with a high mortality rate (21%) (MMWR, 2013). High risk groups include the elderly (≥65 years), pregnant women, immunocompromised, and infants also referred to as YOPI (young, old, pregnant, immunocompromised). Listeriosis can be transmitted between species (zoonotic) and can present itself as invasive and noninvasive as seen in figure 2.3 (Donnelly, 2001). In non-pregnant adults Listeriosis leads to septicemia, meningitis, and meningoencephalitis and in pregnant women this disease can lead to stillbirths and abortion (Slutsker & Schuchat, 1999).

Incubation times vary greatly from known outbreaks which can make it difficult to determine infectious dose. The United States Department of Health and Human Services report incubation times of 3-70 days (foodsafety.gov, 2013). Five investigations of outbreaks in which \textit{L. monocytogenes} has been implicated revealed that the infectious dose was high (1.9 \( \times \) 10\(^5\) – 1.6 \( \times \) 10\(^9\) CFU) (Mead et al., 2005; Graves et al., 2005). The health of the individual, microbial load, and strain type all play a role in causing disease. Healthy individuals (2-6%) were found to be asymptomatic carriers of \textit{L. monocytogenes} (Ramaswamy et al., 2007). A Listeriosis outbreak implicating \textit{L. monocytogenes} and hotdogs in 1998 from a plant in Michigan found that the outbreak strain (serotype 4b), were present in numbers below the quantifiable limit of the three-tube most probable number (MPN) method. Turkey deli meat from this same plant during the time of the outbreak was found to have \textit{L. monocytogenes} serotype 1/2a, present at levels <0.3-2,200 CFU/g (Graves et al., 2005).
2.5 Virulence and Pathogenicity

*L. monocytogenes* is an opportunistic pathogen that infects animals and humans intracellularly (Hamon, M. 2006). Pathogenic strains of *L. monocytogenes* cause the rupture of red blood cells, hemolysis, a major virulence factor (Rocourt & Buchrieser, 2007). Of the 13 serotypes only 3 (4b, 1/2a, and 1/2b) account for the majority (>90%) of human Listeriosis (Dussurget et al., 2004). Table 2.3 lists the identified serotypes of the *Listeria* species.

Human Listeriosis generally occurs from ingestion of contaminated foods (Ramaswamy et al., 2007). *L. monocytogenes* infects epithelial cells by the interaction of internalin A (InlA) on the bacterial surface and E-cadherin (epithelial surface) (Mengaud et al., 1996). Because mice epithelial cadherin (E-cadherin) cells differ by a single amino acid from human E-cadherin cells they are more resistant to infection from *L. monocytogenes* (Lecuit et al., 2001). Guinea pigs and transgenic mice expressing E-cadherin with the same amino acid profile as humans were found to allow *L. monocytogenes* to cross the intestinal barrier (Lecuit et al., 2001).

2.6 Reservoirs

*Listeria* species are wide spread in nature and survive under a wide range of conditions. *Listeria* species can be found in soil, water, food processing plants, and in the intestinal tracts of healthy animals and humans. The Center for Disease Control and Prevention (CDC) state that *L. monocytogenes* can live for years in food processing facilities and is found in a wide variety of raw foods such as uncooked meats and vegetables, fruit, and
dairy products. Farming practices are important so as not to contaminate raw vegetables or silage used as animal feed. Sewage used as fertilizer has been found to contaminate vegetation with *L. monocytogenes* which can add to the risk for both human and animal health (Al-Azawi, 1990). Pasture grass from dairy farms reported 64.7% prevalence of *Listeria* species and 22.7% in silage samples (Husu et al., 1990). Patients with Listeriosis and those that are asymptomatic shed *L. monocytogenes* in their feces (Jensen, 1993).

The CDC has notified the public that RTE meat products like hotdogs and deli meats can be contaminated with *L. monocytogenes* because of post processing contamination. Food processing facilities are likely to have positive environmental samples from drains, floors, food contact surfaces, and condensation found in refrigeration systems of *L. monocytogenes* (Cox et al., 1989). *Listeria* species are able to form biofilms as a monoculture or with other bacteria which can lead to post-lethality contamination of foods. *L. monocytogenes* is able to attach to stainless steel at various temperatures (Herald & Zottola, 1988).

Suboptimal sanitation has been shown to not remove biofilms, leaving pathogens on food contact surfaces (Stone & Zottola, 1985). Pathogens that form biofilms can attach to rubber, glass, and stainless steel all present food processors with the potential for contamination (Zoltai et al., 1981).

### 2.7 *Listeria monocytogenes*: a robust microorganism

*Listeria monocytogenes* can grow over a wide range of temperatures, pH and sodium chloride concentrations as well as survive freezing and drying (Donnelly, 2001). Since *L. monocytogenes* is such a robust organism, ready-to-eat (RTE) meat processors must adhere to strict sanitation and sampling regulations put forth by the Food Safety and Inspection
Service (FSIS). Currently, the FSIS has six open federal cases regarding *L. monocytogenes* with the following foods, frozen meatballs (5/2/2013), Cooked Meat, Poultry, and Deli Products (4/12/2013), fully cooked meat and poultry products (1/19/2013), RTE pork product (6/2/2012), stuffed beef products (3/29/2012), and salad products which contain eggs (2/2/2012) (FSIS, 2013). Combined, the U.S. Food and Drug Administration (FDA) and the FSIS had a total of 100 recalls related to *L. monocytogenes* in 2012.

*L. monocytogenes* of primary concern for RTE establishments because it is widespread in nature and can survive and multiply in environments where most non-spore-forming pathogens cannot. *Listeriae* have no special nutrition requirements and are easily found on wet surfaces in food processing plants at refrigeration temperatures (Tompkin, 2002). *L. monocytogenes* grows slowly between 2°C-5°C but loses certain antigenic qualities at 37°C, can remain virulent in 20% NaCl at 4°C, and on growth media at room temperature (22°C) remains virulent for 3-4 years. *L. monocytogenes* has been shown to survive 6-26 weeks in animal feed that has been inoculated as well as survive 5 minutes at 80°C (Murray, 1955).

Both animals and humans can be asymptomatic carriers of *L. monocytogenes*. Fecal samples from healthy individuals were examined and found that 2-6% were carriers of *L. monocytogenes*, other sources estimate carriers as high as 10% (Ramaswamy et al., 2007). Table 2.2 presents data of fecal carriage rates of *L. monocytogenes* (Painter & Slutsker, 2007). This is potentially dangerous for RTE manufacturers and the reason for strict sanitation guidelines needed in these industries. Since 1999, Listeriosis outbreaks have
declined 40% in the United States (Ryser & Marth, 2007) however, Listeria is still very important to control due to high mortality rates (28%) (Mead et al., 1999).

2.8 Antimicrobial effect of organic acids

Weak organic acids such as acetic acid and lactic acid have been used as a preserving agent in foods since before recorded history (Hirshfield et al., 2003). In solution, acids exist in either a dissociated or undissociated state. Weak organic acids such as acetic acid can either be charged or uncharged; this depends on the protonation state of the acidic group, which is then determined by the pKₐ of that group and the pH of the medium surrounding it (Hirshfield et al., 2003). Acetic acid has a pKₐ of 4.75 (Adams & Hall, 1988) and so if the pH of the surrounding medium is below 4.75, acetic acid will have a higher concentration of undissociated species (lipid permeable) which will allow the passage through the cell wall of a microorganism (Hirshfield et al., 2003). Gram positive organisms (i.e. L. monocytogenes) do not have an outer membrane layer and so acids in the undissociated state are able to pass through the plasma membrane of a cell with less resistance than gram negative organisms (Brul & Coote, 1999). Upon entering the cell, cytoplasmic pH is near neutral which leads to the dissociation of the acid causing an imbalance of anions and protons within the cell. The accumulation of protons within the cell can exceed the buffering capacity of the bacterial cytoplasm and lead to growth inhibition (Booth & Kroll, 1989). Several mechanisms have been described as having an inhibitory effect on microorganisms such as membrane disruption (Bracey et al., 1998), intracellular disruption of pH homeostasis (Salmond et al., 1984), and energetically expensive proton pumping by the organism causing stress and/or
death (Bracey et al., 1998). *L. monocytogenes* has been shown to acquire acid tolerance when exposed to sub-lethal acid stress, also called the acid tolerance response (ATR) (Davis et al., 1996). Although a rapid change in pH, pH 7.0 to pH 3.0 in 10-30 minutes, will result in the destruction of cells of up to a 5 log reduction depending on the organism (Booth & Stratford, 2003).

Acetic acid is a clear colorless liquid with a strong odor and commonly referred to as vinegar (Luttrell, W.E., 2012). Vinegar is the primary ingredient used in sauce formulation of Eastern North Carolina Style BBQ. Ketchup, another common ingredient used for BBQ sauces in other parts of the U.S. also contains vinegar. Breidt et al., (2014) found that at a pH of 4.6 with the primary acidulant being acetic acid in cucumber juice that a 5-log reduction of *L. monocytogenes* is attainable within 14.32 minutes at 66°C (150.8°F). Another study showed a 5-log reduction of *L. monocytogenes* within .6 days in brined cucumbers with a 2.5% acetic acid concentration at a pH of 3.5 (Breidt et al., 2013). USDA-FSIS approved antimicrobial agents such as potassium lactate, sodium lactate, sodium diacetate, sodium benzoate, and potassium sorbates are commonly used to inhibit microbial growth at allowed concentrations of 4.8% (lactates), 0.25%, and 0.1% (benzoates and sorbates) by weight respectively (9 CFR 424.21 (c)). A synergistic effect of sodium lactate along with sodium diacetate at 3% and .25% respectively completely inhibited *L. monocytogenes* growth on pork frankfurters for 120 days at 4°C (Samelis et al., 2002). Sodium lactate used alone on frankfurters at a concentration of 3% by weight showed to have very little effect on the growth of *L. monocytogenes* stored in the same conditions and sodium acetate and sodium diacetate at the allowed limits (.25% by weight) only inhibited *L. monocytogenes* for 35-50
days at 4°C (Samelis et al., 2002). Sodium benzoate in combination with sodium diacetate shows to provide a longer shelf life (less than 1-log growth of \textit{L. monocytogenes}) in products than when used alone (Samelis et al., 2002). Product moisture also plays a role in the effectiveness of the antimicrobials used. A study looking at the inhibition of \textit{L. monocytogenes} in ready-to-eat meat products showed that products with high-moisture content (~75%) had shorter time to growth (TTG) than products with low moisture (~60%) with the use of sodium benzoate and sodium diacetate at varying concentrations (Seman et al., 2008).

### 2.9 Ready-to-Eat (RTE) Meats

The Food Safety Inspection Service (FSIS) defines a Ready-to-Eat (RTE) product as a product that is intended to be consumed without any further processing steps. There are four categories of these foods that are defined under the code of federal regulations (CFR); 1- not heat treated (shelf stable) 9 CFR 417.2(b)(v), 2- heat treated (shelf stable) 9 CFR 417.2 (b)(vi), 3- fully cooked (not shelf stable) 9 CFR 417.2 (b)(vii), and 4- product with secondary inhibitors (not shelf stable) 9 CFR 417.2 (b)(ix). Food producers making RTE meat products must abide by 9 CFR 430, which states that these establishments have sanitation standard operating procedures (SSOPs) plans and hazard analysis and critical control point (HACCP) plans in place to prevent the adulteration of their products by \textit{L. monocytogenes}. RTE meat products are of concern because of outbreaks related to these products. Refrigeration usually inhibits the growth of most pathogens except for \textit{L. monocytogenes}. 
2.10 Post-Processing Contamination

Since *Listeria* species are ubiquitous in nature and have been discovered in food processing plants it is important for RTE establishments to have strict sanitation guidelines in place. *L. monocytogenes* easily transfers from contaminated surfaces to food and can tolerate a wide range of hurdles that usually inhibit other organisms (Murphy et al., 2005). The most obvious indication that *L. monocytogenes* enters the food stream post-lethality is when uninjured cells have been recovered from thermally processed foods such as dairy, meat, poultry, and seafood (Kornacki & Gurtler, 2007). Table 2.4 illustrates contamination sites in food processing plants of *Listeria* species.

USDA-FSIS inspectors sampled 15,000 processed meat products from September 1987 to October 1991 and found during this period 1.6% of products tested positive for *L. monocytogenes* (Kornacki & Gurtler, 2007). Between 1990 and 1991, another study had collected 18,000 environmental samples of RTE meat and poultry plants and found that 40% of these samples tested positive for *L. monocytogenes* (Tompkin, 2002).

2.11 Rationale and Significance

Food processors that make RTE meat products fall under strict guidelines and inspections by the USDA-FSIS in order to guarantee a safe unadulterated product. 9 CFR 430 allows the processor the choice of alternatives including antimicrobial agents (AMA), antimicrobial processes (AMP), and/or sanitation to control or inhibit *L. monocytogenes* growth in the product. For all three alternatives, establishments may use verification testing to ensure the effectiveness of sanitation. Measures used for controlling *L. monocytogenes*
(i.e. sanitation, AMA, AMP) can either be included in the plants HACCP plan, sanitation SOP, or other prerequisite program. The FSIS will conduct verification testing on all RTE establishments, but those that choose alternative 3 are likely to be subjected to product testing more often than those choosing alternative 2 or 1. Alternative 1 will more than likely receive the least testing done by the FSIS.

Although a number of studies have been done on RTE meat products and *L. monocytogenes*, there have not been any specific to reduced pH pork BBQ products with acetic acid as the primary mode of inhibition. BBQ is a popular product not only in North Carolina but throughout the U.S. Eastern North Carolina BBQ specifically uses pork and a vinegar based sauce. BBQ produced in other parts of the U.S. typically use a tomato based sauce which also contains acetic acid. Acetic acid concentrations in a tomato based sauce may contain the concentrations used in this study and so may be able to be applied to other sauce formulations. This study will determine how acetic acid at various concentrations may inhibit the growth of *L. monocytogenes* as well as how optimum temperature (4°C/39.2°F) and abuse temperature (10°C/50°F) affects the growth of *L. monocytogenes* in a BBQ pork product.
References


CHAPTER 3

Abstract

Contamination of ready-to-eat foods, such as pork BBQ, with *Listeria monocytogenes*, is a major concern that needs to be addressed in order to enhance the safety of this product. The objective of this study was to determine the acetic acid concentration that will inhibit *L. monocytogenes* growth to less than 2-logs over the expected shelf life of the product. *L. monocytogenes* inoculated (ca.10⁵ CFU/gram) pork BBQ post-lethality, prepared with apple cider vinegar (5% acetic acid) at different concentrations (0.0-1.00%) and pH values (~4.63-6.11). Samples were stored at 4°C for 80 days and 10°C for 60 days and analyzed for pH and microbial growth. At 1.0% acetic acid *L. monocytogenes* was inhibited over the expected shelf life (p<0.05) at both 4°C and 10°C. These results may allow processors with final a pH of ≤ 4.63 to have fewer environmental and end product testing.

3.1 Practical Applications

This study provides useful information to ready-to-eat pork BBQ processors with final product pH values ≤4.63 using acetic acid on the inhibition of *L. monocytogenes*. The results may allow processors to have fewer environmental and end product testing. Fewer inspections and sampling of the environment and product will save the processor both time and money. This will allow the processor to continue production uninterrupted by inspections and more product being shipped for sale. Additional studies are required to evaluate the effects of combinations of other common ingredients as well as different packaging used for this product.
3.2 Introduction

Data from the CDC released in 2013 estimate that 1 in 6 Americans acquire a foodborne illness annually. Listeriosis is an important public health problem in the United States as *Listeria monocytogenes* can cause serious health issues and even death, usually with the elderly, immunocompromised, infants, and pregnant women (CDC, 2013). Symptoms of Listeriosis vary from person to person, but common signs may be fever, fatigue, nausea, vomiting, muscle aches, diarrhea and more severe illnesses such as meningitis and septicemia (Awaisheh, 2009). The most recent data (June, 2013) from the Centers for Disease Control and Prevention (CDC) show that there are approximately 1600 reported illnesses each year in the U.S., and of these 260 deaths.

Ready-to-eat (RTE) meat products such as deli meats, hotdogs, and BBQ pose potential health issues because of the types of pathogenic growth that can be supported including *L. monocytogenes*. North Carolina eastern-style pork BBQ is a ready-to-eat (RTE) food product that is cooked, treated with a vinegar sauce, cooled, and packaged. There is a concern of *Listeria monocytogenes* (*Lm*) contamination due to post-lethality exposure primarily from food contact surfaces and packaging. Processors who produce RTE meat and poultry products must use one of three Alternatives (Alt) in order to meet requirements stated under 9 CFR Part 430, the *Listeria* guideline to ensure a safe product: Alt 1- post-lethality treatment (PLT) and an antimicrobial agent or process (AMAP), Alt 2 – PLT or an AMAP, Alt 3 – sanitation only. PLT’s can include steam pasteurization, radiant heating, and high pressure processing. Examples of AMAP’s can include any of the following; vinegar, lactates, diacetates, fermentation, drying, and freezing. Many North Carolina BBQ
processors operate under Alt 3, depending on sanitation alone for preventing adulterated product. Operating under Alt. 3 places the processor under increased end product and environmental testing.

The genus *Listeria* is ubiquitous in nature and has unique growth characteristics that give the pathogen the ability to survive and grow in processing facilities. *L. monocytogenes* is naturally found on meats and raw produce, has the ability to grow at refrigerated temperatures and may form biofilms which protect the organisms from sanitizing agents (Pan et al., 2006). Biofilm formation on various surfaces suggests that there is potential for *L. monocytogenes* contamination on food contact surfaces as well as packaging. Biofilm formation occurs in the following sequence; cell deposition on surface, cell adhesion to surface, surface colonization, biofilm formation, and finally biofilm development (Figure 2.4) (Lado & Yousef, 2007). This is especially important for RTE meat producing facilities since the product is handled after the lethality step.

The Food Safety Inspection Service (FSIS) released a revised version of the *Listeria* Guideline in 2014 for controlling this bacterium in facilities that make RTE meat and poultry products as defined under 9 CFR part 430.1. The FSIS will sample a product less frequently for processors who are using Alternative 1 or 2 and show that the antimicrobial agent or process used demonstrates 2-log or less growth over the estimated shelf life (FSIS *Listeria* Guideline, 2014).

Food preservation techniques such as the addition of organic acids, reducing the water activity, addition of salts, and drying have been used for many years. It has also been shown that reducing the pH of a food along with thermal treatment destroys *L.*
monocytogenes (Beuchat et al., 1986). Breidt et al., (2014) found that at a pH of 4.6 with the primary acidulant being acetic acid in cucumber juice that a 5-log reduction of L. monocytogenes is attainable within 14.32 minutes at 66°C (150.8°F). Another study showed a 5-log reduction of L. monocytogenes within .6 days in brined cucumbers with a 2.5% acetic acid concentration at a pH of 3.5 (Breidt et al, 2013). The Center for Red Meat Safety in Fort Collins, Colorado demonstrated that both pH and water activity independently and combined can inhibit growth of L. monocytogenes and that inoculum levels played an important role (Koutsoumanis & Sofos , 2005). Limits for growth of L. monocytogenes as published by the International Commission on Microbiological Specifications for Foods states that listeria can grow at temperatures ranging from -0.4°C to 45°C, pH ranging from 4.1-9.4, and can grow at a water activity as low as 0.92.

Materials and Methods

3.3 Preliminary pH testing of BBQ Pork

Pork shoulders purchased from a local grocery store were stored and prepared in the Meat Science lab at North Carolina State University. Pork Shoulders were cooked in a convection oven (Vulcan model# VC4GD-10, Louisville, KY) at 350°F (176°C) for 3 hours to an internal temperature of 180°F (82.2°C). In a refrigerated (4°C or 39.2°F) environment the fat back and bone was then removed from the pork shoulders and pulled into smaller pieces prior to being placed in a bowl-chopper (Hobart Corporation, model #84145, Troy, Ohio). The pork shoulders were chopped to a consistency typical of retail packaged BBQ. The chopped meat was then separated into 4 bins of 200g. Commercially available apple
cider vinegar (5% acetic acid) was added at concentrations of 0.5%, 0.75%, 1.0%, and 0.0% as a control (w/w acetic acid) and mixed for 3 minutes to create a homogeneous sample.

USDA-FSIS protocol for measuring pH of meat described in the Microbiology Laboratory Guidebook chapter 2.3 was performed in the following steps (1) the pH meter was calibrated, using certified buffers pH 7.00 and 4.00 1, (2) A 1:10 dilution (45mL deionized water was added to 5g meat) was made in a clean blender jar and blended (Waring Laboratory Blender LB10 Model # 38BL54) for 1 minute to a thin uniform consistency for pH measurement (Fisher Scientific Accumet AB15 pH meter).

### 3.4 Strains and Growth Conditions

A six strain *Listeria* cocktail was prepared using the three most common serotypes (4b, 1/2a, and 1/2b) isolated from outbreaks (Dussurget et al., 2004). Each strain was streaked onto brain heart infusion (BHI) agar and blood agar plates then incubated at 37°C for 24 hours to confirm purity. A single colony from each isolate was then separately transferred to 15mL of BHI broth and incubated at 37°C for 24 hours. A 100-µl portion from each tube was then added to 10mL of fresh BHI broth and incubated at 37°C for an additional 20 hours. Absorbance readings at 600nm after the 20 hours incubation at 37°C confirmed ca. 10⁸ cells/mL when compared to plates incubated for the same period at 37°C. Each strain was then centrifuged at 6500 rpm for 5 minutes (Labnet International, Inc., Spectrafuge 6C, Edison, NJ). After centrifugation the supernatant was poured off and 5mL of phosphate buffered solution was added to the tubes and spun again at 6500rpm for an additional 5 minutes. This process was repeated a total of two times to purify the pellet. Each strain was then vortexed and combined into a 50mL centrifuge tube. Absorbance and plating
of the cocktail on BHI agar confirmed ca. $10^8$ cells/mL. The cocktail was then placed into 1mL tubes for freezing at -70°C for future use.

Listeria monocytogenes strains NRRL B-33322 serotype 4b was isolated from pork bbq and NRRL B-33325 serotype 1/2b was isolated from bbq sauce with cooked pork and was obtained from the USDA-Agriculture Research Service culture collection laboratory in Peoria, Illinois. Multiplex polymerase chain reaction (PCR) (Doumith et al., 2004) and Pulsed-Field Gel Electrophoresis (PFGE) was used to verify the serotypes of the L. monocytogenes strains NRRL B-33322 and NRRL B-33325. Both NRRL B-33322 and B-33325 were also tested for arsenic and benzalkonium chloride resistance. L. monocytogenes strain NRRL B-33322 was resistant to arsenic and not resistant to benzalkonium chloride where NRRL B-33325 was not resistant to arsenic or benzalkonium chloride. The four additional L. monocytogenes strains were used in order to make the six strain cocktail. L. monocytogenes Scott A serotype 4b a human isolate, J-0161 serotype 1/2a isolated from sliced turkey, NRRL B-33027 serotype 1/2a isolated from frankfurters, and NRRL B-33028 serotype 1/2b isolated from chicken were obtained from Dr. Kathoriou North Carolina State University.

3.5 Pork BBQ and meat analysis

The product being used for this challenge study was a Ready-to-Eat (RTE) pork BBQ product. This product is traditionally prepared by smoking followed by chopping and/or pulling, depending on the meat particle size desired and finally seasoned with vinegar or a tomato based sauce. Smoking was not used in the preparation of this BBQ so as to not impart antimicrobials (Fellows, 2009). Often the vinegar based sauce is chilled to assist in lowing
the temperature of the meat. Cooked meat products must be cooled from 130°F to 80°F within 1.5 hours and from 80°F to 40°F within 5 hours according to USDA-FSIS Appendix B.

Two hundred grams of cooked BBQ without the addition of apple cider vinegar was sent to Microbac laboratories Inc., Wilson, NC for analysis of protein, moisture, fat, ash, and sodium. Results are as follows and displayed in Table 4.1: Protein 25.3% AOAC 992.15 MOD, moisture 54.9% AOAC 950.46B(b), fat 20.1% AOAC 960.39(b), ash 1.04% AOAC 920.153, and sodium 74.2mg/100g AOAC 990.08C/SW846 6010B.

3.6 Preparation of BBQ samples

Three uncooked pork shoulders were purchased from a local grocery store and immediately transported to the North Carolina State University Food Science Department in Schaub Hall to be prepared. Pork shoulders were cooked in a convection oven (Vulcan model# VC4GD-10, Louisville, KY) at 350°F (176°C) for 3 hours to an internal temperature of 180°F (82.2°C). The following procedure was performed at approximately 40°F (4°C). The fat back and bone was removed from the pork shoulders and pulled into smaller pieces prior to being placed in a bowl-chopper (Hobart Corporation, model #84145, Troy, Ohio). The pork shoulders were chopped to a consistency typical of retail packaged chopped BBQ (small fine texture). Once the meat was chopped and mixed to a homogeneous sample it was then divided into four subsamples of 1800 grams each. Apple cider vinegar (5% acetic acid) was added at 0.5%, 0.75%, and 1.0% (w/w acetic acid) and mixed for 3 minutes. The fourth
1800 gram subsample had no apple cider vinegar added as a control. Meat samples (9.9 gram) were then placed into individual sterile 7oz filtered WhirlPak bags (OTR 0.125 cc/100in²/24 h at 22.8°C and 0% RH; Part#B00992WA; Nasco, Fort Atkinson, Wisconsin) and taken to a pathogen lab for inoculation and incubation.

3.7 Inoculum preparation

Stationary-phase cells ca. $10^8$ CFU/ml were prepared in Brain Heart Infusion Broth (BHI) (Difco, Detroit, Mich., U.S.A.) at 37°C for 20 hours to achieve an optical density (OD) of approximately 600nm (Begley, Maire et al., 2010) (BioMate 3, Thermo Electron Corporation, Waltham, Massachusetts) as to correlate cell density with plate counts (Baty et al., 2002). The individual strains were then combined into 15 mL centrifuge tubes (Eppendorf, Hamburg, Germany) and spun down using a (Spectrafuge 6C, Edison, NJ) centrifuge at 6500 rpm for 5 minutes. The supernatant was discarded and 5mL of 0.01 M phosphate buffered solution (PBS, pH 7.2) was added. The tubes were then spun down an additional two times and rinsed with PBS to purify the pellet (Johnson et al., 1988). The six strain cocktail and pure cultures were stored at -70°C and sub-cultured in BHI Broth at 37°C for 24 hours before being used.

3.8 Inoculation of Ready-to-Eat Pork BBQ

One tenth of a milliliter of the six strain *Listeria monocytogenes* cocktail ca. 5.0 logs CFU/mL (Johnson et al., 1988) was pipetted into each 9.9 gram sample bag of pork BBQ. The sample was then stomached for 2 minutes at 300rpm (Seward Stomacher 400 Circulator, Davie, FL USA) (Johnson et al., 1988). The samples were then placed in the appropriate
incubator 4°C (optimal storage temperature) or 10°C (abuse storage temperature). Bacterial counts for the 4°C samples were then determined on day 0, 48, 64, and 80 and for the 10°C samples counts were determined on day 0, 36, 48, and 60. On the day of sampling each 10 gram sample had 20mL of PBS added and was stomached for 2 minutes at 300rpm (Seward Stomacher 400 Circulator) Serial dilutions were then made and the dilutions were plated onto Modified Oxford Agar (Oxoid CM0856 Listeria selective agar base, Thermo Scientific) with selective supplement (Oxoid SR0140E Listeria monocytogenes selective supplement) containing cycloheximide, colistin sulphate, acriflavine, cefoyetan and fosfomycin, each at 20mg/L) (Oxoid, Hampshire, England) (Johnson et al., 1988). Plates were then incubated at 37°C for 48 hours and colony counts were conveyed as CFU/g. Confirmation of L. monocytogenes was done by using an API Listeria kit (bioMerieux Ref. 10 300).

### 3.9 Shelf-life determination

Each processor determines the shelf life of their product based on quality attributes. It was decided that an average shelf life of RTE Pork BBQ from production day to end of life ranged from 60 to 68 days. An average of the two was used to choose a mid-point for the optimal 4°C samples. From that mid-point we chose a low (75% of the expected shelf life) and high (125% of expected shelf life) for this study. At the abuse temperature of 10°C the mid-point of 48 days was used due to the expectation that at the abuse temperature would see high levels of growth. Low and high levels of days were set at 75% and 125% of the
expected shelf life at abuse temperature.

3.10 Statistical Analysis

The effects of varying concentrations of acetic acid on the *L. monocytogenes* cocktail in pork BBQ at optimal and abuse temperatures were analyzed by statistical comparisons using two-way analysis of the variance (ANOVA) with a random block effect (version 9.3, SAS Institute Inc., Cary, NC, U.S.A). Statistical significance occurs at P<0.05.
References


CHAPTER 4

4.1 Results and Discussion

A critical factor for safety of RTE foods is not allowing recontamination of the food product after the lethality step as defined in 9 CFR Part 430. To assure safety of RTE foods, processors follow one of three alternatives during the processing and formulation of their product including sanitation of the facility. If a product is found to contain \textit{L. monocytogenes} it is considered adulterated and must adhere to the guidelines set forth by FSIS. Processors who rely solely on sanitation for the control of \textit{L. monocytogenes} will have more frequent testing of the facility and end product. Processors who can show that their formulation inhibits the growth of \textit{L. monocytogenes} to less than 2-logs over the expected shelf life with approved antimicrobials will therefore not fall under Alt. 3 and will have fewer inspections.

The meat product and apple cider vinegar used in this study were purchased at a local grocery store (Harris Teeter, Raleigh, NC, U.S.A.). Analysis of the RTE Pork BBQ samples for protein, moisture, fat, ash, and sodium were done by Microbac Laboratories in Warrendale, PA. Table 4.1 shows the results of this analysis. Figure 4.1 shows the estimated means with 95% confidence levels the survival of \textit{L. monocytogenes} after 80 days of storage at 4°C. It is seen that at day 0 the initial inoculum for all treatments are ca. $10^5$ CFU/gram. Sample counts were then done on days 48, 64, and 80. On day 48 the control sample with 0.0% acetic acid saw a 4 log CFU/gram increase in \textit{L. monocytogenes}. The data for the control sample after day 0 was statistically significant (p < 0.05) from the other treatments as seen in table 4.2. Samples with 0.5% and 0.75% treatments were not statistically significant
from each other throughout the entire 80 day shelf life study. It is worth noting that at day 48 both treatments fall within the allowed 2-logs allowable growth of *L. monocytogenes*. On day 64 the 0.5% and 0.75% acetic acid treatments both exceeded the allowable growth of 2-logs over the shelf life of the product but by day 80 both treatments saw decreased levels of *L. monocytogenes*. Day 80 for both of these treatments had final counts ca. $10^6$ CFU/gram. The 1.0% acetic acid treatment on day 48 saw an increase of *L. monocytogenes* counts of ca. 1-log CFU/gram representing a statistically significant ($p < 0.05$) difference between this treatment and the others on each of the days that counts were conducted. At 1.0% acetic acid *L. monocytogenes* does not grow beyond the 2-logs allowable growth over the shelf life of the pork BBQ product.

Figure 4.2 shows estimated means with 95% confidence levels the survival of *L. monocytogenes* during the 60 days of storage at an abusive temperature of 10°C. Counts were taken on days 0, 36, 48, and 60. It is seen that at day 0 the initial inoculum for all treatments are ca. $10^5$ CFU/gram. The control samples which contained 0.0% acetic acid had the highest initial growth and by the first sampling day 36. Viable *L. monocytogenes* were well beyond the 2-logs allowable growth over the shelf life of the product with counts ca. $10^9$ CFU/gram. At day 48 *L. monocytogenes* counts climbed to ca. $10^{10}$ CFU/gram and by day 60 final counts were near $10^9$ CFU/gram. The 0.5% acetic acid treatment samples saw growth beyond the allowed 2-logs with counts ca. $10^8$ CFU/gram on day 36. Counts taken on day 48 for this treatment saw continued growth ca. $10^9$ CFU/gram. From day 48 to day 60 there was a leveling off of growth and final counts showed ca. $10^9$ CFU/gram. The control treatments as well as the 0.5% acetic acid treatments on day 60 are not significantly different as seen in
table 4.3. Treatment samples with 0.75% acetic acid reached the 2-log growth limit by day 36 and continued to see growth throughout the 60 day shelf life study. There was no statistical significant difference between day 36 and day 60 for the 0.75% acetic acid treatment samples. At 1.0% acetic acid *L. monocytogenes* growth was inhibited to no more than 2-logs growth over the 60 shelf life of the RTE Pork BBQ product. On day 36 counts for the 1.0% acetic acid treatments were $3.93 \times 10^6$ CFU/gram, day 48 for this same treatment showed growth to $1.77 \times 10^7$ CFU/gram, and day 60 reveled counts of $8.38 \times 10^6$ CFU/gram.

### 4.2 Total aerobic plate counts

Total aerobic plate counts provided an idea of spoilage and pathogen contamination of the RTE Pork BBQ product over the shelf life at both optimal and abuse temperatures. Initial inoculum levels were ca. $10^5$ CFU/gram and counts were taken on the same days as the *L. monocytogenes* specific counts done on MOX plates at both optimal and abuse temperatures. Similar trends were seen as the MOX plates. The control samples with 0.0% acetic acid had the highest counts where the 1.0% acetic acid treatment had the lowest bacterial counts. Because the number of replicates was limited, statistical significance cannot be attributed to all of the treatments as seen in Figure 4.3. There is statistical significance between 0.0% acetic acid treatment and the 1.0% acetic acid treatment samples by day 80. Figure 4.4 shows the estimated means at 95% confidence levels for samples held at abuse temperature (10°C) for 60 days. Initial inoculum levels were ca. $10^5$ CFU/gram for all treatments. Statistical significance can be seen between the control group (0.0% acetic acid) and the 1.0% acetic acid treatments on day 36. Samples treated with 1.0% acetic acid had
counts ca. $10^6$ CFU/gram on day 36 where all other treatments shown counts ca. $10^9$
CFU/gram. Day 48 and day 60 counts for all treatments were ca. $10^8$-$10^9$ CFU/gram.
Additional replicates would need to be conducted with the total aerobic plates for statistical
significance.

4.3 Conclusions

The six strain cocktail of *Listeria monocytogenes* used in this study grew to high
levels ca. $10^{10}$ CFU/gram in the control samples in both the optimal and abusive temperature
environments. This study along with others has shown that *L. monocytogenes* can grow to
very high levels with RTE meat products. Samelis et al., (2001) saw a 4 to 5-log increase in
*L. monocytogenes* in 20 days at refrigeration temperatures in sliced pork bologna. Buncic et
al., (1991) reports a 3-log increase in *L. monocytogenes* within 20 days at refrigeration
temperatures on the surfaces of frankfurters. Beumer et al., (1996) saw refrigerated luncheon-
meat, ham and chicken breast have 3-logs growth during the shelf life even under modified
atmosphere (30% CO$_2$/ 70%N$_2$) packaging. It was also reported in this study that even the
presence of competitors (*Lactobacilli*) at 100 times the concentration of *L. monocytogenes*
only slightly inhibited the growth of the pathogen.

Our results have shown that at 1.0% acetic acid concentrations using apple cider
vinegar was effective in inhibiting *L. monocytogenes* growth to within the allowed 2-logs
over the expected shelf life of RTE Pork BBQ. However, the ability of *L. monocytogenes* to
grow in RTE Pork BBQ with lower concentrations of acetic acid at refrigeration
temperatures (4°C) was observed. *L. monocytogenes* has been shown to persist in certain
ready-to-eat food products that contain common antimicrobials such as lactates and diacetates over their shelf life (Porto-Fett et al., 2010).

The acetic acid concentration of 1.0% was most likely effective due to the pKa of acetic acid being 4.75 therefore at a pH of ~4.63 there were more undissociated species available to pass through the cell wall of the pathogen. Weak organic acids such as acetic acid are lipid permeable allowing the acid to pass through the cell wall. This pH gradient leads to cell death and/or stress by exhausting the cell through a process called proton pumping (Bracey et al., 1998).

Based on this data recommendations made for RTE pork BBQ processors are to achieve a final equilibrium pH of 4.63 or less. Data confirms a final equilibrium pH of 4.63 inhibits the growth of *L. monocytogenes* over the shelf life (60-80 days) at 10°C and 4°C respectively. Implications for future research include investigating other antimicrobials such as lactates and diacetates that have proved successful at inhibiting bacterial growth. The field may also benefit from researching modified atmosphere packaging (MAP). The populations most vulnerable to Listeriosis should consider reheating RTE foods to reduce the risk associated with these types of food products.

Processors may use the formula $C_1V_1 = C_2V_2$ to calculate the amount of vinegar to use for reaching ~1.0% acetic acid in their sauce formulation. For example, using 50 grain or 5.0% acidity vinegar for 100 lbs of meat the processor would want to add approximately 2.4 gallons of 5.0% acidity vinegar. Using 200 grain or 20.0% acidity vinegar the processor would use approximately 0.60 gallon vinegar.
References


TABLES AND FIGURES

Tables

Table 2.1 Growth limits of *Listeria monocytogenes*.

<table>
<thead>
<tr>
<th></th>
<th>Min</th>
<th>Optimal</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>-0.4</td>
<td>37</td>
<td>45</td>
</tr>
<tr>
<td>pH</td>
<td>4.1*</td>
<td>7.0</td>
<td>9.4</td>
</tr>
<tr>
<td>Water Activity</td>
<td>0.92</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>NaCl (%)</td>
<td>---</td>
<td>---</td>
<td>20.0**</td>
</tr>
</tbody>
</table>

Adapted from “Microorganisms in Foods 5. Characteristics of Microbial Pathogens as well as Acid and NaCl Limits to Growth of *Listeria monocytogenes* and Influence of Sequence of Inimical Acid and NaCl Levels on Inactivation Kinetics (Shabala et al., 2008)*. A Characterization of Listeriosis in man and other animals (Murray, 1955)**.
### Table 2.2  Fecal Carriage Rates of *Listeria monocytogenes*

<table>
<thead>
<tr>
<th>Year</th>
<th>Population</th>
<th>No. Studied</th>
<th>% with <em>L. monocytogenes</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1972</td>
<td>Slaughterhouse workers</td>
<td>1,147</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>Hospitalized adults</td>
<td>1,034</td>
<td>1.2</td>
</tr>
<tr>
<td>1986</td>
<td>Healthy nonpregnant women</td>
<td>59</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>Healthy pregnant women</td>
<td>51</td>
<td>2.0</td>
</tr>
<tr>
<td>1990</td>
<td>Healthy food handlers</td>
<td>2,000</td>
<td>0.8</td>
</tr>
<tr>
<td>1992</td>
<td>Pregnant women with listeriosis</td>
<td>18</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td>Household contacts of pregnant women with listeriosis</td>
<td>60</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>Cheese plant employees</td>
<td>31</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>Household contacts of cheese plant employees</td>
<td>94</td>
<td>10.6</td>
</tr>
<tr>
<td>1993</td>
<td>Household contacts of patients with listeriosis (persons)</td>
<td>82</td>
<td>21.0</td>
</tr>
<tr>
<td></td>
<td>Households of patients with listeriosis with at least one carrier</td>
<td>28</td>
<td>21.0</td>
</tr>
<tr>
<td>2003</td>
<td>Healthy adults</td>
<td>3</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Adapted from Painter and Slutsker, 2007
Table 2.3  *Listeria* species Serotypes

<table>
<thead>
<tr>
<th><em>Listeria</em> species</th>
<th>Serotypes</th>
</tr>
</thead>
</table>
| *L. monocytogenes* | 1/2a, 1/2b, 1/2c  
|                   | 3a, 3b, 3c  
|                   | 4a, 4ab, 4b  
|                   | 4c, 4d, 4e, “7” |
| *L. ivanovii*     | 5         |
| *L. innocua*      | 4ab, 6a, 6b, Un$^a$ |
| *L. welshimeri*   | 6a, 6b    |
| *L. seeligeri*    | 1/2b, 4c, 4d, 6b, Un |

$^a$Un, undefined

Adapted from Seeliger, H.P.R., 1986
Table 2.4  Food Contact and Non-Food Contact Contamination Sites in Food Processing Plants

<table>
<thead>
<tr>
<th>Source</th>
<th>Plant A</th>
<th>Plant B</th>
<th>Plant C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drains</td>
<td>10</td>
<td>10</td>
<td>44</td>
</tr>
<tr>
<td>Floors</td>
<td>4</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>Garbage Bins</td>
<td>4</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Raw Meat Ingredients</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Equipment and table surfaces</td>
<td>15</td>
<td>17</td>
<td>64</td>
</tr>
<tr>
<td>Listeria species</td>
<td>47</td>
<td>45</td>
<td>167</td>
</tr>
<tr>
<td>Total Samples</td>
<td>520</td>
<td>520</td>
<td>520</td>
</tr>
<tr>
<td>Prevalence (%)</td>
<td>9.0</td>
<td>8.7</td>
<td>32.1</td>
</tr>
</tbody>
</table>

Adapted from Kushwaha & Muriana, 2009
Table 2.5  Provisional cases of infrequently reported notifiable diseases—United States, week ending August 31, 2013 (35th week)

<table>
<thead>
<tr>
<th>Disease</th>
<th>Current week</th>
<th>Cumulative 2013</th>
<th>5-year weekly average</th>
<th>Total cases reported for previous years</th>
<th>States reporting cases during current week (No.)</th>
</tr>
</thead>
</table>

Adapted from Centers for Disease Control and Prevention Morbidity and Mortality Weekly Report, 2013
Table 4.1 Analysis of RTE Pork BBQ product

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>25.3%</td>
</tr>
<tr>
<td>Moisture</td>
<td>54.9%</td>
</tr>
<tr>
<td>Fat (Soxhlet)</td>
<td>20.1%</td>
</tr>
<tr>
<td>Ash</td>
<td>1.04%</td>
</tr>
<tr>
<td>Sodium</td>
<td>74.2 mg/100g</td>
</tr>
</tbody>
</table>
## Table 4.2

Recovery of *Listeria monocytogenes* at 4°C for 80 days in RTE Pork BBQ with various concentrations of Acetic Acid

<table>
<thead>
<tr>
<th>Acetic Acid Treatment&lt;sup&gt;b&lt;/sup&gt;</th>
<th>4°C Log CFU/g&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Day 0</th>
<th>S.E.</th>
<th>Day 48</th>
<th>S.E.</th>
<th>Day 64</th>
<th>S.E.</th>
<th>Day 80</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>High 1.0%</td>
<td>3.67 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.16</td>
<td>7.87 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.18</td>
<td>3.86 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.15</td>
<td>2.94 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>Mid 0.75%</td>
<td>4.39 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.16</td>
<td>5.26 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0.18</td>
<td>6.56 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0.15</td>
<td>3.22 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>Low 0.5%</td>
<td>4.21 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.16</td>
<td>2.88 x 10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>0.18</td>
<td>1.28 x 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>0.15</td>
<td>5.23 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.07 x 10&lt;sup&gt;5&lt;/sup&gt;</td>
<td>0.16</td>
<td>1.68 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>0.18</td>
<td>7.10 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>0.15</td>
<td>5.08 x 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>0.11</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Analyses were based on three experiments with mean being averaged of the three determinations. Means within each vertical column followed by the same letter are not significantly different (P≥0.05) from each other. Statistical comparisons of all pairs were analyzed using the Tukey method following a two-way analysis of variance (ANOVA) with a random block effect (SAS Institute Inc., version 9.3, Cary, NC).

<sup>b</sup> Different concentrations of acetic acid from apple cider vinegar were used to treat the inoculated RTE Pork BBQ samples.
Table 4.3  Recovery of *Listeria monocytogenes* at 10°C for 60 days in RTE Pork BBQ with various concentrations of Acetic Acid

<table>
<thead>
<tr>
<th>Acetic Acid Treatment</th>
<th>Day 0</th>
<th>S.E.</th>
<th>Day 36</th>
<th>S.E.</th>
<th>Day 48</th>
<th>S.E.</th>
<th>Day 60</th>
<th>S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>High 1.0%</td>
<td>4.18x 10^5</td>
<td>0.14</td>
<td>3.93x 10^6</td>
<td>0.14</td>
<td>1.78x 10^7</td>
<td>0.18</td>
<td>7.48x 10^6</td>
<td>0.19</td>
</tr>
<tr>
<td>Mid 0.75%</td>
<td>4.30x 10^5</td>
<td>0.14</td>
<td>5.63x 10^7</td>
<td>0.14</td>
<td>1.25x 10^8</td>
<td>0.18</td>
<td>3.01x 10^8</td>
<td>0.19</td>
</tr>
<tr>
<td>Low 0.5%</td>
<td>4.12x 10^5</td>
<td>0.14</td>
<td>1.13x 10^8</td>
<td>0.14</td>
<td>4.03x 10^9</td>
<td>0.18</td>
<td>4.99x 10^9</td>
<td>0.19</td>
</tr>
<tr>
<td>Control</td>
<td>5.70x 10^5</td>
<td>0.14</td>
<td>2.62x 10^9</td>
<td>0.14</td>
<td>1.60x 10^10</td>
<td>0.18</td>
<td>5.68x 10^9</td>
<td>0.19</td>
</tr>
</tbody>
</table>

*a* Analyses were based on three experiments with mean being averaged of the three determinations. Means within each vertical column followed by the same letter are not significantly different (P≥0.05) from each other. Statistical comparisons of all pairs were analyzed using the Tukey method following a two-way analysis of variance (ANOVA) with a random block effect (SAS Institute Inc., version9.3, Cary, NC).

*b* Different concentrations of acetic acid from apple cider vinegar were used to treat the inoculated RTE Pork BBQ samples.
**Figure 2.1** Recalls reported in 2012 by the Food and Drug Administration (FDA) due to *Listeria monocytogenes* contamination
Figure 2.2  Recalls reported in 2012 by the USDA Food Safety and Inspection Service (FSIS) archive
Figure 2.3  Categorization of Listeriosis as invasive and noninvasive

Adapted from Donnelly, 2001
1. Planktonic Cells

2. Cell deposition on surface
   - Hydrophilic interactions
   - Flagella

3. Cell adhesion to surface
   - Hydrophilic interactions
   - ± Fibrils
   - Synthesis of exopolymers

4. Surface colonization
   - Cells monolayer

5. Biofilm formation
   - Layers with variable cell density
   - Homogeneous cell distribution horizontally

6. Biofilm development
   - Biofilm growth
   - Presence of capillary water channels (3-D structure)

Figure 2.4  *Listeria monocytogenes* biofilm formation

Adapted from Lado & Yousef, 2007.
Figure 4.1  *Listeria monocytogenes* survival at 4°C during shelf life of 80 days
Figure 4.2  *Listeria monocytogenes* survival at 10°C during shelf life of 60 days
Figure 4.3  Total aerobic plate count at 4°C during shelf life of 80 days
Figure 4.4  Total aerobic plate count at 10°C during shelf life of 60 days